# CHARACTERISING THE RELATIONSHIP BETWEEN FOWLPOX VIRUS AND THE MAMMALIAN IMMUNE SYSTEM

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# ABSTRACT

Fowlpox viruses (FPV) are attractive platform vaccine vector candidates because their capacity for insertion of multiple heterologous genes makes them favourable for genetic modification. They also have strong adjuvant activity in their own right. As FPV does not replicate in mammalian cells, there is significantly less opposition associated with their clinical application, with a number already in use. However, a thorough understanding of the immunological relationship between FPV and the mammalian immune system is still lacking.

The aim of this thesis was to construct a series of recombinant FPV vectors that co-expressed the nominal antigen chicken ovalbumin (OVA), ($FPV_{OVA}$), and/or murine interleukin-4 (mIL-4). These constructs were used for the characterisation of the relationship between FPV and the mammalian immune system and how this is altered by the co-expression of mIL-4. Immunisation with $FPV_{OVA}$ resulted in rapid and highly localized OVA expression which induced strong $CD8^+$ cytotoxic T cell (CTL) activity but only weak $CD4^+$ T helper and antibody responses. In addition, presentation of FPV-derived antigen and the priming of antigen-specific CTL responses required a permissive bone marrow (BM)-derived cell as the antigen presenting cell (APC). Co-administration with $FPV_{mIL\text{-}4}$ resulted in a dramatic reduction in CTL activity that remained largely non-functional throughout the infection and a skewing of the T helper (Th) response towards Th2 with a reduction in interferon (IFN)-γ production by OVA-specific Th cells. These findings provide a sound basis for further characterization of how FPV interacts with the innate and adaptive arms of the immune system, how these can be manipulated via the co-administration of cytokines, and discovering if future rationally designed modifications result in FPV vectored vaccines that induce durable cellular and humoral immunity.

## THESIS DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Emma Beukema

NOTE:
The comic strip is included on page 12 of the print copy of the thesis held in the University of Adelaide Library.

*This Masters thesis is dedicated to my husband, John.*

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# ABBREVIATIONS

| | |
|---|---|
| **ALB** | Alkaline lysis buffer |
| **ALVAC** | Canarypox virus |
| **ANK** | Ankyrin repeat |
| **APC** | Antigen presenting cell |
| **AT** | Adoptive transfer |
| **BHK** | Baby hamster kidney |
| **BM** | Bone marrow |
| **BP** | Binding protein |
| **bp** | Base pair |
| **BSA** | Bovine serum albumin |
| **CC** | Chemokine |
| **CEA** | Carcinoembryonic antigen |
| **CEF** | Chicken embryonic fibroblast |
| **CFSE** | Carboxyfluoroscein succinimidyl ester |
| **CLR** | C-type lectin receptor |
| **CMI** | Cell-mediated immunity |
| **CP** | Circumsporozoite protein |
| **CPV** | Cowpox virus |
| **crmA** | Cytokine response modifier A |
| **CRV** | Crocodilepox virus |
| **CT** | Connective tissue |
| **CTL** | Cytotoxic T lymphocyte |
| **DC** | Dendritic cell |
| **DNA** | Deoxyribonucleic acid |
| **Ds** | Double stranded |
| **EEV** | Extracellular enveloped virus |
| **EGFP** | Enhanced green fluorescent protein |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EV** | Ectromelia virus |
| **FACS** | Fluorescence-activated cell sorting |
| **FCS** | Foetal calf serum |
| **FDA** | Food and Drug Administration |
| **FL** | Fluorescence |
| **FPV** | Fowlpox virus |
| **GM** | Growth medium |
| **GM-CSF** | Granulocyte macrophage colony-stimulating factor |
| **HAT** | Hypoxanthine-aminopterin-thymidine |
| **HEK** | Human embryonic kidney |
| **HIV** | Human immunodeficiency virus |
| **HSV** | Herpes simplex virus |
| **ICE** | Interleukin-1β-converting enzyme |
| **IFN** | Interferon |
| **IL** | Interleukin |

| | |
|---|---|
| **IMV** | Intracellular mature virus |
| **IND** | Investigational New Drug Application |
| **IRES** | Internal ribosome entry site |
| **LCMV** | Lymphocytic choriomeningitis virus |
| **LN** | Lymph node |
| **LTR** | Long terminal repeat |
| **LU** | Lytic unit |
| **mAb** | Monoclonal antibody |
| **MAPK** | Mitogen-activated protein kinase |
| **MHC** | Major histocompatibility complex |
| **MM** | Maintenance medium |
| **MPA** | Mycophenolic acid |
| **MOI** | Multiplicity of infection |
| **MVA** | Modified vaccinia Ankara |
| **NF- κB** | Nuclear factor κB |
| **NHP** | Non-human primate |
| **NK** | Natural killer |
| **NLR** | NOD-like receptor |
| **NR** | Neutral red |
| **NTA** | Nitrilotriacetic acid agarose |
| **O/N** | Overnight |
| **ORF** | Open reading frame |
| **OVA** | Ovalbumin |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PAMP** | Pathogen-associated molecular pattern |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **pDC** | Plasmacytoid dendritic cell |
| **PFU** | Plaque forming unit |
| **PI** | Proliferation index |
| **p.i.** | Post-infection |
| **PRR** | Pattern recognition receptor |
| **PSA** | Prostate-specific antigen |
| **PVR** | Polio virus receptor |
| **RB** | Roller bottle |
| **RBC** | Red blood cell |
| **rFPV** | Recombinant FPV |
| **REV** | Reticuloendotheliosis virus |
| **RIG** | Retinoic acid-inducible gene |
| **RLR** | RIG-like receptor |
| **RT** | Room temperature |
| **SDS** | Sodium dodecyl sulfate |
| **Serpin** | Serine protease inhibitor |
| **TAA** | Tumor-associated antigen |
| **TAE** | Tris-acetate-EDTA |
| **TAP** | Transporter associated with antigen presentation |

| | |
|---|---|
| **TCR** | T cell receptor |
| **Th** | T helper cell |
| **$T_{reg}$** | Regulatory T cell |
| **TK** | Thymidine kinase |
| **TLR** | Toll-like receptor |
| **TNF** | Tumour necrosis factor |
| **TRICOM** | Triad of co-stimulatory molecules |
| **UV** | Ultra violet |
| **VSS** | Virus seed stock |
| **VSV** | Vesicular stomatitis virus |
| **VV** | Vaccinia virus |

# CHAPTER 1: THE POTENTIAL ROLE OF FOWLPOX VIRUS (FPV) IN RATIONAL VACCINE DESIGN

## 1.1 INTRODUCTION

In 1796, English country physician Edward Jenner discovered that exposure of an individual to a weakly pathogenic variant of an infectious organism prevents disease, and this is now known as vaccination. He demonstrated resistance to smallpox by inoculating a boy with virus from lesions on the hands of an infected milkmaid, presumed to have arisen as a zoonotic infection from poxvirus-infected cows (1). Although this course of action was initially met with alarm and scepticism, it became a widely accepted practice. Eventually, this procedure was superseded by inoculation with a relative of cowpox virus called vaccinia, from the Latin *vaccinus*, for cow. Despite the unclear origin of the vaccinia virus (1), it has provided a generic name for the process of vaccination and the avirulent agents (vaccines) used in this practice.

Jenner's early work on smallpox vaccination encouraged the concept that deliberate protection could be afforded against infectious diseases. Following observations made by Louis Pasteur detailing methods of pathogen attenuation, over a century followed where vaccines were empirically developed (2). The precise mode of action of these vaccines were not understood (3). Recent technological advances have furnished enormous analytical power facilitating an understanding of the mechanisms of immune protection from infection (4). An approach to vaccine development where the immune correlates of protection are defined and vaccines are designed to elicit such responses is known as rational vaccine design. Poxviruses are attractive candidates for this approach due to their large genome, which allows for the insertion of large or multiple genes including antigenic targets and immunomodulatory proteins, in order to selectively induce protective immunity.

**Figure 1.1 Vaccination with smallpox.**

Historical engraving of Edward Jenner (1749-1823) vaccinating a baby against smallpox.

(Adapted from: Smith, GL and McFadden G. Smallpox: anything to declare? *Nature Reviews in Immunology*. 2002: **2**: 521-27).

## 1.2 POXVIRUSES COMPRISE A LARGE FAMILY OF HIGHLY SUCCESSFUL PATHOGENS

### *1.2.1. The poxvirus family*

All of the viruses that figure in the Jenner story are members of the *Poxviridae*, which comprises a large family of complex DNA viruses that infect both vertebrate and invertebrate hosts (5). The poxvirus genome, which is composed of a single, linear double-strand DNA molecule of 130,000 to 300,000 base pairs (bp), is responsible for encoding all the genes necessary for a unique cytoplasmic existence, allowing the virus to replicate almost entirely independent of the nucleus (6). Two forms of infectious virus exist. One form, termed intracellular mature virus (IMV), is found in the cytoplasm of virus-infected cells and represents the majority of infectious progeny. The second form of infectious virus, termed extracellular enveloped virus (EEV) is released from infected cells after the IMV migrates to the cell surface and fuses with the plasma cell membrane, releasing the infectious EEV particles (7).

### *1.2.2. Classification of poxviruses*

The earliest classification of poxviruses was based on the clinical manifestations of a variety of different diseases of humans and their domestic animals, including cows, sheep, horses and pigs. Characteristic pock marks on the skin resulted in their description as poxes. Several of these conditions were indeed caused by poxviruses, but these classifications were based on clinical symptoms rather than genetic identity, resulting in the inclusion of chickenpox (caused by varicella-zoster virus) and the great pox, syphilis, in the same group as the smallpox viruses (8). Being the largest of all animal viruses and clearly visible in stained smears by light microscopy, the poxviruses were the first group of viruses to be described, that is, viruses that were not serologically related but appeared similar in size and other physical characteristics (8). The International Committee on Taxonomy divided the poxvirus family into two subfamilies based on their vertebrate or invertebrate host range and is summarized in Table 1.1.

**Table 1.1 Family *Poxviridae***

| Subfamilies | Genera | Members |
|---|---|---|
| *Chordopoxvirinae* (vertebrate poxviruses) | *Orthopoxvirus* | Buffalopox, camelpox, cowpox, monkeypox, rabbitpox, raccoonpox, taterapox, vaccinia[1], variola, volepox |
| | *Parapoxvirus* | Chamois contagious ecthyma, Orf[1], pseudocowpox |
| | *Avipoxvirus* | Canarypox, fowlpox[1], juncopox, pigeonpox, quailpox, sparrowpox, starlingpox, turkeypox |
| | *Capripoxvirus* | Goatpox, sheeppox[1], lumpy skin disease |
| | *Leporipoxvirus* | Hare fibroma, myxoma[1], rabbit (Shope) fibroma, squirrel fibroma |
| | *Suipoxvirus* | Swinepox |
| | *Molluscipoxvirus* | Molluscum contagiosum |
| | *Yatapoxvirus* | Tanapox[1], Yaba |
| *Entomopoxviridae* (insect poxviruses) | A | Melontha melontha |
| | B | Amasacta moori |
| | C | Chironimus luridus |

[1] Prototypical member

The *Chordopoxvirinae* infect vertebrates ranging from birds to humans and have been placed into eight genera: *Orthopoxvirus, Parapoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Yatapoxvirus, Molluscipoxvirus* and *Avipoxvirus* (5). In addition to the classified members of the *Chordopoxvirinae* subfamily, the genome of crocodilepox virus (CRV) has recently been characterized and similar poxviruses infecting other reptiles have been described [9]. Phylogenetic analyses of CRV indicate that it is quite distinct from other members of the *Chordovirinae* subfamily, representing a new genus (9). Further analysis and characterisation of poxvirus genomes such as CRV may reveal novel mechanisms of poxvirus host range and virus-host interaction.

### *1.2.3. Avipoxviruses are the only classified member of the Chordopoxvirinae genus able to infect non-mammalian hosts*

The Avipoxviruses are the only classified genus of the *Chordopoxvirinae* subfamily able to infect non-mammalian hosts, targeting more than 60 species of wild birds representing 20 families (10, 11). Fowlpox virus (FPV), which infects chickens and turkeys, is the prototypical and best-studied member of the *Avipoxvirus* genus (12). Avipoxviruses are mechanically transmitted by biting arthropods; however, there is no evidence to suggest viral replication in invertebrate vectors (13). Despite the significant economic impact worldwide imposed by poxvirus diseases of poultry and other domestic birds (11), the use of live attenuated FPV vaccines has provided for effective disease control. However, there have been reports of FPV outbreaks in previously vaccinated flocks and the possibility of reticuloendotheliosis virus (REV) sequence integration within the genomes of field strains of FPV being responsible for the enhanced virulence is under investigation (14). The administration of live agents and natural exposure enables multiple infections to occur simultaneously and the exchange of genetic material between pathogens (15). Indeed, the use of Australia's standard FPV strain (designated FPV S) was discontinued because of suspected REV contamination (16). Fortunately, only a remnant of an REV long terminal repeat (LTR) has been found in the FPV vaccine strains FP9, a plaque-purified, highly attenuated, European strain of FPV and FPV M, the widely-used mild Australian vaccine strain (17). Although the presence of REV sequences may pose a safety concern for vaccine FPV strains due to maintained infectivity of the integrated retroviruses, it appears as though REV sequences are only retained by naturally occurring FPV strains and not by those artificially propagated in a laboratory setting such as the vaccine strains (15). Indeed, human clinical trials of recombinant FP9 against liver-stage *P.falciparum* malaria (18) have confirmed that the remnants of REV sequence present in vaccine strains of FPV are of little safety concern.

#### *1.2.3.1. Avipoxvirus vectors undergo abortive replication in the cells of non-avian species*

Fowlpox virus is able to bind and enter both permissive and restrictive cells (19), but the ability of a given poxvirus to fully complete the replication cycle varies markedly between cells of different lineages or species, as downstream intracellular events are specifically

inhibited in restrictive cells (20). Interestingly, it has recently been demonstrated that vaccinia virus (VV) and canarypox (ALVAC) virus show a strong bias towards monocyte infection, infecting leukocyte subsets in human bone marrow, peripheral blood, and monocyte-derived dendritic cells (DCs) (21). Transmission of the avipoxviruses is limited to avian species and infection of mammalian cells results in an abortive replication, although viral gene expression can persist for up to fourteen days (22). Infection at high multiplicities of infection (MOI) causes cytopathic effects in mammalian cells, but no evidence of productive viral replication can be detected (23). These findings have been confirmed via the evaluation of a wide range of mammalian cell lines for permissivity to three different avipoxvirus strains, FPV and two newly isolated strains from sparrow and pigeon (24). However, a remarkable discovery of this study is that Syrian baby hamster kidney (BHK-21) cells were equally permissive to all virus strains as compared to avian cell lines , without interruption in virus morphogenesis (24). This is the first description of efficient multiplication of an avipoxvirus in a cell type of mammalian origin and adds another layer of complexity to the issue of host range restriction related to the poxvirus family. *In vivo*, inoculation of six different non-avian species with recombinant FPV did not produce a single replicative infection or any evidence of overt disease (13). There exists a single report of an avipoxvirus isolate found in a mammal. In 1969, viable FPV was detected in an already terminally ill, and probably immunosuppressed captive rhinoceros (23). This isolate was identified as a somewhat atypical FPV, based on pathological, virological and serological characteristics (23). Thus, it may be accepted that while the host range restriction of avipoxviruses is not fully understood, they are extremely unlikely to cause disease in mammalian hosts.

### *1.2.4. Fowlpox virus tropism and immune surveillance evasion strategies*

The specific interaction of a virus with its cellular receptor determines the host range restriction (tropism) of the virus (20). This interaction is needed to initiate infection. Indeed, comparative analysis of tropism between ALVAC and VV has shown that the preferential infection of monocyte-derived cells directly correlates with the level of virus binding (21). Furthermore, a study examining VV tropism for primary haematolymphoid cells has demonstrated that the key determinant for infection is virus binding to the cell surface (25).

Indeed, the susceptibility of primary human cells to VV infection was found to be determined by restricted expression of a cellular receptor that is induced *de novo* upon T cell activation (25). However, it has more recently been discovered that VV particles that could bind to cells could not enter them or mediate cell-cell fusion in non-permissive conditions, indicating that fusion with the plasma membrane is also necessary for viral entry (26). The ability of VV to enter cells by both neutral-pH plasma membrane and low-pH endosomal pathways may contribute to its wide host range in cell culture (27). Although the cellular targets of FPV in a mammalian immune system have not been delineated, these findings suggest that poxvirus tropism for cells encountered *in vivo* including FPV is far more complex than has been previously realised.

In addition to poxvirus-specific cellular receptors, the development of disease is influenced by the intracellular milieu, kinetics of viral replication, cytopathogenicity and the rate of spread of infection (28). The analysis of the FPV genomic sequence and its comparison with those of other *Chordopoxvirinae* members has predicted putative functions for a diverse complement of host range genes functioning in immune evasion and modulation, and other cellular functions (12). Their goal is to create an intracellular environment that promotes productive virus infection (29). Potential FPV host range proteins include ankyrin repeat (ANK) proteins (30), NK cell receptors, chemokines (CC), serine protease inhibitors (serpins) and homologues of those involved in apoptosis, cell growth, tissue tropism and avian host range (11). The wide array of gene homologues with likely host range functions in FPV suggests significant viral adaptation to the avian host. The design of safer and more effective FPV vaccines and FPV-based expression vectors will require complete information on viral genes associated with viral virulence and host range as well as a more complete understanding of how these genes function in viral pathogenesis, immune evasion and avian host range (11). There is a wide body of work identifying and describing the mode of action of the many different immunomodulatory proteins encoded by poxviruses (31-33) some of which are illustrated in Figure 1.2. Only examples of inhibitors of apoptosis and the various cellular receptor homologues used by FPV will be briefly discussed in this introduction.

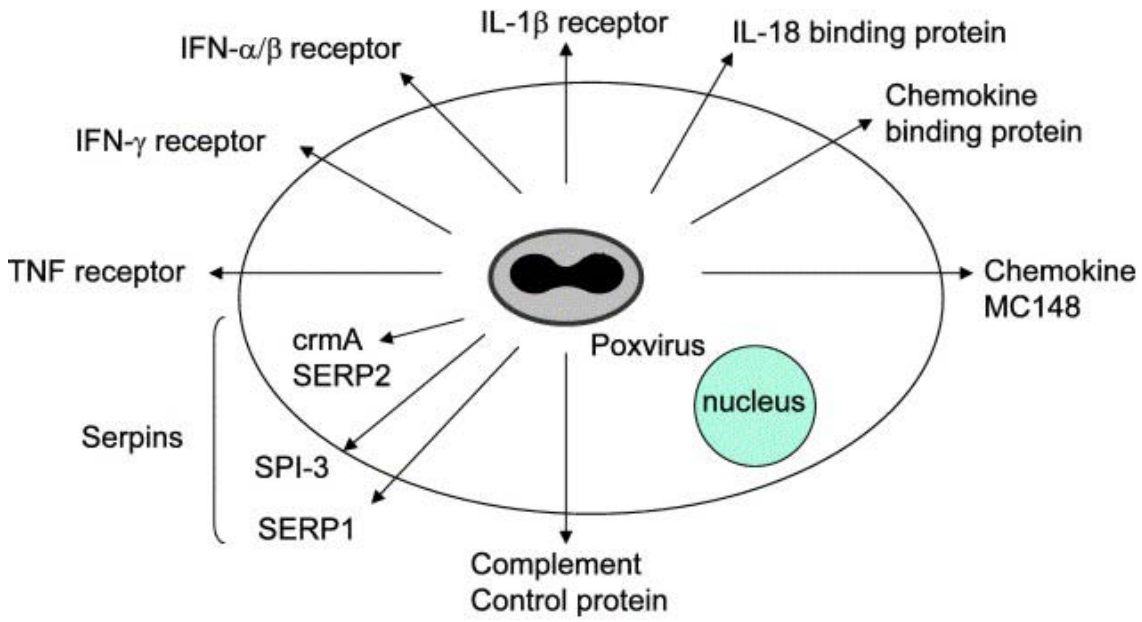


**Figure 1.2 Production of secreted and intracellular immune modulators by a poxvirus-infected cell.**

Secreted poxviral host range proteins function as either soluble or cell surface decoy receptors that bind host cytokines or chemokines or agonistic or antagonistic ligands for host cellular receptors. A number of intracellular poxvirus proteins function to modulate apoptosis, cytokines processing, and host range.

(Adapted from: Turner, PC and Moyer, RW. Poxvirus immune modulators: functional insights from animal models. *Virus Research*. 2002; **88**:35-53)

### *1.2.4.1 Inhibition of apoptosis by FPV*

The first host range gene products to be identified in the poxviruses were the serine protease inhibitors (serpins) which inhibit programmed cell death or apoptosis. Cytokine response modifier A (crmA, also known as SPI-2), identified in cowpox virus (CPV), was the first of these to be cloned (20). Five homologues of serpins are encoded by FPV and all exhibit 21 to 29% amino acid identity to each other (11). Serpin genes are encoded by most chordopoxviruses and inhibit apoptosis by blocking both the cysteine protease interleukin-1β-converting enzyme (ICE), also known as caspase-1, and the serine proteinase granzyme B (6). Caspases are key components of the cell death machinery that turn off protective pathways and turn on downstream activities leading to cellular destruction (34). The IL-1β precursor, a key mediator of inflammation, is proteolytically cleaved by caspase-1 during

apoptosis and it is this activation that is inhibited by serpins (35). Their second anti-apoptotic activity is the inhibition of cytotoxic T lymphocyte (CTL)-mediated apoptosis, induced by granzyme B (36). Granzyme B is crucial for the killing activity of CTLs and is thought to mediate apoptosis through direct activation of caspases. CrmA expression renders target cells resistant to CTL killing which would be expected to prolong virus replication in natural infections (37). While these serpins have been shown to be non-essential for virus growth, they most likely contribute a selective advantage by providing a safe cellular haven conducive to viral replication (37).

*1.2.4.2. Fowlpox virus encodes receptors with target specificity for host proteins*

One of the most prominent groups of poxvirus host range genes are those that encode cellular receptors homologous to the host. These proteins act as decoy receptors and scavenge ligands that promote antiviral immunity or inflammatory processes (29, 38). Mammalian poxvirus-encoded cytokine-binding proteins which are specific for a diverse range of host cytokines have been identified, including tumour necrosis factor (TNF), interleukin (IL)-1β, IL-2, IL-18, interferon (IFN)-α/β, IFN-γ, and granulocyte macrophage colony-stimulating factor (GM-CSF) (39).

Interferons are potent immune response modifiers that are produced by mammalian lymphoid cells following viral infection and the central role of IFN-γ in the host defense against poxviruses probably explains why this cytokine is one of the main targets of the poxviral immune evasion strategy (40, 41). Poxviral IFNγ-binding proteins are important virulence factors that can act as decoy receptors, binding induced IFNγ and impairing its role in the host response to infection (42). This is reflected in the finding that disruption of the signaling cascade of extracellular signal-regulated kinase1/2 (Erk1/2), type I interferon and STAT1, which mediates the expression of an array of antiviral cytokines including IFN-γ, renders normally resistant cells fully permissive to myxoma virus replication (43). A gene encoding a soluble IFN-γ-binding protein has been identified in most chordopoxviruses including FPV (44). Interestingly, unlike previously known cellular and poxviral IFN-γ receptors, the IFN-γ-binding protein of FPV contains an immunoglobulin domain and exhibits no significant homology to any known viral or cellular protein (11). Soluble IFNγ-binding proteins of

various poxviruses differ in their specificity for IFNγ from different animal species and this host species specificity reflects the host range and evolutionary history of a particular poxvirus family (12, 39).

## 1.3 POXVIRUS-BASED VECTORS AS CANDIDATES FOR RATIONAL VACCINE DESIGN

The aim of rational design is to define the correlates of protection and develop vaccines that induce such responses. Effective vaccines should generate humoral immunity and produce protective antibodies, as well as establish lasting cell-mediated T cell responses (45). Although CTL are important in mediating immunity to various types of microbial pathogens, it has proven extremely difficult to develop vaccines that elicit effective cell-mediated responses. Recombinant poxviruses are capable of inducing helper T cells, CTL and high levels of antibodies concurrently (46, 47). This ability makes poxviruses ideal vector vaccines (48). In addition, a number of other properties of poxviruses make them attractive for *rational* vaccine vector development. These include high-level gene expression, the capacity for insertion of large or multiple genes and cytoplasmic gene expression (49). Despite the fact that early application of poxviruses as gene expression vectors concentrated on VV, FPV is intrinsically a more suitable vector due to its safety profile, i.e. it has a restricted host range, lack of adverse side effects and an inability to replicate within mammalian hosts.

### *1.3.1. Development of host-restricted poxvirus vectors*

*1.3.1.1. The impact of poxviral-specific immunodominance on their potential application as vaccine vectors*

Pre-existing immunity to VV from smallpox vaccination and immunodominant VV-specific CTL responses have somewhat limited their effectiveness in recombinant vaccination strategies (50). For instance, mice infected with recombinant VV expressing a CTL epitope of lymphocytic choriomeningitis virus (LCMV) generate massive virus-specific T cell immunity, with the VV-specific CD8 T cell response being over 20-fold greater than that directed towards the LCMV epitope incorporated in the vaccine vector (51). In addition,

human phase I/IIa clinical trials of a highly attenuated strain of VV, NYVAC, for *P.falciparum* malaria have demonstrated that although repeated immunization with the vaccine elicited both humoral and cell-mediated immune responses, anti-VV immunity in volunteers previously exposed to VV did have a significant effect on the magnitude of the antibody response (52). Consequently, the level of immunity generated by this vaccine did not confer protection against subsequent malaria infection (52). What has probably been the most striking finding is that pre-existing vector-specific immunity can increase susceptibility to infection as demonstrated in the recent adenovirus STEP trial (53). While the high prevalence of adenovirus-specific antibodies as a result of prior exposure to the virus and resulting elimination of the vaccine vector was anticipated to be an impediment to the development of a T cell response against the inserted antigen, the enhanced susceptibility to HIV infection was not. These results provide strong incentive for understanding the nature and consequences of vector-specific host responses as well as raise new questions about the safety of virally vectored vaccines.

As well as pre-existing immunity, immunodominant vector-specific immune responses following repeated administration also limit their effectiveness in recombinant vaccination strategies. Repeated administration of the NYVAC vaccine for *P.falciparum* malaria observed that all individuals displayed little CTL activity after the third dose, with no difference with respect to VV immune status (52). Clinical studies using highly attenuated replication-deficient strains such as modified vaccinia Ankara (MVA) expressing melanoma tumour antigen epitopes have also shown that vaccinia-specific CTL dominate the outcome of the overall CTL responses and outnumber the target antigen-specific CTL, even in vaccinia-naïve patients (50). Interestingly, human clinical trials of MVA and FPV prime-boost regimens against the liver stage *P.falciparum* malaria have also demonstrated powerful anti-vector responses following 3 successive vaccinations, but only a low to moderate overall anti-vector responses after a single immunization and at late time points (54). Therefore, immunodominant anti-vector responses may be reduced by using such heterologous prime-boost strategies and/or boosting at later time points such as 6 or 12 months.

Because a number of the immunodominant epitopes of VV have been identified and well characterised in both mice and man, it has been possible to design studies to specifically quantify the anti-vector responses as compared to those directed against the heterologous target antigen (55-58). In contrast, few antigenic epitopes for FPV have been identified, although three structural immunodominant proteins have been cloned (10). Accordingly, the ability to quantify FPV-specific responses remains elusive. It is imperative that effort is directed towards identifying and characterising the immunodominant epitopes of FPV, not only to gain a basic understanding of the cellular immune responses to FPV, but to facilitate quantification of the ratio of anti-vector to anti-target antigen-specific responses of recombinant FPV vaccine candidates. The quantification of the FPV-specific responses coupled with experimental approaches to modulate them will enable the design of vaccines where epitope hierarchies could be subverted to significantly enhance the strength, breadth and duration of the immune response to the vaccine target antigens.

*1.3.1.2. The impact of poxviral safety concerns on their potential application as vaccine vectors*

Initial exposure to VV commonly results in fever and flu-like symptoms as well as inadvertent inoculation, in which VV translocates from the primary site to other parts of the body or to other individuals (59). More serious adverse effects associated with VV vaccination include generalized vaccinia, which is the systemic spread of the virus, encephalitis, myopericarditis and even death (22, 59). Indeed, if VV was still prepared as it was prior to 1982, which was by collecting and clarifying viral exudate from the torso of scarified cows, it would be unlikely to receive FDA approval under today's standards (59). Even though an improved tissue culture-derived VV (designated ACAM1000) has been developed to overcome some of the problems associated with the current calf lymph smallpox vaccine (Dryvax), both represent replication-competent viruses. This always imposes an inherent health risk in hosts that have unknown or undisclosed immunodeficiencies at the time of vaccination (59). These safety concerns have been addressed somewhat by the use of highly attenuated replication-deficient strains such as MVA. However, the effect of prior exposure to vaccinia virus from smallpox vaccination on

the ability to elicit primary immune responses to extrinsic immunogens limits its potential for recombinant vaccine vector construction (60).

## 1.4 FPV IS A SAFE AND EFFECTIVE PLATFORM FOR RECOMBINANT VECTOR VACCINE CONSTRUCTS

### *1.4.1. Clinical and experimental applications of recombinant FPV vector vaccines*

The potential of recombinant FPV vectors as vaccines for infectious diseases has received significant attention, and one of the earliest applications of recombinant FPV in a vaccine setting was the use of a variant expressing the rabies virus glycoprotein. Immunisation with this FPV recombinant provided protection against live rabies virus challenge in mice, cats and dogs and there was no evidence of replicative FPV infection (13). Similarly, a canarypox-based rabies recombinant has been evaluated for safety and immunogenicity in human clinical studies and was found to be well-tolerated with only mild and short-lived skin reactions at the inoculation site (61).

Infectious diseases such as human immunodeficiency virus (HIV) and malaria which are affecting large proportions of the global population are naturally frontline targets for vaccine development. Clinical trials are currently being conducted for a range of poxviral HIV and malaria vaccines and most of these are part of heterologous prime-boost regimens incorporating combinations of DNA, MVA, and FPV (62-66). Early phase I clinical trials of FPV and MVA HIV vaccines in humans are ongoing and predominantly in the recruiting phase (66, 67). However, in mice, homologous prime-boosting with an FPV vector expressing a multi-epitope polypeptide composed of the central 15 amino acids of the V3 loop from six different HIV-1 isolates induces potent CD8 T cell responses to HIV antigens (68). Comparable findings have been made in a number of non-human primate models of both HIV and SIV infection (69-72). Recent work with humans has shown promising results for the use of heterologous prime-boost vaccination regimes for multi-epitope malaria vaccines. Indeed, priming with DNA followed by MVA resulted in unprecedented levels of immune responses and a significant delay in the time to patent parasitemia in a sporozoite challenge model (73). When FP9 was used as the priming vector instead of DNA, complete

protection could be achieved in some individuals (54, 64, 74). In contrast, regimes with vectors encoding the full-Length *Plasmodium falciparum* circumsporozoite protein (CP) have only induced modest immune responses, with no evidence of efficacy in a sporozoite challenge (62). In summary, consecutive immunization involving recombinant FPV vector vaccines encoding HIV and malaria antigens elicit enhanced CTL responses that protect against experimental infectious challenge. These combination strategies could represent the basis of safe and effective vaccines.

A range of tumor-associated antigens (TAAs) have been incorporated into FPV vectors to attempt to elicit effective anti-tumoral immunity. Examples of TAAs inserted into FPV include carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), melanoma antigens and human papillomavirus antigens. The US Federal Clinical Trials website currently lists 24 clinical trials that are either recruiting, underway or completed that have involved the application of FPV recombinants targeting cancers including ovarian, breast, prostate, colorectal and bladder cancer
(http://www.clinicaltrials.gov/ct2/results?term=fowlpox+virus+AND+cancer).
A proportion of these recombinant FPV vector vaccines contain a triad of co-stimulatory molecules (designated TRICOM) normally found on the surface of APCs in order to increase T cell cytokine production and activation (75). Results from clinical trials of poxviral vaccines incorporating TRICOM have been promising, reporting no significant toxicity, enhanced CD8 and CD4 immune responses and in some studies durable (18 months) clinical responses (76-79). There are a broad range of strategies that can be used to enhance the efficacy of recombinant FPV vector vaccines and some of these will be discussed in more detail below.

### *1.4.2. Strategies for enhancing the efficacy of recombinant FPV vector vaccines*

#### *1.4.2.1. Controlled and enhanced heterologous gene expression through promoter design*

Poxviral promoters are important elements in FPV vector vaccine design. Fowlpox virus replicates in the cytoplasm using its own transcription machinery and the time and level of viral gene expression is regulated by early, intermediate or late promoters. Early promoters drive gene expression before viral DNA replication, followed by the intermediate and late

promoters, which are sequentially activated (80). The choice of promoter clearly influences the timing and level of heterologous gene expression (81). In a permissive host, gene expression driven by intermediate and late promoters can give rise to over 10-fold higher protein levels than those under early promoter control, due to the relative abundance of nascent viral DNA templates and transcription factors following DNA replication (80). However, despite their lower intrinsic activity, early promoters have been used in recombinant FPV in order to elicit heterologous gene expression prior to virus-induced cytotoxic effects and the abortive replication process which occurs in mammalian cells (80). Indeed, human dendritic cells (DCs) infected with FPV expressing a lacZ marker gene show no evidence of expression of the transgene when under control of late viral promoters, indicating that FPV-encoded gene expression arrests at an early stage (19). More recently, mutagenesis studies have identified minimal promoters capable of driving optimal levels of gene expression and these powerful synthetic early/late promoters have become highly favored (80). Of technical concern, it is important to note that within several hours post-infection, host protein synthesis is abrogated and early viral mRNA transcripts are terminated downstream of the termination sequence TTTTTNT (1). Therefore, when using early promoters in the construction of recombinant FPV, one must be sure that such early terminations sequences are not present within the heterologous gene. There is currently no indication that onerous insert size restrictions apply in FPV, allowing any number of heterologous genes to be included in the FPV vector. The inclusion of multiple heterologous genes, co-stimulatory molecules and cytokines necessitate stringent control of expression levels of the incorporated genes. Exquisite control is particularly important if immunomodulatory genes are to be included in the vaccine vector, as the timing and levels of their expression will impact on the type of immune response that is elicited.

*1.4.2.2. The use of prime boost strategies reduces anti-virus specific responses and enhances target antigen-specific humoral and cell-mediated immunity*

Strategies involving priming with DNA vaccines and boosting with recombinant poxviruses, in particular FPV, generates unparalleled levels of specific humoral and cell-mediated immunity (48). This use of unrelated vectors bearing the same antigenic determinants has proved effective at focusing the immune response on the common (hopefully protective)

pathogen-specific target antigen rather than on the scaffold antigens, which can be the case with traditional homologous boosting regimens (82). Indeed, prime-boosting has been shown to stimulate high levels of antigen-specific CTL with high lytic capacity that recognize targets expressing very low levels of specific antigen, and these CTL expand rapidly upon challenge with replicating virus vectors encoding the heterologous gene (83). It has been demonstrated that DNA appears to be most effective at priming immunity, greatly increasing the immunogenicity of a recombinant poxvirus-based vaccine resulting in a greater immune response with partial protection (72, 84, 85). In particular, DNA priming broadens the CTL response and significantly increases antigen-specific $CD4^+$ T helper cells (86). The recombinant poxvirus booster immunization amplifies this response, both by the expression of higher levels of recombinant antigen and the adjuvant activity of the poxvirus itself (72). A wide array of *in vivo* prime boost studies have been performed, comparing the immune responses elicited when using combinations of DNA, FPV, MVA and VV and combinations of FPV and MVA show the greatest promise due to their safety profiles as well as ability to efficiently induce the highest numbers of CTL (87, 88). Prime boost strategies that include recombinant avipoxvirus vectors have produced promising results in animal models for a range of diseases including HIV/SIV (84, 87, 89), *Plasmodium berghei* (88) and cancer (90) Further refinements of prime-boost regimens should help to identify and predict the optimal combinations of the types of vehicles for antigen delivery.

*1.4.2.3. Harnessing the innate arm of the immune system to modulate vaccine responses*

Understanding the mechanisms underlying the innate and adaptive immune response to a pathogen is essential in order to undertake a rational design approach to develop a vaccine capable of providing protective immunity to subsequent pathogenic challenge. In a remarkably insightful article, Janeway argued that components of the innate immune system, specifically DCs, require the microbial stimuli often used in empirically-developed vaccine concoctions to become activated and acquire the capacity to induce productive responses from antigen-specific lymphocytes (91). He proposed that evolutionarily conserved features of infectious organisms, termed pathogen-associated molecular patterns (PAMPs), are detected by the immune system through a set of pattern recognition receptors (PRRs) which induce subsequent production of anti-viral cytokines such as type I interferons (92). Type I

IFNs induce the expression of hundreds of IFN-stimulated genes that have direct anti-viral actions and modulate adaptive immunity by enhancing natural killer (NK) cell function, activating immature DCs, and priming the survival and effector functions of T and B cells (93). The profound influence of PRRs on the strength, duration and quality of adaptive immune responses has made the parameters of innate immunity a central theme in rational vaccine design (94).

The first family of PRRs studied in detail was the Toll-like receptor (TLR) family. The members of the TLR superfamily are key players that mediate responses to the presence of non-self products from host molecules (95). One of the central features of the TLR signaling system is that they link microbial recognition to the activation of the specialized cells involved in T cell activation and initiation of adaptive immunity, namely DCs (96). The selective drive of adaptive immune responses, resulting from DC exposure to different TLR-mediated signals, provides a mechanistic framework linking the recognition of the type of invading organism to the class of antigen-specific response that ensues (97). For example, DCs triggered via TLRs 3,4 and 7-9 yield both IL-12 p70, which is the bioactive form of IL-12 and induce strong Th1 and CTL responses (98). In contrast, DCs triggered via TLR2 heterodimers (TLR2/1 or TLR2/6) produce relatively little IL-12 p70, but abundant IL-10, and can shift the balance towards the Th2 and a T-regulatory spectrum (3). Given that triggering DCs through different TLRs results in this induction of distinct responses, then theoretically, different TLR ligands could be administered in vaccine formulations to selectively skew the immune response towards appropriate protective immunity. The administration of TLR7/8 agonists and TLR9 ligands as adjuvants with the HIV Gag protein in non-human primates (NHPs) significantly increases antibody titre and the number of Th1 cells, compared with animals immunized with HIV Gag protein alone (99, 100). Accordingly, the specificity of different TLRs for pathogens and their ability to selectively drive adaptive immune response via differential DC activation makes TLRs and their ligands attractive candidates for rational vaccine design.

Recently, three other families of PRRs have been described: the C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and the retinoic acid-inducible gene (RIG)-like

receptors (RIRs). The membrane-associated CLRs recognize glycan structures expressed by host cells of the immune system or on specific tissues, which upon recognition allow cellular interactions between DCs and other immune or tissue cells (101). CLRs act as PRRs via the recognition of carbohydrate structures present on pathogens or pathogenic structures, which upon binding, internalize antigen for presentation onto MHC class I or II molecules for presentation to T cells (102). In addition, CLRs can function as signaling molecules that trigger specific cytokines. In the presence of TLR signaling, co-stimulatory molecules are up-regulated, and antigen uptake and presentation through CLRs can initiate immunity through T cell stimulation (Th1, Th2 or Th17). In the absence of any danger signaling and co-stimulation uptake of antigen through CLRs leads to presentation to T cells and the development tolerance through regulatory T cells ($T_{reg}$) (102). While TLRs detect ligands exposed either in the extracellular milieu or in the lumen of endocytic vesicles, NLRs are intracellular PRRs that survey the cytoplasm for signs that broadcast the presence of intracellular invaders (103). While the biological significance of most of the NLRs remains to be determined, the two most characterized family members, NOD1 and NOD2, have been shown to detect bacterial peptidoglycan, and drive activation of mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF- κB) (104). RIG-like receptors (RLRs), of which RIG-I is the prototypical member, have been shown to recognize viral RNA as non-self PAMPs (105). Unlike TLRs, which are found either on the cell surface or within membrane-bound vesicles, RLRs are found in the cytoplasm where cellular RNA is also present. Similar to TLR signaling, NLR and RLR signaling activate IFN regulatory factors (IRFs) and nuclear factor κB (NF- κB), resulting in the expression of type I IFN and pro-inflammatory cytokines. While we are only just beginning to understand the basic principles surrounding PRRs, it is clear that the interplay among innate signals and shaping of the adaptive responses is the consorted action of many players that influence each other. The ultimate outcome is dictated by the signatures present on the pathogens that determine which set of innate receptors are involved in shaping the immune repertoire of the DC. Understanding the processes of PAMP ligand recognition within each microbial niche will provide a foundation for the design of appropriate vaccines, adjuvants, and immunotherapies.

*1.4.2.4. The deletion of host range genes to enhance safety and immunogenicity*

Ironically, the emerging role of TLRs in the host response to viral infection is strongly supported by the targeting of TLR signaling by poxvirus proteins in order to usurp anti-viral defences. Two proteins encoded by VV, A46R and A52R, are antagonists of host IL-1 and TLR signaling (106). Multiple pathways activated by IL-1R/TLR are blocked, increasing the range of signaling pathways that VV can inhibit (107). Accordingly, immunomodulatory genes encoded by poxviruses can be viewed as a double-edged sword with respect to the design of new vaccines. Whilst the inclusion of some such as the IFN-γ binding proteins may be beneficial to a vaccine vector due to reduced anti-viral responses, the inclusion of others such as the TLR antagonists may prove detrimental as a result of their ability to downregulate innate recognition of the virus and subsequent activation of DCs. To date, no TLR antagonists have been identified in FPV. Although they are important virulence factors, poxviral immunomodulatory genes aren't usually essential for viral replication. In these cases, it may be possible to selectively delete such undesirable genes to further customize poxviral vectors to enhance vaccine potency. This has been illustrated clinically with the use of a highly attenuated strain of VV, NYVAC. The NYVAC strain was derived by deleting 18 open reading frames (ORFs) from the Copenhagen strain of VV and is highly debilitated in its *in vitro* replicative capacity of a number of mammalian cells including those of mouse and human origin (108). Importantly, NYVAC's highly attenuated phenotype is evident in a range of animal systems used to assess the virulence of vaccinia strains and serves as an example that the deletion of virulence and host range genes can increase the safety and immunogenicity of poxvirus vectors (108).

*1.4.2.5. Targeting antigen presentation pathways to enhance host immune responses to the vaccine*

Induction of primary $CD8^+$ T cell responses require that viral peptides are presented by MHC class I molecules on professional APCs (pAPCs), particularly DCs. CTL priming is also dependent on CD4 T helper cells, which activate APCs via CD40-CD40 ligand (L) interactions (109). Peptides can be generated from viral proteins synthesized by infected pAPCs themselves (direct presentation) or from proteins originally synthesized by other infected cells, and subsequently presented by pAPCs (cross-presentation) (110). Cross-

presentation in viral infection involves phagocytosis of the remnants of infected cells by DCs and through pathways not clearly understood, viral peptides are eventually expressed in the context of MHC class I molecules on the surface of these pAPCs (111). Potential mechanisms proposed for antigen access to the cross-presentation pathway include the uptake of apoptotic cells, antigen transfer via heat shock proteins, nibbling of live cellular material and exosome uptake (112). Another possible mechanism involves the transfer of peptides through gap junctions (113). This offers a way for extending the cell-mediated destruction of infected tissue to immediately surrounding cells (114). Although the exact mechanism of antigen cross-presentation remains unresolved, it presumably functions to prevent viruses from escaping immune recognition by avoiding the need for them to directly infect DCs in order to undergo T cell recognition (112).

It remains contentious as to whether cross-presentation has physiological significance or is merely an artifact that, under special conditions, can be exploited therapeutically to induce responses that would otherwise be difficult to generate (115), but regardless, it is clear that it is influenced by TLR signaling. *In vivo* maturation of DCs by systemic TLR ligand administration results in decreased DC phagocytosis and a failure to cross-present subsequently encountered cell-associated antigens (116). In this scenario, DCs are incapable of inducing CTL responses by cross-presentation but maintain full capacity for direct antigen presentation and the ability to induce T cell proliferation (117). These findings raise issues as to why DC activation is associated with diminished antigen cross-presentation. It has been suggested that diminished cross-presentation may allow DCs to focus on presenting antigens associated with the signal that initially triggered their activation (117). As described earlier, TLR ligands are already being included in vaccine formulations in order to induce more robust effector and memory responses. The downregulation of cross-presentation by DCs following systemic TLR ligand administration raises some concerns regarding their use as adjuvants and also the use of live viral vectors, given that preactivation of DCs with TLR ligands substantially impairs the cross-presentation of viral antigens. Therefore, viruses that do not infect DCs and rely heavily on the ability of DCs to cross-present viral antigens and prime CTL will generate significantly impaired anti-viral immunity (117). As a result, the optimal use of TLR ligands in vaccines will require more thought than simply adding them to

the formulations. Indeed, it may be that they should be introduced to the DCs only after a suitable interval following antigen exposure (116).

The use of viral vector vaccines such as FPV therefore raises the key question of whether they target pAPCs and thus the direct presentation pathway, or whether cross presentation pathway is important for immune recognition. To date, the roles of direct and cross presentation of FPV and antigens encoded by recombinant variants of FPV following immunization have not been resolved. Studies with VV have demonstrated that virally infected non-haematopoietic cells are unable to stimulate CTL-mediated immunity and that bone marrow-derived cells are required as APCs to initiate anti-viral CTL responses (118). Furthermore, it is the alternative exogenous MHC class I pathway that is the mechanism for the initiation of virus-specific CTL responses in these APCs (118). Other work has shown that the direct and cross presentation pathways contribute differentially to the induction of VV-specific CD8 T cells depending on the route of immunization (119) although a significant proportion of infected cells appear to be DCs (120). *In vitro,* FPV has been shown to infect human DCs that have been cultured *ex vivo* from peripheral blood monocytes (19). Under the control of a VV early/late promoter, greater that 80% of DCs expressed the heterologous FPV-encoded gene and the level of expression continued to rise post-infection until the termination of the experiment at 5 days (19). However, FPV has also been shown to infect other mammalian cell types *in vitro* such as African green monkey kidney (Vero), monkey fibroblast (CV-1) and human embryonic lung (MRC-5) cells (23). Similar to VV, it is probable that both mechanisms are involved, possibly with one predominating over the other depending on the level of antigen expression, cell type specificity of the vector and other defined factors relating to the nature of the antigen (112). Since the efficiency of antigen presentation influences the number of memory CD8 T cells following immunization with viruses, identifying which presentation pathway dominates is clearly essential (121, 122). Defining these factors, deciphering approaches that can measure the contribution of both pathways and subsequently designing immunization strategies targeting the optimal presentation pathway will be important steps for successful vaccine design.

*1.4.2.6. Co-expression technologies can be used to enhance vaccine immunogenicity and efficacy*

Cytokine co-expression by recombinant FPV vector vaccines has the potential to provide immunomodulatory activity to enhance vaccine potency (123). More complex vaccine design strategies are likely to require the simultaneous expression of different heterologous genes to manipulate the microenvironment to favour the development of appropriate protective immune responses (124). One of the earliest and most extensively studied co-expression systems used to improve vaccine efficacy and antigen-specific T cells responses is the FPV-TRICOM vaccine. The molecules expressed in the FPV-TRICOM vector (B7-1, ICAM-1 and LFA-3) are normally found on the surface of antigen presenting cells (APCs) and mediate the activation of signal transduction pathways that lead to T cell cytokine production and activation (125). Peptide pulsed, FPV-TRICOM-infected human DCs have been shown to be extremely effective at activating T cells to relatively weak immunogens such as carcinoembryonic antigen (CEA) and PSA *in vitro* (125). *In vivo*, intratumoral injection of FPV-TRICOM in mouse models of mesothelioma elicit a systemic anti-tumor immune response that results in prolonged survival and in some cases complete tumour regression (126). Interestingly, it has been observed that T cell activation and antigen-specific immunity are enhanced only when all three co-stimulatory molecules are expressed as compared to when any one or two of these co-stimulatory molecules are expressed individually (127-129).

However, the turn taken by the 2006 phase I clinical trial of the monoclonal superagonist targeting CD28 (TGN1412) which rapidly caused a life-threatening "cytokine storm" in all six healthy volunteers suggests that extra precaution be taken when using new therapies designed to stimulate rather than dampen functionality of the immune system (130). The CD28 co-receptor is constitutively expressed on almost all mouse, human CD4 and 50% of human CD8 T cells (131). The B7-1 molecule included in the TRICOM vaccines is a ligand for CD28, which in turn strongly co-stimulates T cell receptor (TCR)-mediated cell signaling, enhancing overall T cell activation. Subsequent research has revealed that the reason why the massive systemic inflammatory response that occurred in the participants of the trial wasn't reflected in animal studies (using Cynomolgus macaques). While TGN1412 is a superagonist in humans, it is not in Cynomolgus macaques, although

it is not known which T cell subsets, if any, are in involved in regulating the different responses of human and macaque T lymphocytes (132). In addition the *in vitro* protocols used before the study failed to predict toxicity *in vivo* because they did not present the Ab to white blood cells in a manner that mimicked its presentation in vivo, which when rectified did indeed stimulate the striking release of cytokines and profound lymphocyte proliferation that occurred *in vivo* in humans (132). Regardless of the exact reasons as to why this occurred, the TGN1412 human trial was launched hastily, barely a year after the structure of the CD28 receptor was deduced, and its outcome has been a valuable lesson. If co-stimulation is to be included in vaccine formulations and immunotherapies, a detailed understanding of the signaling pathways involved, the cell types stimulated and all possible physiological outcomes are essential. Although the non-replicative nature of FPV in mammalian cells limits the distribution of FPV-infected cells and the co-stimulatory molecules and cytokines encoded by FPV such as B7-1 do not circumvent TCR binding and widespread T cell activation, a detailed understanding of the pathways and cell types involved and caution are still required.

*1.4.2.7. Cytokine co-expression modulates the immune response to the vaccine*

The generation of protective immunity is dependent on the induction of specific immune responses and the type required is going to be pathogen-dependent. For example, defence against infectious intracellular micro-organisms such as viruses and some types of bacteria and protozoans tends to be dominated by cell-mediated forms of immunity characterised by cellular cytolytic activity and the production of cytokines including IFN-γ (133). In contrast, resistance to extracellular pathogens, for example helminths, is often associated with humoral responses in which high levels of pathogen-specific antibodies are generated in an attempt to neutralise the foreign organism (133). Co-expression of cytokines by recombinant FPV vector vaccines can dramatically modulate resulting immunity towards the type that is required for long-term immunogenicity and protective efficacy (69). For instance, an FPV vector expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) as an immune adjuvant vaccine administered in conjunction with an FPV-LacZ recombinant induced strong CTL responses directed against β-gal, the lacZ gene product (134). Granulocyte-macrophage colony-stimulating factor is a potent immune stimulant, inducing the proliferation and maturation of pAPCs (professional APCs). In contrast, although a DNA prime rFPV boosting regimen targeting HIV stimulates high levels of HIV-specific Th1 cells in macaques which persist for up to 9 months (69), the co-expression of IFN-γ or IL-12 by the rFPV boost results in significantly lower T cell immunity and a nearly complete loss of protective efficacy (70, 135). The negative immunomodulatory effect seen with co-expression of either IFN-γ or IL-12 may have been due to an enhanced elimination of the FPV vector by FPV-specific T cells, resulting in decreased FPV and HIV antigen expression and consequently poor immunogenicity (135). One of the most profound effects of cytokine co-expression in poxviral vectors has been found to occur with interleukin-4 (IL-4). Co-expression of murine IL-4 in recombinant Ectromelia virus (EV), or mousepox, renders it lethal in normally resistant strains of mice (136). Recombinant viral expression of IL-4 had previously been shown to delay viral clearance presumably through immune deviation from a Th type 1 to type 2 responses and overall modulation of CTL activity (137, 138). All of these findings underscore the importance of establishing the ideal combination of vaccine and adjuvant to obtain the most potent host immune response to the vaccine. The effect of ectopically expressed IL-4 will be discussed in more detail below in section 1.5.

## 1.5 THE EFFECT OF ECTOPICALLY EXPRESSED IL-4 ON THE HOST IMMUNE RESPONSE TO POXVIRUSES

In 1986, TR Mosmann and RL Coffman provided initial proof at a clonal level that antigen-specific $CD4^+$ T helper (Th) cells could be divided into two major subsets, Th1 and Th2, based strictly upon secretion patterns of cytokines (139). In general, Th1 cells produce IFN-γ and IL-2 and favour cell-mediated immunity (CMI) and the eradication of intracellular pathogens such as viruses. Th2 cells produce interleukins -4, -5, and -13 and thus contribute to the elimination of extracellular pathogens as well as provide B cell help to develop humoral immunity (140, 141). During Th differentiation, one set of genes is epigenetically activated and the other is silenced: Th1 cells transcribe *Ifng* and silence the *Il4* locus, whereas Th2 cells exhibit the converse pattern of activation and silencing (142). In addition, cytokine secretion from one Th subset negatively regulates the other, reinforcing epigenetic effects on the maintenance of Th1 or Th2 gene expression programs (143-145). Cross regulation by IL-4 and IFN-γ provides balanced and limited immune responses, which may be perturbed during disease states as Th1 and Th2 cytokines come to dominate. Therefore, resistance or susceptibility to pathogens is strongly determined by the balance of Th1 and Th2 cytokines during infection.

### *1.5.1. IL-4 expression by recombinant viruses significantly diminishes virus-specific CTL responses and exacerbates disease*

The role of Th1 versus Th2 responses in poxvirus infection has been extensively studied in the mouse using VV where mice genetically deficient in type Th1 cytokines such as IL-2, IFN-γ and IL-12 are more susceptible to infection whereas a deficiency in Th2 cytokines like IL-4 or IL-5 has little effect (146). In contrast to studies in mice lacking cytokines, significant attention has been given to the introduction of cytokines into the viral genome and examining the effect on concurrent primary infection. Studies of recombinant viruses engineered to express IL-4 including EV (136, 147), VV (148-151), respiratory syncytial virus (RSV) (147, 152-156) or influenza (157) demonstrate that IL-4 diminishes virus-specific CTL activity *in vivo* in association with a reduced number of IFN-γ-producing $CD8^+$

T cells. Furthermore, there was weak production of Th1 cytokines such as IL-12 and IFN-γ and an increase in the proportion of virus-specific IgG1 antibodies, indicating that the diminished cytolytic response is presumably through immune deviation from a Th type 1 to type 2 response as well as overall modulation of CTL activity. Consequently, mice infected with these viruses had delayed viral clearance and exacerbated disease. Most dramatically, C57BL/6 mice genetically resistant to EV were lethally susceptible to IL-4 expressing EV as a result of uncontrolled viral replication (158).

### *1.5.2. IL-4 diminishes perforin-mediated and increases Fas ligand-mediated cytotoxicity*

The down-regulation of CTL activity by IL-4-expressing viruses and elucidating the mechanism(s) involved has received significant attention as a result of its potential to create "killer viruses" with biological warfare applications. *In vitro* clonal culture systems have demonstrated that exposure to IL-4 during primary T cell activation induces a subpopulation of $CD8^+$ T cells that are non-cytolytic and express low levels of CD8 ($CD8^{low}$), perforin and granzyme (159-161). In addition, the IL-4-dependent down-regulation of CD8 expression was not a transient response to activation but was acquired during progressive differentiation and commitment to produce effector cells with a stable, poorly cytolytic $CD8^{low}$ phenotype, as CD8 expression on long term clonal and polyclonal cultures of $CD8^{low}$ or $CD8^{high}$ cells was not subsequently influenced by exogenous IL-4 or anti-IL-4 Ab (161).

That IL-4 down-regulates the expression of perforin and granzymes by $CD8^+$ T cells *in vitro* is also observed *in vivo*. Infection with poxviruses of mice with genetic disruption of components of cellular cytotoxicity reveal that IL-4 switches the cytolytic mechanism of CTL from the $Ca^{2+}$-dependent perforin/granzyme-mediated pathway to the $Ca^{2+}$-independent Fas (CD95)/ Fas ligand (CD95L) mediated pathway (162). The perforin/granzyme exocytosis pathway is important for the elimination of virus-infected and tumour cells, whereas Fas/FasL-mediated killing is important for the elimination of autoreactive T cells, although some studies suggest that in certain virus infections both pathways are operative (163-165). Mice infected with IL-4-expressing VV have an increase in the expression of FasL on $CD4^+$ and $CD8^+$ T cells and lyse $Fas^+$ cells with a 10-fold greater magnitude than mice infected with wild type VV (147). In addition to IL-4-mediated increased FasL expression on $CD8^+$ T

cells, poxviruses such as EV and VV also encode proteins related to the serpin family of proteinase inhibitors, such as the cytokine response modifier (crmA) of cowpox virus (CPV), that prevent target cell apoptosis mediated largely by the Fas/FasL pathway (166-169). The ability of poxviruses such as EV to block the Fas pathway of cytotoxicity provides an explanation for the observed increase in virulence seen with recombinant EV expressing IL-4. The fact that IL-4 expression during CTL induction preferentially activates the Fas mediated pathway, blocked by EV infection, and down regulates the perforin-dependent exocytosis pathway of cytotoxicity, may account for the increased virulence of EV encoding IL-4 (162). The observation that RSV infection can increase Fas expression on epithelial cells further supports the potential role for Fas/FasL interactions in mediating viral clearance. *In vivo*, FasL may function to de-escalate the immune response but if IL-4 production is high then increased FasL expression on $CD8^+$ T cells may result in increased bystander lysis to enhance immunopathology and disease severity (137).

An overview of some of the effects of ectopically expressed IL-4 on the host immune response are summarised in Figure 1.3 (170).

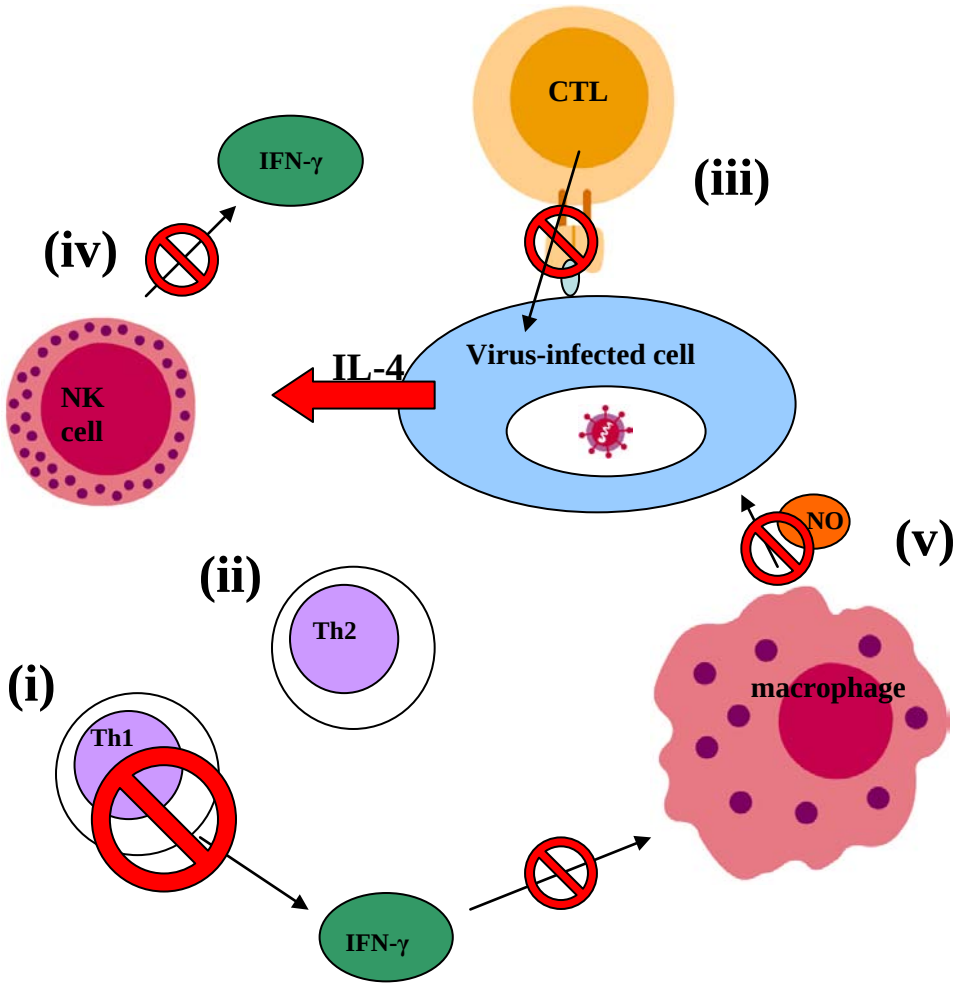


**Figure 1.3 The effect of ectopically expressed IL-4 on the host immune response.**

Poxviruses encode many proteins that modulate the host immune response. The introduction of IL-4 into the poxvirus genome (red arrow) adds another layer of complexity to host immune modulation (red circles with a line through them). IL-4 inhibits the development of Th1 cells (i), resulting in a Th2-dominated immune response (ii) and inhibiting the development of virus-specific CTL effector responses (iii). In addition, there is suppression of IFN-γ production by NK cells (iv) and a decrease in the cytotoxic activity of macrophages (v).

While the expression of IL-4 in the context of a poxviral infection, in which the virus itself encodes a variety of its own repertoire of gene products that significantly inhibit the host immune response, should be a selective advantage for the virus many key questions remained unanswered. These include whether IL-4 expression has effects on other components of the immune system or contributes to an immunopathology separate from inhibiting anti-viral CTL responses. This issue merits further attention because the most robust defense against

the possible exploitation of recombinant viruses for malicious purposes is further research into the basic mechanisms of how viral pathology can be manipulated, and, hopefully, interdicted.

## 1.6 RATIONALE AND OBJECTIVES

Interest in FPV has increased enormously in recent years due to its potential as a vaccine vector. In addition, technological advances have facilitated a better understanding of the mechanisms of immune protection from infection and enabled a more rational approach to vaccine design. Customised vaccines incorporating features such as heterologous genes under tight promoter control and expression of immunomodulatory proteins, all in conjunction with effective prime boost regimes, are resulting in the generation of vaccines and vaccination strategies that elicit powerful cell-mediated and humoral immunity. Fowlpox virus is an attractive candidate for such regimes due to its large genome, which allows for the inclusion of these features, coupled with an inability to replicate in mammalian cells. The potential of recombinant FPV vector vaccines is reflected by the number of clinical trials for diseases including HIV, malaria and a number of different types of cancer. Despite their promise, intricate details regarding FPV and how it interacts with the host immune system including antigen presentation pathways, vector-specific antigenic epitopes, host-specific immunomodulatory proteins and route and dose of infection have not been resolved. These are all areas of exploration not only for the development of future vaccines based on FPV, but to also gain a better understanding of how our best empirically-designed vaccines work.

The aim of this project is to construct a series of rFPV vectors that express the nominal antigen chicken ovalbumin (OVA), ($FPV_{OVA}$), and/or murine interleukin-4 (mIL-4) for the characterization of the relationship between FPV and the mammalian immune system, and how this is altered by the co-expression of mIL-4. This should give insight into how manipulating the formation of the immune response within the context of poxvirus infection can help tip the balance in the favor of either host or pathogen.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 MATERIALS

### *2.1.1 Mice*

All animal studies were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Pathogen-free, 6-8 week old male C57BL/6 (H-2K$^b$) mice were purchased from the University of Adelaide Laboratory Animal Services Division (SA, Australia). TCR transgenic C57BL/6 OT-I mice (171) respond to the OVA peptide SIINFEKL (residues 257-264) when presented in the context of H-2K$^b$ MHC class I and were provided by Dr W Heath (Walter Eliza Hall Institute (WEHI), Vic, Australia). TCR transgenic C57BL/6 OT-II mice respond to the OVA peptide $OVA_{323-339}$ when presented in the context of MHC class II and were purchased from the Animal Resource Centre (ARC) (WA, Australia). C57BL/6$^{bm1}$ mice were purchased from the Animal Resource Centre (ARC) (WA, Australia) and have a mutation that involves the substitution of three amino acids in one of the alpha helixes of the class I H-2Kb molecule, resulting in an inability to present the OVA peptide SIINFEKL in the context of MHC class I.

### *2.1.2 Antibodies and reagents*

Synthetic chicken ovalbumin peptide $OVA_{257-264}$ (SIINFEKL) was synthesised on an ABI 431A peptide synthesiser using standard Fmoc chemistry. Whole chicken ovalbumin Grade V protein (Sigma-Aldrich, MO, USA) was purified to homogeneity by ion exchange chromatography to remove any associated peptides. Recombinant FPV proteins FPV058L, FPV120L, FPV140L and FPV168L were produced via cloning into the protein expression vector pTrcHisA (Invitrogen Life Technologies, CA, USA) and purified using $Ni^{2+}$ charged nitrilotriacetic acid agarose (NTA) by Erin Lousberg (Experimental Therapeutics Laboratory, Hanson Institute, SA, Australia).

Cell supernatant containing recombinant mIL-4 (3.3 U/ml) derived from Sf9 insect cells infected with murine IL-4 cDNA-encoding baculovirus (172) was a kind gift from Dr N. Kienzle (Queensland Institute of Medical Science (QIMR), Qld, Australia).

All antibodies used for direct immunofluorescent staining including PE-conjugated CD4 (clone RM4-5) and CD45.1 (clone A20) and PE-Cy5-conjugated CD8α (clone 53-6.7) were purchased from Pharmingen (BD Biosciences, CA, USA).

Tissue culture reagents including RPMI media, HEPES, penicillin/streptomycin solution, L-glutamine, trypsin, xanthine, hypoxanthine-aminopterin-thymidine (HAT), neutral red (NR) solution and mycophenolic acid were purchased from Sigma-Aldrich (MO, USA). Gentamicin and TrypLE select solutions were purchased from Gibco-Invitrogen (CA, USA).

## 2.2 GENERAL MOLECULAR BIOLOGY METHODS

### *2.2.1 Polymerase chain reaction (PCR)*

Polymerase chain reactions (PCR) were performed using ampliTaq Gold (Promega, WI, USA) and touchdown cycling parameters (see Figure 3.1), where the annealing temperature is sequentially reduced by 2°C to increase sensitivity and maximise the product yield.

94°C 1 min
60°C 1 min } 2X
72°C 1 min

94°C 1 min
58°C 1 min } 2X
72°C 1 min

94°C 1 min
56°C 1 min } 2X
72°C 1 min

94°C 1 min
54°C 1 min } 2X
72°C 1 min

94°C 1 min
52°C 1 min } 2X
72°C 1 min

94°C 1 min
50°C 1 min } 2X
72°C 1 min

94°C 1 min
48°C 1 min } 30X
72°C 1 min

**Figure 2.1 Touchdown PCR cycle parameters.**

Touchdown PCR cycles are more sensitive due to the gradual decrease of the annealing temperature, maximising the amount of product.

### *2.2.2 Agarose gel electrophoresis*

Electrophoresis was performed using specified volumes of DNA diluted in Orange G DNA loading buffer. A pre-stained 1 kb molecular weight marker (New England Biolabs, MA, USA) was always run in conjunction with DNA solutions. All samples were run on set 1% agarose gels containing 1 μg/ml ethidium bromide at 90 volts (v) in 1X Tris-acetate-EDTA (TAE) buffer. Gels were visualised under UV light using Alphaimager® equipment (Alpha Innotech, CA, USA).

### *2.2.3 Extraction of DNA from agarose*

This was performed using Qiagen's (Hilden, Germany) QIAquick Gel Extraction Kit as per the manufacturer's instructions. Briefly, DNA of interest was separated via agarose gel electrophoresis (Section 3.2.2.) and the bands of interest were excised using a razor blade under a hand-held ultra violet lamp. The excised gel slices were then weighed and incubated at 50°C in an appropriate volume of solubilisation and binding buffer until the agarose had dissolved completely. The DNA of interest was subsequently isolated and purified on provided spin columns and eluted with 50 µl of $dH_2O$.

### *2.2.4 DNA ligation*

A mixture was prepared in a final volume of 10 µl $dH_2O$ containing: 1 µl of 100 ng/µl vector DNA, 1, 3 or 7 µl of 100 ng/ µl insert DNA, 1 µl 10X ligation buffer and 1 µl of 3 Weiss units/µl T4 DNA Ligase (Promega, WI, USA). Reactions were incubated at 4°C O/N and stored at -20°C until further use.

### *2.2.5 Transformation of competent Escherichia coli (E.coli)*

Recombinant DNA constructs were transformed into the electrocompetent DH5α strain of *E.coli* by electrotransformation. Briefly, 40 µl of DH5α cells were incubated on ice with 1 µl of ligated DNA for 1 min, transferred to chilled 0.2 cm electroporation cuvettes and pulsed once at 2.5 kV in a BioRad MicroPulser® (Biorad, CA, USA). Immediately after electroporation, cuvettes were removed from the electroporation chamber and cells were gently resuspended in 1 ml of YENB medium. Cell suspensions were transferred to 1.5 ml eppendorf tubes, incubated at 37°C without shaking for 1 hr to allow synthesis of the plasmid-encoded ampicillin resistance protein and then 100 µl of the transformed cells were plated onto 100 µg/ml ampicillin agar plates with 100 µl YENB medium. In the case of pGEM-T constructs, the agar plates also contained 10mM IPTG and 20 mg/ml X-Gal for blue-white colour selection. All plates were incubated O/N at 37°C.

### *2.2.6 Restriction enzyme digestion*

For analytical restriction enzyme digestion, such as the screening of clones for the presence of the correct insert, mixtures were prepared in a final volume of 20 µl $dH_2O$ containing 5 µg

DNA, 1 µl of each restriction enzyme (1000 U/ml) and 2 µl of the compatible 10X restriction enzyme buffer. Digestions of crudely prepared plasmids using TENS buffer (see section 2.2.7) also included 1 µl RNase A to digest any RNA remaining from the crude plasmid prep. Reactions were incubated at 37°C for 1-2 hr and analysed via agarose gel electrophoresis (section 3.2.2.). For preparative restriction digestion, the final volume was increased to 100 µl $dH_2O$ containing 25 µg DNA and reactions were incubated at 37°C O/N.

### *2.2.7 Plasmid preparation*

Crude plasmid preparations for analytical restriction enzyme digestion were done using TENS buffer and 3M sodium acetate (NaOAc) pH 5.2. Briefly, 1 ml of O/N bacterial culture was centrifuged at 13,000 rpm for 1 min and the cell pellet was resuspended in 100 µl of supernatant. 300 µl of TENS buffer was added to the sample and mixed gently by inversion followed by the addition of 150 µl 3M NaOAc (pH 5.2) and centrifugation at 13,000 rpm for 5 min. The supernatant was removed to a fresh tube and 900 µl of chilled 100% EtOH was added. After being incubated on ice for 10 min, the samples were centrifuged for 10 min at 4°C and pellets were washed with 200 µl 70% EtOH and air-dried. Dried DNA pellets were resuspended in 20 µl restriction digestion mix.

Plasmids for sequencing, sub-cloning and transfections were purified on spin columns using Qiagen (Hilden, Germany) Mini and Maxiprep Kits as per the manufacturer's instructions. Optical density readings were measured via UV spectroscopy at absorbance 260 and 280 nanometres (nm) to calculate DNA purity, yield and concentration. Stock solutions at a concentration of 1µg/µl were prepared.

### *2.2.8 BigDye™ terminator sequencing*

DNA was sequenced by the Molecular Pathology Department of the Institute of Medical and Veterinary Sciences (IMVS) (SA, Australia) on an ABI 3700 sequencer (Applied Biosystems, CA, USA). Sequencing reactions were made to a final volume of 20 µl in $dH_2O$ and contained 4 µl of BigDye™ terminator mix version 3, 1 µg template DNA, 2 µl of 2.5 µM primers for the universal promoters T7 (forward) and SP6 (reverse) and 4 µl 5X sequencing buffer. Cycling parameters were 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min

repeated 25 times and then stored at 4°C. Reactions were precipitated with fresh 75% isopropanol. 80 µl of 75% isopropanol was added to the reactions which were then vortexed and incubated at RT for a minimum of 15 min. These were centrifuged at 13,000 rpm for 20 min, the supernatant was aspirated and the tubes were spun for another 2 min. Pellets were washed with 250 µl of 75% isopropanol, air-dried until all traces of isopropanol had been removed and submitted for sequencing.

## 2.3 CLONING AND EXPRESSION VECTORS

### *2.3.1 Bioinformatics tools*

The sequence of all cDNAs were obtained from the online National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). The software Gene Construction Kit® (version 2.5 by Textco BioSoftware, NH, USA) was used to draw plasmid maps and detect restriction sites on DNA stretches. The Angis BioManager program (www.angis.org.au) was used for the alignment of protein sequences.

### *2.3.2 Construction of the pKG10a –OVA-IRES-EGFP plasmid*

The full-length, secreted form of chicken ovalbumin (OVA) was PCR amplified from the plasmid pBR322-OVA (kindly supplied by Prof P. Chambon, Institute for Genetics and Cellular and Molecular Biology, Strasbourg, France). However, the OVA sequence did not include the start codon and first 15 base pairs (bp) so these were included in the forward PCR primer. Primers also contained a FPV bidirectional early/late promoter, restriction digestion sites and a Kozak sequence. Restriction digestion sites included are: *Nhe*I and *Xho*I for insertion into the pGEM-T-IRES-EGFP vector, an upstream *Xba*I site for insertion of the OVA-IRES-EGFP sequence into the mammalian expression vector pcDNA3.1(-)/Hygro (Invitrogen Life Technologies, CA, USA) and a *Bgl*II site for its insertion into the FPV shuttle vector pKG10a (a kind gift from Dr D. Boyle, CSIRO, Australia).

The OVA primer sequences are as follows:

**Forward primer:**

**5'**- TCG **G▼CT AGC** *TCT AG▼A* A▼GA TCT **ATT TAG TAT CCT AAA ATT**
***Nhe*I** *XbaI* *Bgl*II **FPV early/late bidirectional**

**GAA TTG TAA ATT GAA TTG TAA TTA TCG ATA ATA AAT** *GCC ACC*
**promoter** *Kozak*

**ATG** GCC TCC TCC ATC GGT GCA GCA AGC ATG GAA TTT TGT **-3'**
OVA start (OVA start in pBR322)

**Reverse primer:**

**5'**- AAT *CTC GA▼G* **TTA** AGG AGA AAC ACA TCT GCC – **3'**
*XhoI* OVA

The internal ribosome entry site (IRES) from the encephalomyocarditis virus and the enhanced green fluorescent protein (EGFP) were PCR amplified from the pIRES-EGFP vector (Clontech, CA, USA). The IRES-EGFP primers contained a Kozak sequence, poxvirus T5NT transcription termination sequence and restriction digestion sites. Restriction digestion sites included are: *Nhe*I and *Xho*I for the insertion of OVA, a *Not*I site downstream of IRES-EGFP for insertion of the OVA-IRES-EGFP sequence into pcDNA3.1(-)/Hygro and a *Sal*I site for its insertion into pKG10a.

The IRES-EGFP primer sequences are as follows:

**Forward primer:**

**5'–** TCG *G ▼CT AGC* **C ▼TC GAG** TCC GCC CCT CTC CCT CCC CCC CCC CTA
*NheI* ***Xho*I** IRES

ACG – **3'**

**Reverse primer:**

5' – **AAT <u>GC ▼G GCC</u> GC*G TCG A ▼C*A CAA AAA <u>**TTA CTT GTA CAG CTC GTC**</u>
***<u>Not</u>*I** ***Sal*I** T5NT **EGFP**

**CAT –** 3'

An overview of the construction strategy for the pKG10a-OVA-IRES-EGFP plasmid is illustrated in Figure 2.2.

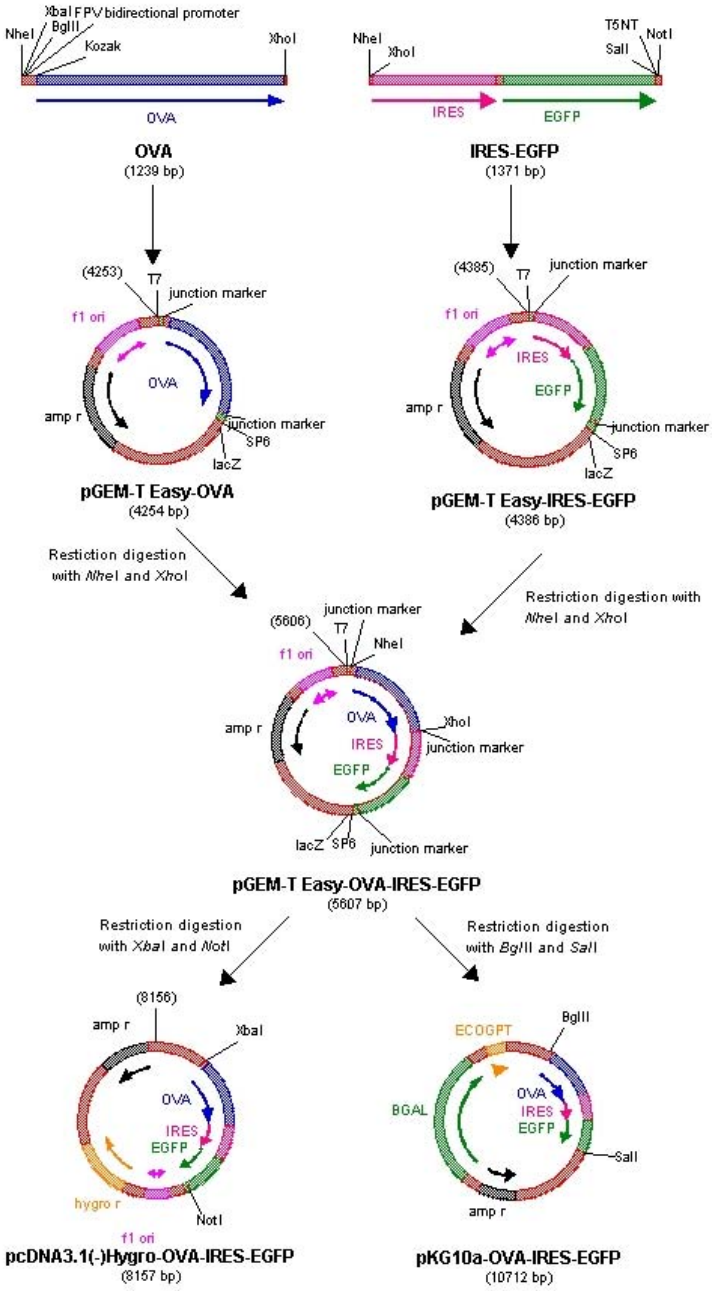


**Figure 2.2 Cloning strategy for the construction of the pKG10a-OVA-IRES-EGFP shuttle vector.**

OVA and IRES-EGFP were PCR amplified to contain the correct restriction sites as well as a FPV bidirectional promoter, Kozak sequence and a poxviral T5NT transcription termination sequence. The OVA gene was cloned directly upstream from IRES-EGFP in the pGEM-T Easy vector and the OVA-IRES-EGFP cassette was then cloned into the pcDNA3.1(-)/hygro vector to test for *in vitro* expression and the FPV shuttle vector pKG10a for insertion into the F7-F9 region of the FPV genome.

PCR was done using touchdown cycling parameters as described in section 2.2.1 and products were purified via 1% agarose gel electrophoresis (described in section 2.2.2) and extracted using Qiagen's (Hilden, Germany) QIAquick Gel Extraction Kit (described in section 2.2.3). The OVA and IRES-EGFP inserts were cloned into the eukaryotic expression vector pGEM-T Easy (Promega, WI, USA) for sequence verification using T4 DNA ligase (described in section 2.2.4). pGEM-T-OVA and pGEM-T-IRES-EGFP were transformed into the electrocompetent DH5α strain of *E.coli* as described previously in section 2.2.5 and transformed cells were plated onto agar plates containing 100 μg/ml ampicillin, 10mM IPTG and 20 mg/ml X-Gal for blue-white colour selection and incubated O/N at 37°C. White clones were believed to be positive for the OVA or IRES-EGFP inserts due to the disruption of the *lacZ* gene. *LacZ* gene expression is inducible by IPTG and the product of the *lacZ* gene, β-galactosidase, forms a blue precipitate with X-Gal, making insert-negative clones easily identifiable.

Individual white clones were sub-cultured in 10 ml of 2X YT medium containing 100 μg/ml ampicillin and incubated O/N at 37°C with shaking at 200 rpm. Crude plasmid preparations were made using TENS buffer (see section 2.2.7) and clones were screened for the correct sized inserts via analytical restriction enzyme digestion with *Eco*RI as described previously in section 2.2.6. *Eco*RI, which does not cut either OVA or IRES-EGFP, cuts the pGEM-T vector in the multiple cloning site (MCS) on either side of the cloned insert. Positive clones were then purified on spin columns and sequenced using primers for the T7 and SP6 promoters and BigDye$^{TM}$ terminator technology as described in section 2.2.8. Glycerol stocks consisting of 500 μl of O/N bacterial culture and 500 μl of 30% glycerol were made from the positive clones and stored at -70°C.

Following sequence verification, pGEM-T-OVA and pGEM-T-IRES-EGFP were amplified in bacterial culture and purified on maxiprep spin columns as described in section 2.2.7. OVA was then cloned directly upstream from IRES-EGFP in the pGEM-T Easy-IRES-EGFP construct by restriction digestion with *Nhe*I and *Xho*I. Ligation, transformation and restriction digestion reactions were done as described previously. Plasmid preparations and the screening for positive clones were done as described previously except that transformed

cells were plated onto agar plates only containing 100 μg/ml ampicillin as the blue-white colour selection had been lost. Positive clones were screened via *EcoR*I digestion and subsequently sequenced as described above. However, due to the size of the insert, an internal primer was included in the sequencing reaction in addition to T7 and SP6 in order to sequence the OVA-IRES junction. The internal OVA-IRES primer was designed to bind 50 bp upstream from the OVA-IRES junction and its sequence is as follows:

**Internal primer:**

**5'-** TTC CTC TTC TGT ATC AAG **– 3'**

Sequencing of the OVA-IRES-EGFP cassette revealed that the *Nhe*I and *Xba*I sites upstream of OVA were each missing a codon, and while the *Nhe*I restriction site was no longer required, the *Xba*I site was needed in order to be able to insert the OVA-IRES-EGFP cassette into the pcDNA3.1(-)/Hygro vector. Site-directed mutagenesis was used to insert the missing 6 base pairs (bp).

The QuickChange® Primer Design program (Stratagene, CA, USA) was used to design the mutagenesis primers and their sequences are as follows:

**Sense primer:**

**5'**-CGAATTCACTAGTGATTTCG **G▼CT *AGC TCT*** *AG▼A* A▼GA TCT

pGEM-T vector ***Nhe*I** *XbaI* *Bgl*II

ATTTAGTATCCTAAAA -**3'**

FPV early/late bidirectional promoter

**Anti-sense primer:**

**5'**- TTTTAGGATACTAAAT A▼GA TCT *TCT* ***AG▼A GCT*** **AGC** CGAAATCACT

*Bgl*II *XbaI* ***Nhe*I** pGEM-T vector

FPV early/late bidirectional promoter

AGTGAATTCG – **3'**

Location of the inserted sequence is in ***bold and italics***. Stratagene's (CA, USA) QuickChange II XL Site-Directed Mutagenesis Kit was used for site-directed mutagenesis as per the manufacturer's instructions. Briefly, mutant strand synthesis reactions containing 10 ng pGEM-T-OVA-IRES-EGFP, 125 ng of each of the sense and anti-sense primers, 1 µl dNTP mix, 1 µl *Pfu* Ultra high fidelity DNA polymerase and provided reaction buffers were set up and run through the cycling parameters outlined in Figure 2.3.

95 °C 1 min

95 °C 50 sec
60 °C 50 sec
68°C 1 min/kb plasmid = 6 min for pGEM-T-OVA-IRES-EGFP } 18X

68°C 7 min

**Figure 2.3 Site-directed mutagenesis cycling parameters.**

Cycling parameters used for the synthesis of mutant strands.

After mutant strand synthesis, the amplification products were chilled on ice for 2 min and digested with *Dpn*I at 37°C for 1 h in order to digest non-mutated DNA. Mutagenesis reactions were then transformed into ultra-competent XL-10 cells via hot-cold shock (ice 40 min, 42°C 30 sec, ice 2 min, 37°C 1 h), plated onto agar plates containing 100 µg/ml ampicillin and incubated at 37°C O/N. Colonies were subcultured and screened for the inserted sequence (AGC TCT) via BigDye$^{TM}$ terminator sequencing as described in section 2.2.8.

The complete OVA-IRES-EGFP cassette was cloned into the mammalian expression vector pcDNA3.1(-)/Hygro and FPV shuttle vector pKG10a by restriction digestion with the restriction endonucleases *Xba*I and *Not*I or *Bgl*II and *Sal*I, consecutively. All ligation, transformation and restriction digestion reactions were done as described previously. Plasmid preparations and the screening for positive clones were also done as described previously.

### *2.3.3 Construction of the pAFtd-mIL-4 plasmid*

Murine IL-4 (mIL-4) was PCR amplified from mouse cDNA that had been previously prepared from whole mouse blood. Primers contained the FPV bidirectional early/late promoter, a T5NT transcription termination sequence, restriction digestion sites and a Kozak sequence. Restriction digestion sites included are: *Bam*HI and *Xba*I for insertion into the mammalian expression vector pcDNA3.1(-)/Hygro (Invitrogen Life Technologies, CA, USA)

and *Hind*III and *Sal*I for insertion into the FPV shuttle vector pAFtd (a kind gift from Dr D. Boyle, CSIRO, Australia). The mIL-4 primer sequences are as follows:

**Forward primer:**

**5'**- TCG TCT AG▼A A ▼*AG CTT* **ATT TAG TAT CCT AAA ATT GAA TTG TAA**

*Xba*I *HindIII* **FPV early/late bidirectional promoter**

**TTA TCG ATA ATA AAT** *GCC ACC* | ATG GGT CTC AAC CCC CAG CTA **-3'**

*Kozak* mIL-4 start

**Reverse primer:**

**5'**- AAT *G ▼GA TCC* GTC GA▼C **ACA AAA A**CT | **CTA** CGA GTA ATC CAT TTG

*BamH I* Sal I **T5NT** mIL-4

CAT –**3'**

PCR was done using touchdown cycling parameters as described in section 2.2.1 and products were purified via 1% agarose gel electrophoresis (described in section 2.2.2) and extracted using Qiagen's (Hilden, Germany) QIAquick Gel Extraction Kit (described in section 2.2.3). mIL-4 was cloned into the eukaryotic expression vector pGEM-T Easy (Promega, WI, USA) for sequence verification and subsequently into the mammalian expression vector pcDNA3.1(-)/Hygro and FPV shuttle vector pAFtd. All ligation, transformation and restriction digestion reactions were done as described previously. Plasmid preparations and the screening for positive clones were also done as described previously.

An overview of the construction strategy for the pAFtd-mIL-4 plasmid is illustrated in Figure 2.4.

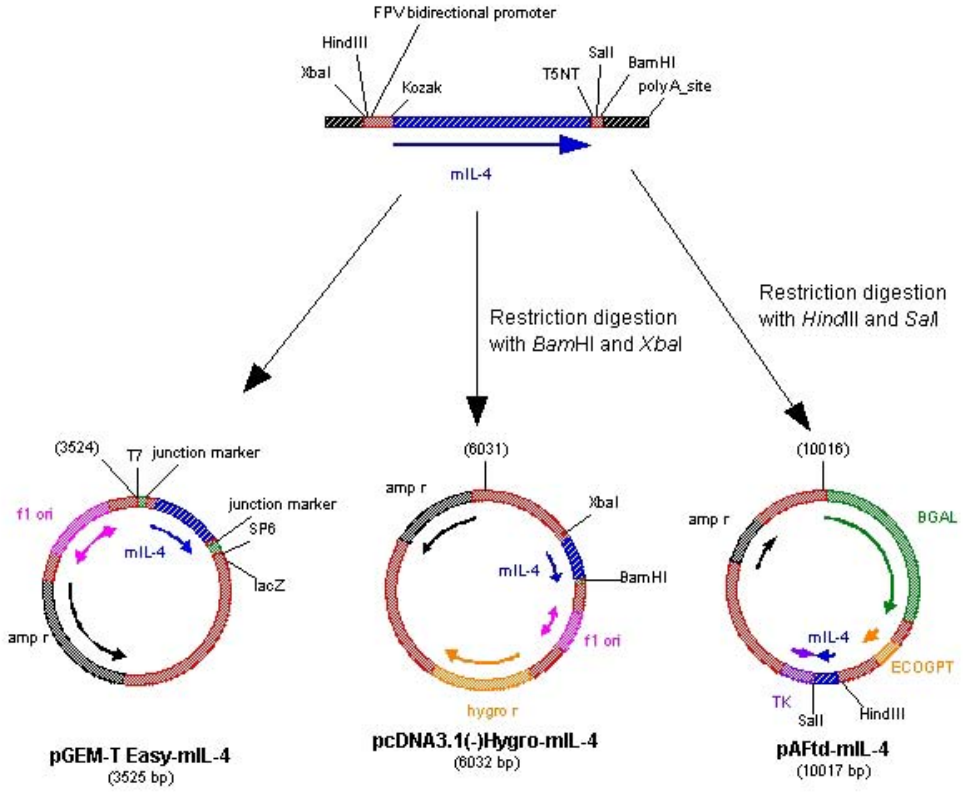


**Figure 2.4 Cloning strategy for the construction of the pAFtd-mIL-4 shuttle vector.**
mIL-4 was PCR amplified to contain the correct restriction sites as well as a FPV bidirectional promoter, Kozak sequence and T5NT termination sequence and cloned into the pGEM-T vector for sequence verification. mIL-4 was then cloned into the pcDNA3.1(-)/Hygro vector to test for *in vitro* expression in mammalian cells and the FPV shuttle vector pAFTD for insertion into the region between the thymidine kinase (TK) gene and the next open reading frame (ORF) of the FPV genome.

## 2.4 TISSUE CULTURE AND ANALYSIS

### *2.4.1 Cell lines*

Chicken embryonic fibroblast (CEF) cells were isolated from day 10 embryos of SPF eggs and used in the construction and propagation of the rFPVs. CEF cells were maintained in either growth medium (GM) (MEM medium (JRH Biosciences, KS, USA) supplemented with 5% foetal calf serum (FCS), 2 mM glutamine, 10 mM HEPES, 0.05 mg/ml gentamicin) or maintenance medium (MM) (same as for GM but 2% FCS). All other cell lines were maintained in complete RPMI 1640 medium (Sigma-Aldrich, MO, USA) supplemented with 10% FCS, 2 mM glutamine, 10 mM HEPES, $5x10^{-5}$ M 2-mercaptoethanol and 20 ml/L antibiotics (penicillin-streptomycin) at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Cell number and viability were determined by trypan blue exclusion and visualisation in a haemocytometer. Adherent cell lines were maintained as monolayer cultures and passaged at confluency with Trypsin-EDTA. The quail fibroblast carcinoma cell line QT35, which is permissive to FPV replication and propagation (173), was infected with the recombinant FPVs and used for the expression analysis of EGFP, OVA and mIL-4 by these recombinant viruses. The human embryonic kidney (HEK) 293 cell line was used for transfection and expression analysis of the recombinant pcDNA3.1(-)/Hygro constructs, as it has a 99% transfection efficiency. The murine cytotoxic T cell cell line CTLL-2 which is dependent on IL-2 for growth and proliferates sub-optimally to mIL-4 (174), was used in a proliferation assay to determine the biological activity of mIL-4. CTLL-2 cells were maintained in complete RPMI 1640 medium supplemented with 100 U/ml of recombinant human IL-2 (r huIL-2). The mouse lymphoma cell line EL-4 was used as target cells in the direct cytotoxicity assay. The HEK 293T cell line stably transfected to express the C57BL/6 class I MHC molecule Kb or Db, were a kind gift from Dr D. Tscharke (QIMR, Qld, Australia) and also used as target cells in the direct cytotoxicity assay.

### *2.4.2 Transient transfection of HEK 293 cells*

The cationic lipid formulation Lipofectamine™ 2000 reagent (Invitrogen Life Technologies, CA, USA) was used for all transient and stable transfections. HEK 293 cells were seeded at $5x10^4$/well in 24-well plates and incubated at 37ºC in 5% $CO_2$ for 24 hours, by which time

cells were 90-95% confluent (approximately $2x10^5$ cells) and ready for transfection. On the day of transfection, cells were washed and culture medium was replaced with 0.5 ml serum and antibiotics-free RPMI medium (1 M HEPES, 2 mM glutamine, $5x10^{-5}$ M 2-ME). For each well to be transfected, 2 µl of Lipofectamine™ 2000 reagent was diluted in 50 µl of serum and antibiotics-free RPMI medium and incubated at RT for 5 min. 0.8 µg of each DNA plasmid was diluted in 50 µl of serum and antibiotics-free RPMI medium, combined with the diluted Lipofectamine™ 2000 reagent and incubated for 20 min at RT to allow the DNA-Lipofectamine complexes to form. The 100 µl DNA-Lipofectamine complexes were then added drop-wise to the cells and incubated at 37ºC in 5% $CO_2$. After 48 hours, the supernatant from each well (600 µl) was removed and cell lysates were collected in 200 µl of NP40 lysis buffer. All samples were stored at -20ºC until needed.

### *2.4.3 CTLL-2 bioassay*

CTLL-2 cells were washed 3 times in complete RPMI medium and incubated at 37ºC in 5% $CO_2$ for 1.5 h so as to internalize all surface-bound IL-2. Cells were then plated at $5x10^4$/well in 50 µl ($10^6$/ml) in round-bottom 96-well plates and co-incubated with 1/5 dilutions of r huIL-2 or IL-4-containing supernatant from either HEK 293 cells transiently transfected with pcDNA3.1(-)/hygro-mIL-4 or Sf9 insect cells infected with murine IL-4 cDNA-encoding baculovirus (positive IL-4 control). r huIL-2 concentrations ranged from 100 U/ml to 0.005 U/ml and IL-4-containing supernatant dilutions ranged from 0 to 1/3125. All concentrations were done in triplicate. Cells were incubated at 37ºC in 5% $CO_2$ for 18 h and pulsed with 1 µCi/well of [$^3$H] thymidine for a further 4 hours. Incorporation of [$^3$H] thymidine was measured by harvesting the cells onto Filtermate™ glass fibre filters (PerkinElmer, MA, USA) and counting on a Topcount instrument (PerkinElmer, MA, USA).

## 2.5 PROTEIN ANALYSIS METHODS

### *2.5.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was performed using gels composed of 12% resolving and 4% stacking gels. Resolving gel containing 12% acrylamide in 0.1% SDS, 0.1% APS, 0.0005% TEMED and 1.5 M Tris pH 8.8 was poured and set. Stacking gel containing 4% acrylamide in 0.1% SDS, 0.1% APS, 0.0005% TEMED and 0.5 M Tris pH 6.8 was poured on top of the set resolving gel and a comb was inserted to form wells. Samples were prepared by diluting 50 µl of cell supernatants and lysates 1:2 in Laemmli sample buffer (reducing) and boiling at 100°C for 5 min. A final volume of 20 µl of the prepared samples was loaded onto the gel as well as pre-stained molecular weight markers (Invitrogen Life Technologies, CA, USA). Electrophoresis was carried out at 40 mAmps until the dye front reached the bottom of the gel.

### *2.5.2 Western blot analysis*

Nitrocellulose membranes were pre-soaked in methanol and rinsed with transfer buffer.
Following PAGE, a sandwich of the gel and soaked nitrocellulose membrane was constructed between layers of pre-soaked (transfer buffer) 3MM Wattman filter paper and sponges. Resolved proteins were transferred onto the nitrocellulose membrane at 350 mAmps for 1 hour with the transfer tank being connected to a water source to keep the tank and transfer buffer cool. The membranes were transferred to a blocking solution of 5% skim milk at RT and incubated on an orbital rotator for 30 min or O/N.

Blocked nitrocellulose membranes containing the resolved proteins were washed 3X with TTBS and OVA protein was detected with 10 µg/ml rabbit biotinylated anti-OVA IgG (Rockland, PA, USA) and 200 ng/ml streptavidin-alkaline phosphatase (Rockland, PA, USA). Both antibodies were diluted in 0.1% BSA/TTBS, incubated on an orbital rotator for 30 min and the membrane was subsequently washed 3X with TTBS. Bound antibody was detected via a chromogenic method with BCIP/NBT substrate (Sigma-Aldrich, MO, USA). Briefly, 3 ml of BCIP/NBT substrate was added to the surface of the membrane, incubated at RT for 5 min and washed extensively with water to stop colour development.

### *2.5.3 Enzyme-linked immunosorbent assay (ELISA)*

OVA and mIL-4 expression by rFPV-infected cells and transiently transfected HEK 293 cells were detected via ELISA.

To detect OVA, high protein binding ELISA plates (Costar, Jomar, SA, Australia) were coated with 2 μg/ml of goat IgG fraction to chicken egg albumin (Cappel, MP Pharmaceuticals, Jomar, SA, Australia). After blocking with PBS/1% BSA, dilutions of cell supernatants and lysates were added and incubated at 37°C for 2 h. Bound protein was detected by the addition of 2 μg/ml biotinylated rabbit anti-ovalbumin IgG (Rockland, PA, USA) and 50 ng/ml streptavidin-horse radish peroxidase (Rockland, PA, USA). The plates were developed with OPD substrate (Sigma-Aldrich, MO, USA) and the reaction was stopped after 20 min using 3 M HCL. Plates were read at 490 nm.

To detect mIL-4, a commercial ELISA kit was used according to the manufacturer's instructions (Quantikine®, R&D Systems, MN, USA). Briefly, dilutions of cell supernatants and lysates were added to the wells of provided mouse IL-4 microplates, which were pre-coated with a mIL-4 monoclonal antibody (mAb), and incubated at RT for 2 hours. Bound protein was detected with the provided mIL-4 conjugate and substrate and plates were read at 450 nm.

## 2.6 RECOMBINANT FPV CONSTRUCTION, PROPAGATION AND VACCINATION

### *2.6.1 Construction of the rFPVs by transdominant selection*

The rFPVs were constructed by Sonja Tingay (Virax Holdings Ltd, Vic, Australia) and Anastasia Yu (Experimental Therapeutics Laboratory, Hanson Institute, SA, Australia).

An overview of the steps involved in the construction of rFPVs is illustrated below in Figure 2.5.

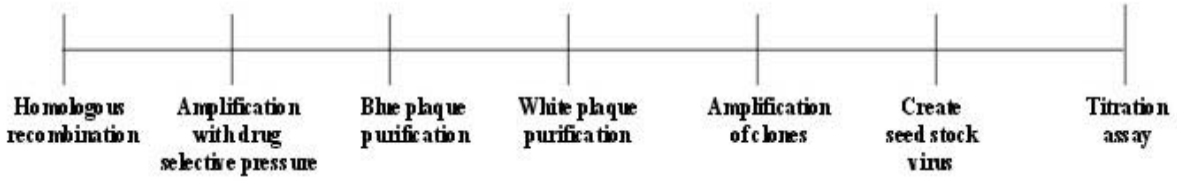


**Figure 2.5 The construction of rFPV by transdominant selection.**

*2.6.1.1 Preparation of primary chicken embryonic fibroblast (CEF) cells from SPF egg embryos*

Chicken embryonic fibroblast (CEF) cells were prepared from 10 day chicken embryos using TrypLE select solution. Eggs were positioned with the air space up and the top of the shell and membrane were removed with sterile forceps. Embryos were subsequently removed from eggs and rinsed with 5 ml PBS. Head and feet were then detached and the rest of the body (trunk) was placed into an additional 5 ml PBS. Trunks were minced by placing up to 10 in a 20 ml syringe and gently squeezing through the syringe into a sterile digestion flask.

Cells were dissociated with TrypLE select solution (5 ml per embryo) at RT for 30 min with stirring and fluid was passed through 2 stainless-steel 40 mesh sieves. The filtrate was centrifuged at 1000 g for 10 min at 20°C and harvested cells were washed in 10 ml PBS per embryo. Cells were then resuspended in 3 ml/embryo GM and ready for use. Primary CEF cells can be stored at 4°C for up to 7 days.

*2.6.1.2 Homologous recombination*

$5x10^6$ CEF cells were seeded in T25 flasks and incubated at 35°C/5% $CO_2$ until they were subconfluent (70-80%). $10^7$ pfu of wild type FPV M3 was incubated with 100 µl of 1 mg/ml trypsin at 37°C for 30 min and diluted in growth medium (GM) to yield a final concentration of $1x10^5$ pfu/ml, which gives a multiplicity of infection (MOI) of 0.01 pfu/cell. GM was then removed from the flask and 500 µl of diluted virus was added and incubated at 35°C/5% $CO_2$

for 1 h to allow the virus to be absorbed into the cells. Inoculum was removed from the cells and replaced with 4 ml GM, ready for immediate transfection.

Infected CEF cells were transfected with 2 μg of either of the rFPV shuttle vectors, pKG10a-OVA-IRES-EGF or pAFtd-mIL-4, using Qiagen's (Hilden, Germany) Effectene kit according to the manufacturer's instructions. The transfection mix was then added to the flask already containing the 4 ml of GM and incubated at 35°C/5% $CO_2$. Medium was changed to MM 2 days post-infection (p.i.). Once substantial cytopathic effects or plaquing within the monolayer could be seen (6 or 7 days p.i.), cells were harvested, pelleted at 1000g for 10 min, resuspended in 1 ml PBS and sonicated until an even homogenate could be seen. Homogenates were stored at -80°C until needed for amplification.

*2.6.1.3 Amplification with drug selective pressure*

$30x10^6$ primary CEF cells in 50 ml GM were seeded into T150 flasks and incubated at 35°C/5% $CO_2$ until the cell monolayer had reached 100% confluency. At 100% confluency, the medium was removed and replaced with 50 ml maintenance medium (MM) supplemented with 25 μg/ml mycophenolic acid (MPA), 250 μg/ml xanthine and 1X HAT (MM plus MXHAT) and incubated at 35°C/5% $CO_2$ O/N. On the day of infection, homologous recombination viruses from section 2.6.1.2 were thawed and trypsinated with 200 μl of 1 mg/ml trypsin at 37°C for 30 min to release viral particles from host cell membranes. 5 ml of MM plus MXHAT was then added to stop the reaction and the diluted trypsinated viruses were added to the flasks after removing MM and incubated at 35°C/5% $CO_2$ for 1 h. 50 ml of MM plus MXHAT was subsequently added to each flask which were incubated at 35°C/5% $CO_2$ for 5 to 7 days until plaquing or substantial cytopathic effects within the cell monolayer could be seen. Viral extracts were harvested via pelleting and sonication as described in section 2.6.1.2 and resuspended in 2 ml PBS.

The presence of rFPV in the amplified viral extract was analysed via PCR using primers for targeting the foreign sequence (OVA-IRES-EGFP or mIL-4) inserted into the FPV. Their sequences are the same as the initial PCR primers used to amplify the sequences from the original DNA plasmids for their insertion into the pKG10a and pAFtd shuttle vectors as

described in sections 2.3.2 and 2.3.3. Therefore, their sequences (non-annotated version) are as follows:

**For screening rFPV transfected with pKG10a-OVA-IRES-EGFP:**

*Forward primer (original OVA forward primer)*

**5'-** TCG GCT AGC TCT AGA AGA TCT ATT TAG TAT CCT AAA ATT GAA TTG TAA ATT GAA TTG TAA TTA TCG ATA ATA AAT GCC ACC ATG GCC TCC TCC ATC GGT GCA GCA AGC ATG GAA TTT TGT **-3'**

*Reverse primer (original IRES-EGFP reverse primer)*

5' – **AAT GCG GCC GCG TCG ACA CAA AAA TTA CTT GTA CAG CTC GTC CAT –** 3'

**For screening rFPV transfected with pAFtd-mIL-4:**

*Forward primer (original mIL-4 forward primer)*

**5'-** TCG TCT AGA AAG CTT ATT TAG TAT CCT AAA ATT GAA TTG TAA TTA TCG ATA ATA AAT GCC ACC ATG GGT CTC AAC CCC CAG CTA **-3'**

*Reverse primer (original mIL-4 reverse primer)*

**5'** -AAT GGA TCC GTC GAC ACA AAA ACT CTA CGA GTA ATC CAT TTG CAT –**3**'

DNA for PCR was extracted from 200 µl of viral homogenate using the Qiagen (Hilden, Germany) DNeasy tissue kit according to the manufacturer's instructions and PCR reactions were carried out for 10 to 15 cycles of amplification. The presence of the target sequence was detected via agarose gel electrophoresis as described in section 2.2.2.

In order to minimize the amount of contaminating wild type FPV M3 virus, the viral extracts from 2.6.1.3 were used in two consecutive rounds of amplification with drug selective pressure and performed exactly as described above.

*2.6.1.4 Blue plaque purification*

After three rounds of amplification with drug selection, the best viral extracts were used for clone purification. 60 mm dishes were seeded with 5 ml of secondary CEF cells at $8.4x10^5$ cells/ml and incubated at 35°C/5% $CO_2$ until cells had reached 100% confluency. 100 µl of the viral extracts from 2.6.1.3 were digested with 900 µl of 1 mg/ml trypsin at 37°C for 30 min and then serially diluted in MM from a $10^{-2}$ to $10^{-7}$ dilution. Four dishes per dilution starting at $10^{-3}$ were infected with 1 ml of serially diluted virus/dish after removing GM and incubated at 35°C/5% $CO_2$ for 1 h. Plates were mock-infected with PBS as a negative cell control. After an hour, 4 ml MM was added to each dish and incubated at 35°C/5% $CO_2$ until plaques started to develop (4 to 6 days p.i.).

Once plaques started to develop, an Xgal agarose overlay was added. Three 50 ml tubes containing 40 ml MM, 750 µl neutral red (NR) solution and 300 µl 50 mg/ml Xgal were warmed to 37°C in a water bath and topped up to 50 ml with 5% molten agarose. Tubes were mixed by gentle inversion and allowed to cool at RT until warm to the touch. 5 ml of agarose solution was then added to each dish and left at RT until the agarose had set. Plates were incubated at 35°C/5% $CO_2$ for 24 h until blue plaques could be clearly seen. Blue plaques were removed from the highest dilution plate containing the smallest number of well separated blue plaques using a plugged Pasteur pipette. 20-24 blue plaques were picked per viral extract purified. Picked clones were dispensed into 1.5 ml microfuge tubes containing 500 µl PBS, briefly sonicated (approx 3 sec burst) and stored at -20°C.

*2.6.1.5 Amplification of blue plaque clones*

Secondary CEF cells were seeded at $4x10^5$ cells/ml in 1 ml/well of 24-well plates and incubated at 35°C/5% $CO_2$ until cells had reached 100% confluency. For each blue plaque clone, 100 µl was digested in 100 µl 1 mg/ml trypsin at 37°C for 30 min. Reactions were stopped by adding 800 µl MM. Medium was removed from the wells, the 1 ml of each digested clone was added to separate wells and plates were incubated at 35°C/5% $CO_2$ until signs of infection could be seen (4 to 6 days p.i.). Viral extracts were harvested via pelleting and sonication as described in section 2.6.1.2, resuspended in 500 µl PBS and stored at -20°C.

Isolated blue plaque clones were further amplified in larger vessels (T25 flasks or 60 mm dishes). Secondary CEF cells were seeded in 5 ml GM at $1x10^6$ cells/ml and incubated at 35°C/5% $CO_2$ until cells had reached confluency. The 500 µl of each virus clone from the 24-well plate amplification were digested with 500 µl of 1 mg/ml trypsin at 37°C for 30 min. 4 ml MM was added to each digestion and one diluted clone was added per flask/plate. Plates were incubated at 35°C/5% $CO_2$ until signs of infection could be seen (4 to 6 days p.i.). Viral extracts were harvested via pelleting and sonication as described in section 2.6.1.2, resuspended in 1 ml PBS and stored at -20°C.

*2.6.1.6 White plaque purification*

Following PCR analysis, the best clone, ie the one with the lowest amount of wild type viral contamination, proceeded to the white plaque purification step. This step is identical to the blue plaque purification described in section 2.6.1.4 except that positive clones are now white due to the lack of drug selection. Following their purification, white plaque clones were amplified in 24-well plates and then subsequently in T25 flasks or 60 mm dishes exactly as the blue plaque clones, described in section 2.6.1.5. The presence of rFPV in the amplified viral extract was analysed via PCR as in section 2.6.1.3, however, primers to detect parental virus were also included in order to detect the presence of contaminating parental vector in the amplified viral extract. The parental vector PCR primer sequences are as follows:

**Screening for empty pKG10a vector:**

*Forward primer*

**5`**-CTTCGGATTTTGCTATAAGCGTAATAAG-**3`**

*Reverse primer*

**5`**-GTCATTTCAAGTTCTTTAAAAGACTCCTG-**3`**

**Screening for empty pAFtd vector:**

*Forward primer*

**5`**-GAACTAATATCGAACTCCATTCCGTG-**3`**

*Reverse primer*

**5`**-GTCCAGTTTGACAGCTATTTGCG-**3`**

The clone containing the lowest amount of parental vector underwent a second cycle of white plaque purification and amplification after which 5 clones from this second purification step proceeded into a third and final cycle of white plaque purification and amplification. These repeated cycles of purification and amplification were done to ensure that >90% of rFPV contained the foreign sequence (OVA or mIL-4).

*2.6.1.7 Production of virus seed stock (VSS)*

The VSS was produced by amplifying all clones positive for the insertion and negative for parental virus in individual 50 ml roller bottles (RBs) and further expanding the single best clone in 50 RB. Secondary CEF cells were cells were seeded at $1x10^8$ cells/ml in 200 ml GM per RB and incubated at 35°C/5% $CO_2$ until cells reached confluency. For each clone, 500 µl of virus was digested with 500 µl of 1 mg/ml trypsin and incubated at 37°C for 30 min. Reactions were stopped by adding 25 ml MM and the medium in the bottles was replaced with the diluted viruses, with one clone per bottle. Roller bottles were incubated at 35°C/5% $CO_2$ for 4 to 6 days and infected cells were harvested in 250 ml centrifuge tubes, pelleted at 1000 g for 10 min, resuspended in 5 ml PBS and sonicated. The presence of rFPV and contaminating parental vector were analysed via PCR using the primers from sections 2.6.1.3 and 2.6.1.6.

As already mentioned, the single best clone was then expanded in 50 RBs. Initially, 13 RBs were seeded with $2x10^8$ cells/ml of primary CEF cells in 200 ml and incubated rolling at 35°C/5% $CO_2$ until cells reached confluency. This primary culture was then expanded into 50 RBs by a 1:4 split ratio to form the secondary cultures, which were incubated rolling at

35°C/5% $CO_2$ until cells reached confluency. 1 ml of the best virus clone was digested with 1 ml 1 mg/ml trypsin at 37°C for 30 min and the reaction was stopped with 1 L MM. The medium from each RB was replaced with 20 ml of diluted virus, incubated at 35°C/5% $CO_2$ for 1 h to allow adsorption of the virus and then topped up with 200 ml MM. Bottles were incubated at 35°C/5% $CO_2$ for 4 to 6 days and then the cell culture was recovered, spun down at 2000 g for 5 min and resuspended in 200 ml TrypLE select solution. Cells were sonicated until an even homogenate could be seen and incubated for 45 min at 37°C to release viral particles from host cell membranes. Insoluble cell debris was pelleted (1000 g, 10 min at 4°C) and the clarified product was sucrose purified. 150 ml of 36% sucrose that had been prechilled to 4°C was added to 4 250 ml tubes and gently layered with 50 ml of virus-containing supernatant per tube. Viral particles were pelleted by centrifugation at 4000 g for 8 h at 6°C and resuspended in a total volume of 50 ml with 10 mM TrisHCl pH 8. Virus was then aliquoted and stored at -80°C. PCR analysis for the detection of the foreign sequence inserted (OVA or mIL-4) and parental virus was performed as previously described.

*2.6.1.8 Titration of VSS*

Titration of the rFPVs in pfu/ml was done using secondary CEF cells cultured in 60 mm dishes. For each virus to be titred, 30 dishes were seeded with $2x10^6$ primary cultured CEF cells per dish in 4 ml of GM and incubated at 35°C/5% $CO_2$ until the secondary CEF cultures reached 100% confluency. The VSSs were then serially diluted in 10 fold and half log dilutions ranging from $10^{-1}$ to $10^{-10}$ in MM. All serial dilutions were done in triplicate (3 dishes per dilution) and 1 ml of each serial dilution was added to each dish after the GM had been removed. Virus was adsorbed for 45 min at 35°C/5% $CO_2$ and then 4 ml/plate of MM was added. Dishes were incubated at 35°C/5% $CO_2$ for 4 to 6 days until plaques could be seen. Medium was removed and cells were fixed with methanol (2-5 ml/dish, RT 10 min). Methanol was then removed, cells were air-dried and all the dilutions containing more than 1 plaque were counted up to dilutions that were uncountable. The calculation of titration in pfu/ml was done using the WHO/BS/03.1977 and WHO/BS/03.1985 method of standardization of smallpox reference stocks and Yellow fever vaccine and takes into account all counts and linearity of dilutions.

The formula is as follows:

*(av1 + av2 + av3) / (dil 1 + dil 2 + dil 3) X plating vol in ml*

Where *av* is the average plaque count and *dil* is the corresponding dilution.

### *2.6.2 Infection of QT35 cells with rFPV in vitro*

QT35 cells were seeded at $10^6$ cells/well in flat-bottom 6-well plates and incubated at 37ºC in 5% $CO_2$ until cells were over 90% confluent (~ $3x10^6$ cells). Complete medium was removed and 500 µl of diluted rFPV was added to each well via pipetting along the side of the wells, to avoid disturbance of the cell layer. To achieve overall infection, cells were infected at a MOI of 10 pfu/cell, which is $3x10^7$ pfu/well. Plates were incubated at RT for 45-60 min and rocked gently every 10-15 min to avoid the cells drying out. 4 ml of MM was added to each well and cells were incubated at 35ºC in 5% $CO_2$ until signs of infection appeared.

### *2.6.3 Immunisation of mice with rFPV*

For the adoptive transfer experiments, mice were immunised with $5x10^6$ pfu of rFPV ($2.5x10^6$ pfu $FPV_{OVA}$ + $2.5x10^6$ pfu FPV M3 or $FPV_{mIL\text{-}4}$) in the right footpad. For all other experiments, mice were vaccinated with $2x10^7$ pfu rFPV ($1x10^7$ pfu $FPV_{OVA}$ + $1x10^7$ pfu FPV M3 or $FPV_{mIL\text{-}4}$) administered intraperitoneally (i.p.).

## 2.7 METHODS FOR THE CHARACTERISATION OF CELLULAR AND HUMORAL IMMUNE RESPONSES

### *2.7.1 Preparation of single cell suspensions from whole organs*

Lymph nodes and spleens were removed, cut into small sections and stored in RPMI medium on ice. Single cell suspensions were prepared by gently homogenizing the sections between frosted glass slides until only connective tissue (CT) remained. Cells were pelleted by centrifugation at 4°C, 1200 rpm for 5 min. Splenocytes were resuspended in 5 ml/spleen in alkaline lysis buffer (ALB) and incubated at RT for 5 min in order to lyse red blood cells (RBC). An equivalent volume of RPMI medium was added and cells were spun for 5 min at 4°C, 1200 rpm. All cell preparations were washed twice in 10 ml RPMI and resuspended as required, with a 30 μl sample taken for trypan blue viability staining and cell counting.

### *2.7.2 Adoptive transfer (AT) of OT-I and OT-II TCR transgenic T cells*

OT-I TCR transgenic T cells were prepared from the lymph nodes of OT-I and OT-II mice as described in section 2.7.1. and cells were incubated for 10 min at 37°C with 0.35 μl of 10 mM carboxyfluoroscein succinimidyl ester (CFSE)/$10^7$ cells/ml. Cells were washed, resuspended in incomplete RPMI medium and passed through a 70 μM filter. $10x10^6$ cells in a 200 μl volume were delivered intravenously (i.v.) via the lateral tail vein into each recipient. Spleen, draining and non-draining lymph nodes (LN) were recovered 3 days later and single cell suspensions were prepared for direct immunofluorescence staining as described in section 2.7.1.

### *2.7.3 Flow cytometry*

The extent of OT-I and OT-II T cell proliferation was analysed via flow cytometry. To distinguish donor OT-I T cells from endogenous T cells, samples were stained with 2 μg/ml PE-Cy5-conjugated anti-CD8α (clone 53-6.7, PharMingen, BD Biosciences, CA, USA) and PE-conjugated anti CD45.1 (clone A20, PharMingen, BD Biosciences, CA, USA) at 4°C for 30 min. After direct staining, cells were washed three times in FACS buffer and resuspended in a final volume of 500 μl FACS Fix. Three colour analysis was performed on a FACScan (BD Biosciences, CA, USA) where profiles were gated on $CD8^+$, $CD45.1^+$, $CFSE^+$ cells. To

distinguish donor OT-II T cells from endogenous T cells, samples were stained with 2 μg/ml PE-conjugated anti-CD4 (clone RM4-5, PharMingen, BD Biosciences, CA, USA) at 4°C for 30 min. After direct staining, cells were washed and prepared as for OT-I T cells. Two colour analysis was performed where profiles were gated on $CD4^+$, $CFSE^+$ cells. For both OT-I and OT-II T cells, the extent of division was determined by calculating the proliferation index (PI), which is a ratio of cells recovered: cells transferred. The PI formula is:

$$PI = (n_{PP} + n_{G1} + n_{G2})/(n_{PP}/2^{PP} + n_{PP}/2^{G1} + n_{PP}/2^{G2})$$

Where n = cell number in the peak, PP = parent peak, G1 = generation 1, G2 = generation 2 etc. Data was analysed using Weasel (WEHI FACS lab, Vic, Australia) and Summit (DakoCytomation, Glostrup, Denmark) software programs.

### *2.7.4 Direct cytotoxicity assay*

Splenocytes were prepared as described in section 2.7.1 and depleted of adherent APC populations by culturing cells at $2x10^6$ cells/ml in incomplete RPMI medium in 10 $cm^2$ tissue culture dishes at 37°C in 5% $CO_2$ for 90 min. Cytolytic activity of these effector cells was assessed by incubating the harvested cells with EL-4 target cells that had been incubated with $^{51}Cr$ (50 μCi/$1.5x10^6$ cells) +/- 2.5 μM SIINFEKL peptide at 37°C in 5% $CO_2$ for 90 min. Labelled EL-4 target cells were washed three times and distributed in U-bottom 96-well plates at $10^4$ cells/100 μl per well and splenic effector cells ($2x10^6$/100 μl in complete RPMI medium) were added at an effector/target (E:T) ratio of 200:1 and serially diluted to 25:1 in duplicate. The plate was centrifuged at 200 x g for 30 s before incubation at 37°C in 5% $CO_2$ for 18 h. The cells were gently pelleted and 100 μl of the supernatant was counted in a gamma counter (Packard). Spontaneous and maximum release was measured by treating the targets with complete RPMI medium or NP40 detergent respectively.

Alternatively, assays assessing anti-vector CTL responses used HEK 293 T cells stably transfected to express the C57BL/6 class I MHC molecule Kb or Db, and were either additionally stably transfected to express OVA or FPV open reading frames (ORFs), or

infected prior to the assay with FPV M3. The rest of the CTL assay was performed as for the EL-4 target cells.

The percentage of specific release of $^{51}Cr$ was determined by the standard equation:

*% specific lysis = (experimental release-spontaneous release)/ (maximum release-spontaneous release) x 100*

The number of lytic units per $10^7$ effectors was calculated using the formula (175):

$$LU/10^7 \text{ effectors} = 10^7/(T-X_p)$$

Where $T$ is the number of target cells ($10^4$), $p$ is the reference lysis level (20%) and $X_p$ is the E:T ratio required to lysis p% of the targets.

### *2.7.5 Helper T cell proliferation assay*

Splenocytes were harvested 5 days after immunization and following red blood cell lysis and depletion of adherent APC populations were seeded at $2x10^5$cells/well of flat-bottom 96-well plates. Cells were co-incubated with either 400-6.25 μg/ml of OVA protein, $2x10^6$ PFU (MOI = 10) UV-inactivated FPV M3 or ConA at 37°C in 5% $CO_2$ for 3 days. All proliferation assays were performed in triplicate wells and in complete RPMI medium. Cell proliferation was assessed by adding 1 μCi/well of [$^3$H] thymidine for 16 hours. Incorporation of [$^3$H] thymidine was measured by harvesting the cells onto Filtermate™ glass fibre filters (PerkinElmer, MA, USA) and counting on a Topcount instrument (PerkinElmer, MA, USA).

### *2.7.6 Antibody ELISAs*

The detection of anti-OVA and anti-FPV antibody were determined via ELISA. High protein binding ELISA plates (Costar, Jomar, SA, Australia) were coated with either 10 μg/ml of soluble OVA or FPV protein or $10^6$ PFU/well of FPV M3. After blocking with PBS/1% BSA, dilutions of serum samples were added and incubated at 37°C for 2 h. Bound antibody was

detected by the addition of 40 ng/ml biotinylated donkey anti-mouse IgG (Rockland, PA, USA) and 67 ng/ml streptavidin-alkaline phosphatase (Rockland, PA, USA). The plates were developed with pNPP substrate (Sigma-Aldrich, MO, USA) at 37°C and the reaction was stopped after 60 min using 1 M NaOH. Plates were read at 405 nm. OVA-specific Ig (IgA, IgG, IgM) and IgM antibodies were detected similarly using biotinylated goat anti-mouse Ig (40 ng/ml) or anti-mouse IgM (35 ng/ml) and streptavidin-alkaline phosphatase (67 ng/ml).

### *2.7.7 Generation of bone marrow chimaeras*

To generate bone marrow (BM) chimaeras, donor BM was harvested from the tibia and fibia of adult B6 or $B6^{bm1}$ mice and depleted of T and NK cells by complement-mediated depletion. Cells were incubated with anti-Thy1 (TIB 107 hybridoma) and anti-NK.1.1 (HB 191 hybridoma) antibodies at 4°C for 30 mins followed by exposure to 250 μL (1/5 dilution) of guinea pig serum/$12.5x10^6$ cells for 60 mins at 37°C. $B6^{bm1}$ and B6 recipient mice were lethally irradiated (9Gy) and reconstituted with $5x\ 10^6$ of the T cell-depleted BM cells from $B6^{bm1}$ or B6 donor mice i.v.. All possible combinations were made, B6 reconstituted with B6 (B6 →B6), B6 reconstituted with $B6^{bm1}$ (B6 → $B6^{bm1}$), $B6^{bm1}$ reconstituted with B6 ($B6^{bm1}$ → B6) and $B6^{bm1}$ reconstituted with $B6^{bm1}$ ($B6^{bm1}$ → $B6^{bm1}$). These mice were then checked after 8 weeks for complete reconstitution of the bone marrow compartments by FACS analysis of peripheral blood. The bone marrow chimaeras were generated by Lachlan Moldenhauer (Research Centre for Reproductive Health, University of Adelaide, SA, Australia).

## 2.8 STATISTICAL ANALYSIS

Statistical comparisons were performed using GraphPad Prism v4 (GraphPad Software, CA, USA). Generally, analysis of variance (ANOVA) was used to deduce significant differences among the results. The Bonferroni post-test comparison was used to report P values. P values are denoted thus: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

# CHAPTER 3: RESULTS

## 3.1 CONSTRUCTION OF THE RECOMBINANT FOWLPOX VIRUS (rFPV) SHUTTLE VECTORS

### *3.1.1. Construction of the pKG10a –OVA-IRES-EGFP plasmid*

The model antigen, Chicken ovalbumin (OVA), was cloned directly upstream from enhanced green fluorescent protein (EGFP) and separated by an internal ribosome entry site (IRES). IRES functions as a ribosome-landing pad for the efficient internal initiation of translation ensuring coordinated expression of several genes, in this case OVA and EGFP. Both genes of interest are translated from a single bicistronic mRNA, resulting in two individual proteins. EGFP is a variant of wild type GFP that has been optimised for brighter fluorescence and higher expression in mammalian cells and was included as a fluorescent marker for cell infection and gene expression.

Cloning into the transient dominant selection plasmid pKG10a required that OVA-IRES-EGFP be cloned into the shuttle vector as a promoter-gene-terminator fusion. This was achieved by the PCR addition of a FPV bidirectional early/late promoter, which results in high levels of protein expression in mammalian cells (176), and a poxviral T5NT transcription termination sequence. The T5NT sequence is recognized by poxviral RNA polymerase for termination of early RNA transcription (177) and was added at the ‘3 end of the continuous coding sequence. It was crucial that the OVA and IRES-EGFP sequences themselves did not contain internal T5NT motifs as these disrupt early gene expression during the abortive replication cycle of the rFPVs in non-avian cells, however, scanning of the OVA and IRES-EGFP sequences revealed no internal T5NT motifs. The pKG10a-OVA-IRES-EGFP expression vector also included a Kozak sequence to facilitate the initial binding of the small subunit of the ribosome to the mRNA. The presence of the OVA-IRES-EGFP cassette (2.6 kb) in the pKG10a shuttle vector (8.2 kb) was confirmed via both restriction digestion and touchdown PCR as illustrated in Figure 3.1.

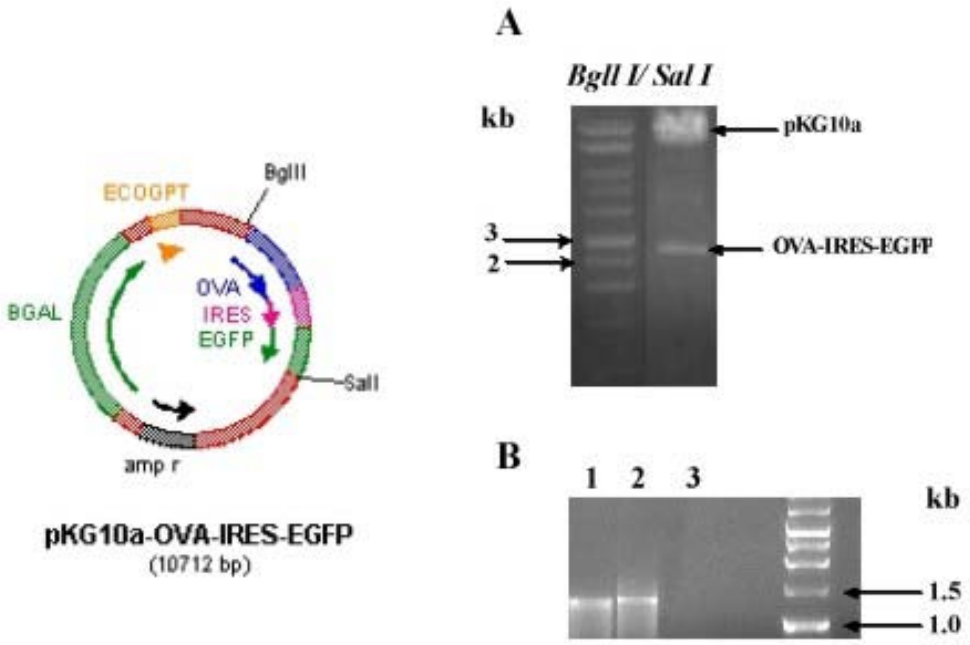


**Figure 3.1 Confirmation of the OVA-IRES-EGFP insert in the pKG10a shuttle vector.**

**(A)** The presence of the entire OVA-IRES-EGFP cassette (2.6 kb) in the pKG10a shuttle vector (8.2 kb) was confirmed by restriction digestion with the restriction enzymes *Bgl*II and *Sal*I at 37°C for 1 hour. **(B)** The 1.2 kb OVA and 1.4 kb IRES-EGFP products were obtained from 1 ng of target DNA, pKG10a-OVA-IRES-EGFP, by PCR with forward and reverse OVA (**B.1**) or IRES-EGFP primers (**B.2**) consecutively. Due to its large size (2.6 kb), the entire OVA-IRES-EGFP cassette was not able to be PCR amplified as a single product using the OVA forward and EGFP reverse primers (**B.3**).

### *3.1.2. Construction of the pAFtd-mIL-4 plasmid*

Murine interleukin-4 (mIL-4) was cloned into the transient dominant selection plasmid pAFtd, which is inserted into the region between the thymidine kinase (TK) gene and the next open reading frame (ORF) the uncharacterized gene ORF X (FPV086 & FPV087). As for pKG10a-OVA-IRES-EGFP, the cloning of mIl-4 into pAFtd required that it be cloned into the shuttle vector as a promoter-gene-terminator fusion. Therefore, mIL-4 was expressed under the control of the same FPV bidirectional early/late promoter as OVA-IRES-EGFP and Kozak and T5NT termination sequences were inserted. The T5NT terminator was added at the 3' end of the continuous coding sequence. Scanning of the mIl-4 sequence revealed no internal T5NT motifs. The presence of mIL-4 (0.5 kb) in the pAFtd shuttle vector (9.5 kb) was confirmed via both restriction digestion and touchdown PCR as illustrated in Figure 3.2.

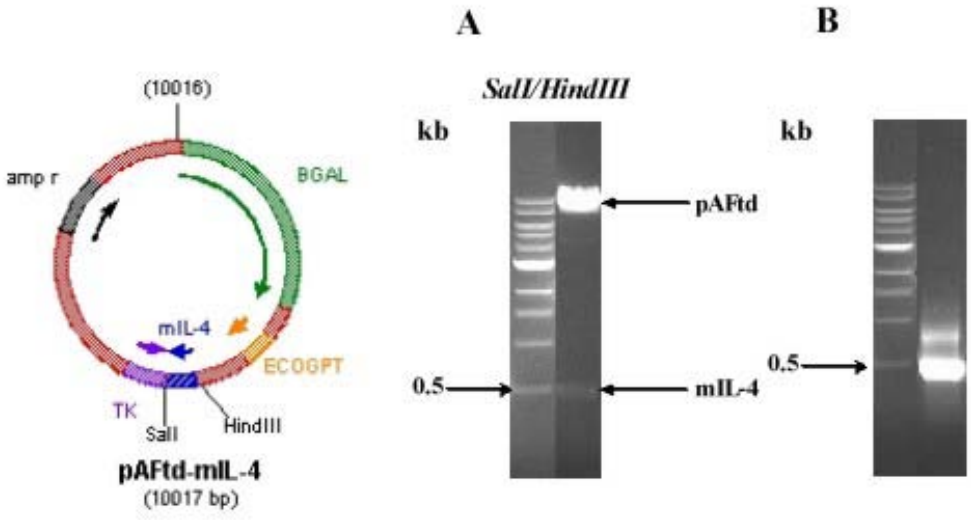


**Figure 3.2 Confirmation of the mIL-4 insert in the pAFtd shuttle vector.**

The presence of mIL-4 in the pAFtd shuttle vector was confirmed via restriction digestion **(A)** and touchdown PCR **(B)** and products were analysed on a 1% agarose gel. **(A)** mIL-4 was dropped out of the 9.5 kb pAFtd vector by restriction digestion with the restriction enzymes *Sal*I and *Hind*III at 37°C for 1 hour. **(B)** The 0.5 kb mIL-4 product was obtained from 1 ng of target DNA, pAFtd-mIL-4, by touchdown PCR.

Prior to their insertion into the FPV genome, restriction digestion profiles of pKG10a-OVA-IRES-EGFP and pAFtd-mIL-4 were done using the restriction enzymes *EcoR*V and *Hind*II for final confirmation that the vectors were intact and contained the correct sized inserts. Comparison of the obtained profiles with predicted digestion profiles confirmed that this was indeed the case, as illustrated in Figure 3.3.

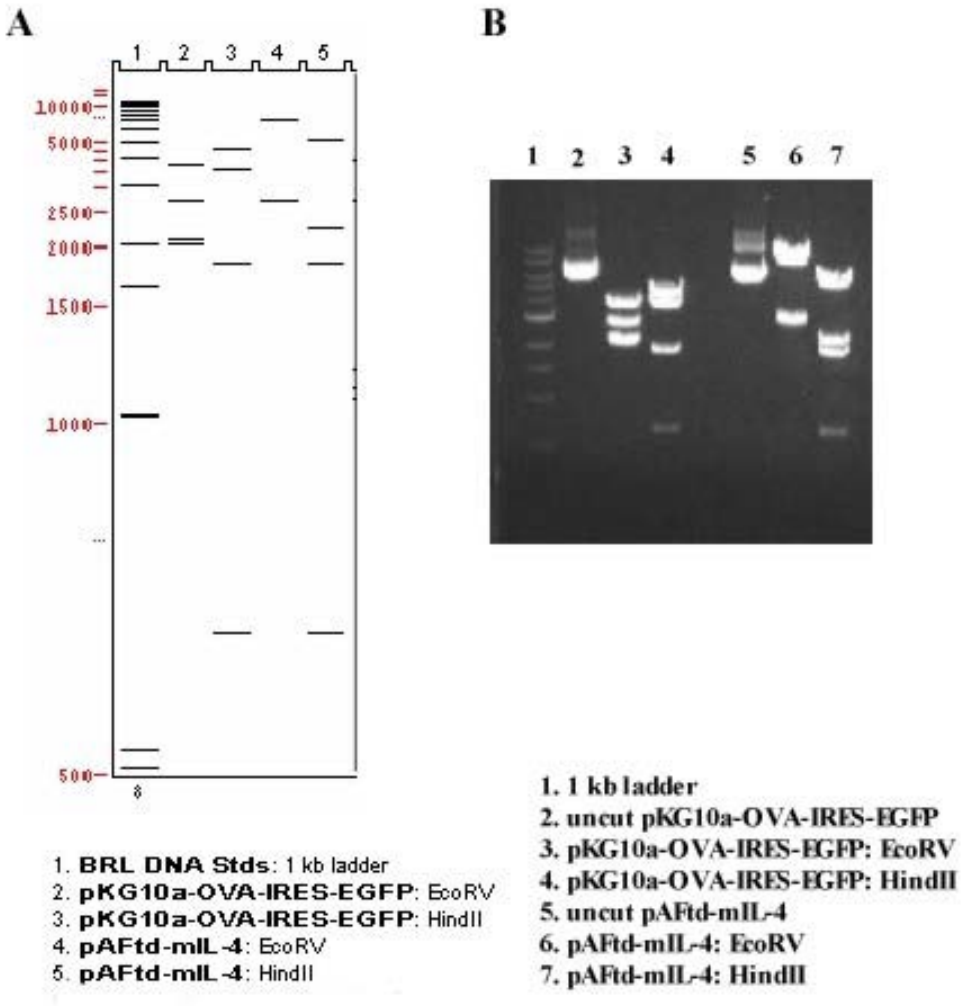


**Figure 3.3 Restriction digestion profile of the rFPV shuttle vectors.**

The presence and correct size of the inserted genes into the two FPV shuttle vectors were confirmed by restriction digestion profile. **(A)** The predicted restriction digestion profile for each of the rFPV shuttle vectors with two different restriction enzymes. **(B)** The actual restriction digestion profile for each of the rFPV shuttle vectors with the same enzymes. 1 µg of pKG10a-OVA-IRES-EGFP or pAFtd-mIL-4 DNA was incubated each enzyme at 37°C for 1 hour and products were analysed on a 1% agarose gel.

## 3.2 *IN VITRO* EXPRESSION ANALYSIS OF PLASMIDS

To confirm that the cloned OVA, EGFP and mIL-4 genes would actually be expressed *in vivo*, they were inserted into the mammalian expression vector pcDNA3.1(-)/Hygro and used to test for *in vitro* expression by transient lipid-mediated transfection of HEK 293 cells (section 2.4.2).

### *3.2.1. HEK 293 cells transiently-transfected with pcDNA3.1(-)/Hygro-OVA-IRES-EGFP express both OVA and EGFP*

EGFP expression was used as a fluorescent marker for cell transfection and was detected via fluorescent microscopy and flow cytometry, where approximately 35-40% of cells had been transfected (expressed EGFP). As illustrated in Figure 3.4, cells transfected with pcDNA3.1(-)/Hygro-OVA-IRES-EGFP and the pcDNA-IRES-EGFP positive control vector had high levels of EGFP expression 48 hours after transfection (Figure 3.4A and B), while cells transfected with empty pcDNA3.1(-)/Hygro vector showed no detectable fluorescence (Figure 3.4C).

Western blotting of transfected cell lysates and supernatants using rabbit biotinylated anti-OVA IgG showed that full length OVA was expressed by cells transfected with pcDNA3.1(-)/Hygro-OVA-IRES-EGFP as opposed to those transfected with empty vector (see Figure 3.5). OVA protein expressed by HEK 293 cells was the expected size of 50 kDa and was present in both the cell supernatant and lysate, as while OVA is a secreted protein, some will remain cell-associated.

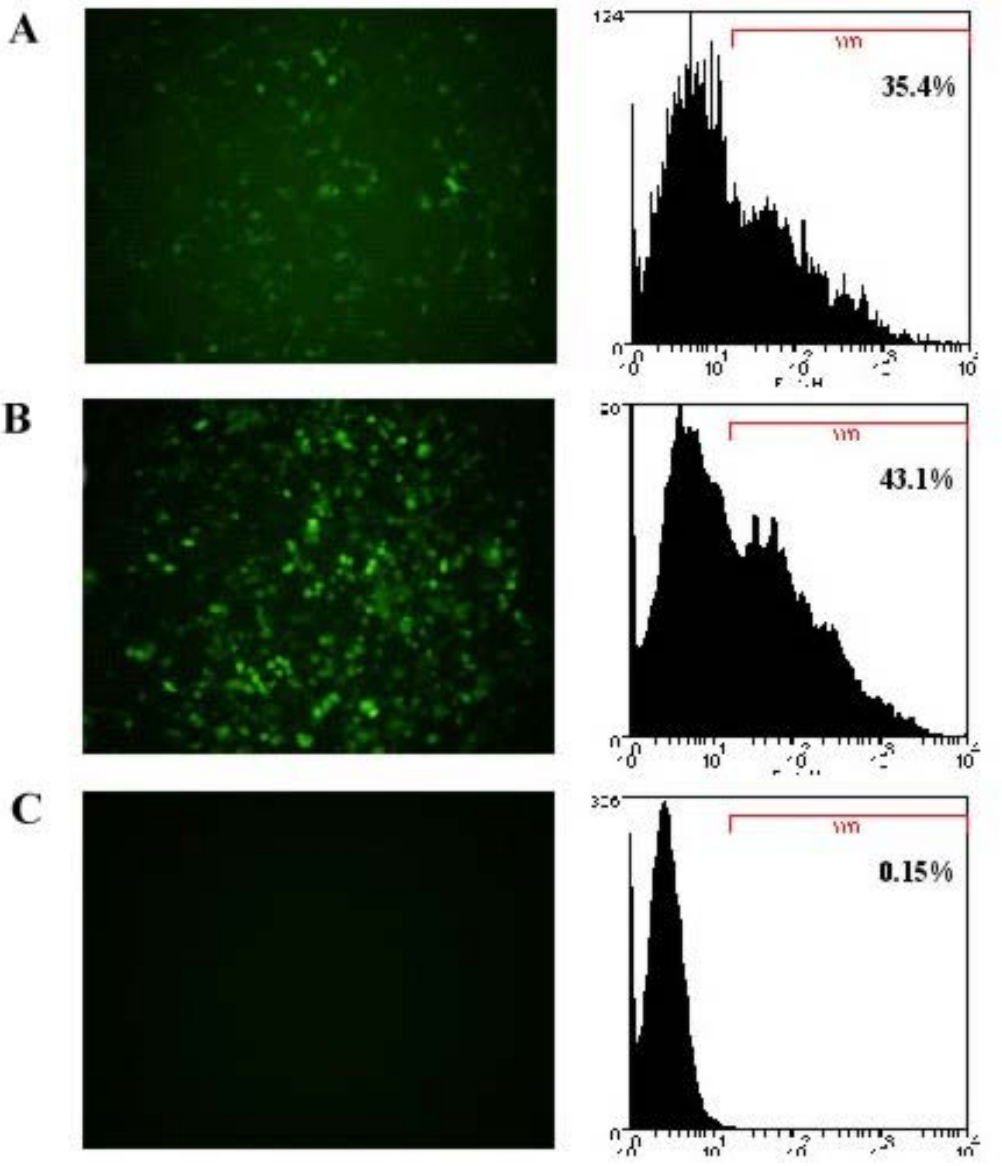


**Figure 3.4 EGFP expression by HEK 293 cells transiently-transfected with pcDNA3.1(-)-OVA-IRES-EGFP.**

HEK 293 cells were transiently transfected with pcDNA3.1(-)-OVA-IRES-EGFP using Lipofectamine reagent and EGFP expression was analysed 48 hours later via both fluorescent microscopy and flow cytometry (**A**). HEK 293 cells transfected with pcDNA-IRES-EGFP (**B**) were used as an EGFP, transfection positive control and HEK 293 cells transfected with empty pcDNA3.1(-)/Hygro vector (**C**) were used as a negative control for autofluorescence. Approximately 35% of HEK 293 cells transfected with pcDNA3.1(-)/Hygro-OVA-IRES-EGFP were positive for EGFP expression.

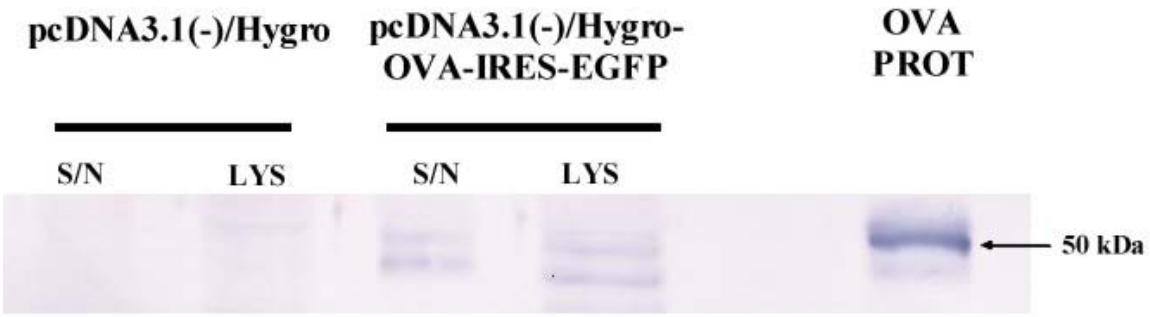


**Figure 3.5 Western Blot analysis of OVA expression by HEK 293 cells.**

OVA is present in the cell supernatant and lysate of HEK 293 cells transiently transfected with pcDNA3.1(-)/Hygro-OVA-IRES-EGFP. The nitrocellulose membrane containing the resolved proteins from the cell lysates and supernatants was blocked overnight with 3% skim milk. OVA was detected using a rabbit biotinylated anti-OVA antibody and alkaline phosphotase conjugated with streptavidin. The resolved OVA protein is 50kDa in size and was detected with BCIP/NBT substrate.

### *3.2.2. HEK 293 cells transiently-transfected with pcDNA3.1(-)/Hygro-mIl-4 express biologically-active mIL-4*

mIL-4 expression by transiently transfected HEK 293 cells was detected via cytokine ELISA (see Figure 3.6). As expected, mIL-4 was present predominantly in the cell supernatant of pcDNA3.1(-)/Hygro-mIL-4-transfected cells and no mIL-4 was detected in samples from cells transfected with empty pcDNA3.1(-)/Hygro vector.

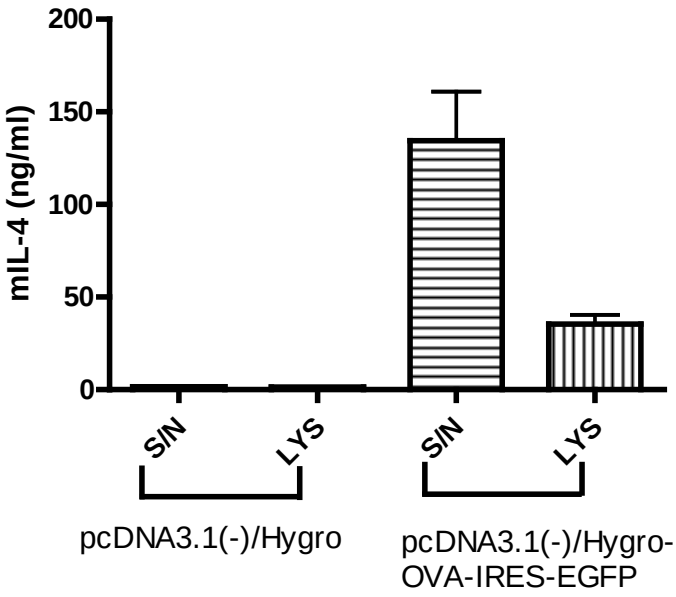


**Figure 3.6 mIL-4 is predominantly present in the cell supernatant of HEK 293 cells transiently-transfected with pcDNA3.1(-)/hygro-mIL-4.**

HEK 293 cells were transiently transfected with pcDNA3.1(-)/Hygro-mIL-4 using Lipofectamine reagent and mIL-4 expression was analysed 48 hours later. Cytokine ELISA was used to detect mIL-4 present in the cell supernatants and cell lysates. Results are expressed as mean +/- SEM of duplicate wells.

The biological activity of mIL-4 was determined using a proliferation assay with CTLL-2 cells and the results are represented in Figure 3.7. CTLL-2 cells proliferate maximally in response to human IL-2 and sub-maximally to mIL-4. Therefore, it was not surprising that cells incubated with r huIL-2 had the highest amount of proliferation (60,000 CPM +/- 4,500). Some background proliferation (10,000 CPM +/- 5,000) was observed in the negative controls, complete medium (cRF-10) and supernatant from empty vector pcDNA3.1(-)/Hygro)-transfected HEK 293 cells, as a result of the FCS in the tissue culture medium containing a small amount of interleukins including IL-2. However, the proliferation of CTLL-2 cells incubated with supernatant from pcDNA3.1(-)/Hygro-mIL-4-transfected HEK 293 cells was still three times higher than that of the negative controls (30,000 CPM +/- 3,700). CTLL-2 cells were also incubated with cell supernatant containing recombinant mIL-4 (3.3 U/ml) derived from Sf9 insect cells infected with mIL-4 cDNA-encoding baculovirus as a positive IL-4 control, however, proliferation was not significantly above the negative

controls (data not shown). This is possibly due to the presence of hypoxanthine-aminopterin-thymidine (HAT) in the supernatant, which suppresses the proliferation of TK-negative mammalian cells.

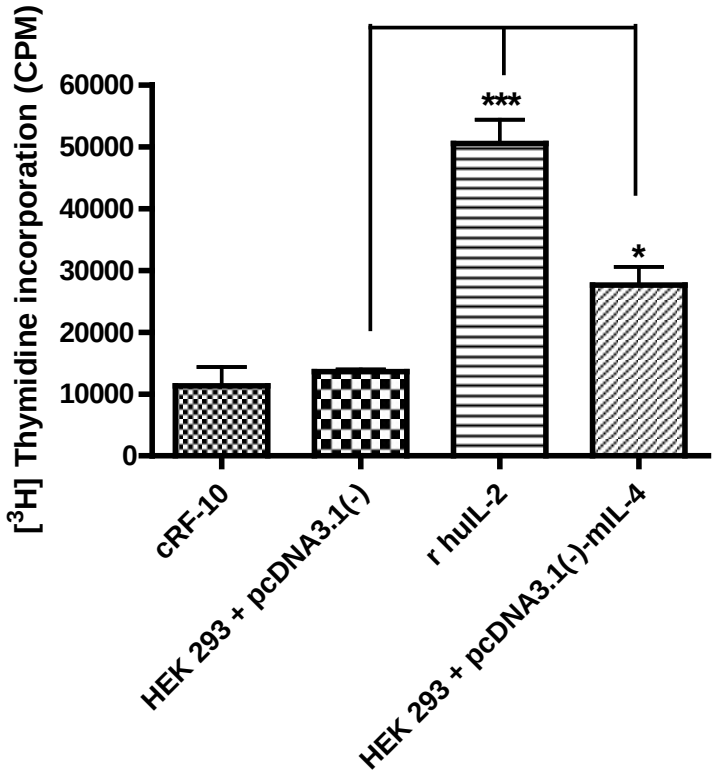


**Figure 3.7 CTLL-2 cells proliferate in response to rhuIL-2 and mIL-4.**

Cells were incubated for 18 hours with either recombinant huIL-2 or cell supernatant from HEK 293 transiently transfected with pcDNA3.1(-)-mIL-4 or empty pcDNA3.1(-) vector. Cell proliferation was assessed by adding 1 μCi/well of [$^3$H] thymidine for 4 hours. Incorporation of [$^3$H] thymidine was measured by harvesting the cells onto Filtermate glass fibre filters and counting on a Topcount instrument. All determinants were performed in triplicate and measured as the mean counts per minute ± SD. Counts were considered significant when $p < 0.05$ and p values are denoted thus: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

## 3.3 THE CONSTRUCTION OF rFPV BY TRANSDOMINANT SELECTION

The source of FPV used to construct the rFPVs was a derivation of the fowlpox M strain that had been plaque purified and amplified three times and hence designated “FPV M3”. The FPV M strain is a mild strain of FPV that is tissue culture propagated as opposed to a virulent field strain produced in embryonated eggs (Websters' vaccine strain S). The derivation of FPV M3 from the parental fowlpox vaccine strain obtained from Arthur Webster Pty Limited (Northmead, NSW, Australia) has been reported in the literature (178).

Recombinant FPVs were constructed using transient dominant selection protocols for the insertion of genetic material into multiple sites in the viral genome of FPV M3 based on those described by Boyle *et al.* (179). Three rFPVs were constructed; FPV expressing OVA ($FPV_{OVA}$), FPV expressing mIL-4 ($FPV_{mIL\text{-}4}$) and FPV expressing both OVA and mIL-4 ($FPV_{OVA+mIL\text{-}4}$). In order for OVA and mIL-4 to be inserted into the same rFPV, they were cloned into different shuttle vectors that are inserted into different regions on the FPV genome. pKG10a was used for the insertion of the OVA sequence at the F7-F9 locus in FPV M3 (Figure 3.8), while pAFtd was used for the insertion of the mIL-4 sequence between the thymidine kinase (TK) gene and the uncharacterized gene ORF X (FPV086 & FPV087) (Figure 3.9). Both vectors contained the β-gal and Ecogpt markers within their plasmid backbones, external to the regions used for homologous recombination into the FPV genome. Hence recombinants generated in CEF cultures by homologous recombination followed by infection with FPV and transfection with plasmid were amplified by selection for Ecogpt expression (blue plaque purification) and then plaque-purified on the basis of the loss of β-gal expression (white plaque purification). The presence of insertions of the predicted size in the viral genome were verified by PCR using primers to the inserted genes and the stability of these recombinants was further assessed by the preparation of viral DNA and its PCR after each of two additional rounds of passage and amplification in CEF cells (data not shown).

As mentioned above, the pKG10a-OVA-IRES-EGFP and pAftd-mIL-4 plasmids were used to construct rFPVs carrying either one (OVA or mIL-4) or two (OVA and mIL-4) insertions. These can be inserted sequentially and in any order into the FPV genome because both

pKG10a and pAftd are transient dominant selection vectors. In the case of the co-expressing $FPV_{OVA+mIL-4}$ virus, pAftd-mIL-4 was to be inserted into the recombinant $FPV_{OVA}$ virus as illustrated in Figure 3.10A. However, while it has previously been demonstrated with HIV and SIV antigens that pKG10a and pAftd can be used in both orders to insert recombinant genes into FPV M3, the presence of mIL-4 in $FPV_{OVA}$ was undetectable by PCR. In contrast, pKG10a-OVA-IRES-EGFP was successfully inserted into the recombinant $FPV_{mIL-4}$ virus as illustrated in Figure 3.10B.

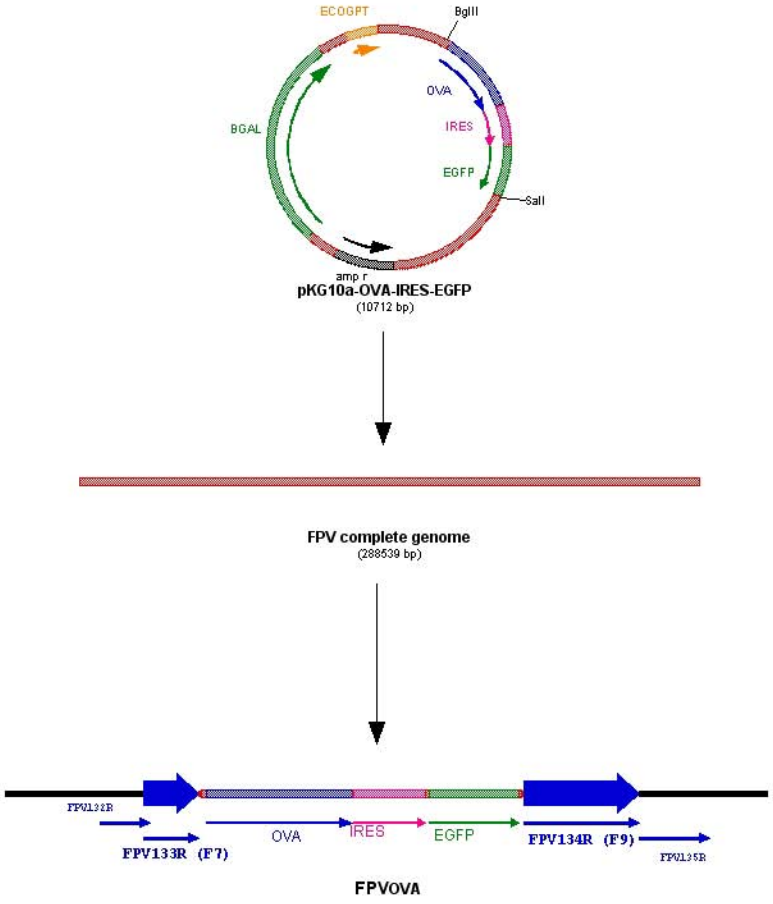


**Figure 3.8 Construction of the $FPV_{OVA}$ virus.**

The transient dominant selection vector pKG10a was used for the insertion of the OVA sequence at the F7-F9 locus in FPV M3. $FPV_{OVA}$ was generated in CEF cultures by homologous recombination followed by infection with FPV M3 and transfection with pKG10a-OVA-IRES-EGFP. Recombinant viruses were amplified by selection for Ecogpt expression (blue plaque purification) and then plaque-purified on the basis of the loss of β-gal expression (white plaque purification). The presence of OVA-IRES-EGFP in the viral genome was verified by PCR.

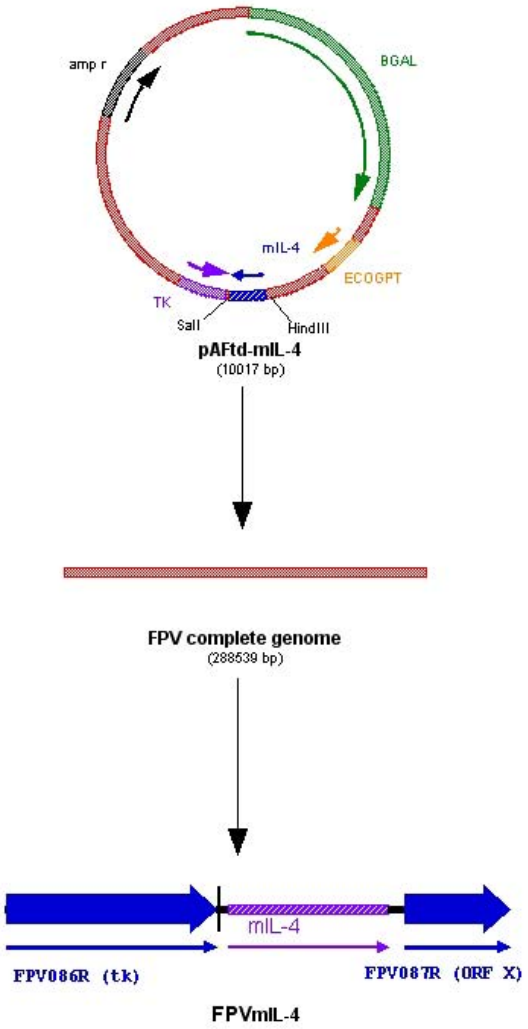


**Figure 3.9 Construction of the $FPV_{mIL-4}$ virus.**

The transient dominant selection vector pAFtd was used for the insertion of the mIL-4 sequence between the thymidine kinase (TK) gene and the uncharacterized gene ORF X (FPV086 & FPV087). $FPV_{mIL-4}$ was generated in CEF cultures by homologous recombination followed by infection with FPV M3 and transfection with pAFtd-mIL-4. Recombinant viruses were amplified by selection for Ecogpt expression (blue plaque purification) and then plaque-purified on the basis of the loss of β-gal expression (white plaque purification). The presence of mIL-4 in the viral genome was verified by PCR.

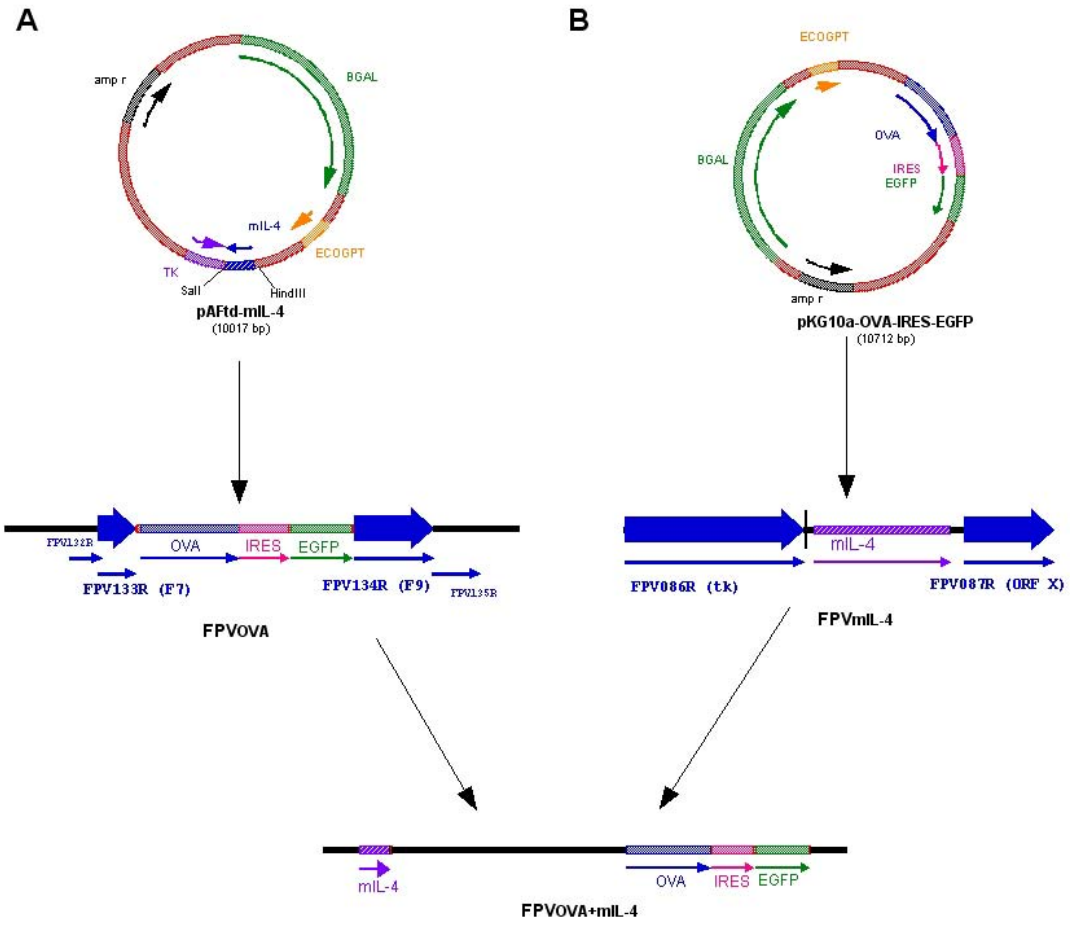


**Figure 3.10 Construction of the $FPV_{OVA+mIL-4}$ virus.**

$FPV_{OVA+mIL-4}$ was constructed by the sequential insertion of pKG10a-OVA-IRES-EGFP and pAftd-mIL-4 into the FPV genome. (**A**) The insertion of pAftd-mIL-4 into the recombinant $FPV_{OVA}$ virus. (**B**) The insertion of pKG10a-OVA-IRES-EGFP into the recombinant $FPV_{mIL-4}$ virus.

## 3.4 CELLULAR TROPISM AND EXPRESSION PATTERNS OF $FPV_{OVA}$, $FPV_{mIL\text{-}4}$ AND $FPV_{OVA+mIL\text{-}4}$ *IN VITRO*

### *3.4.1 Expression of OVA and mIL-4 by FPV recombinants*

For quantitative analysis of OVA and mIL-4 expression, supernatants from rFPV-infected QT35 cells were tested in direct capture ELISA. EGFP expression, present in the rFPVs containing OVA, was used as a fluorescent marker for successful cell infection and effective gene expression and was detected via fluorescent microscopy 24 hours after infection (Figure 3.11A). As expected, EGFP expression was only detectable in cells infected with $FPV_{OVA}$ and not FPV M3-infected or uninfected cells. Analysis by ELISA of cell supernatants taken from these cells (Figure 3.11B) revealed high levels of OVA expression by $FPV_{OVA}$ (125 pg/ml) as opposed to uninfected and FPV M3-infected cultures which did not exhibit any significant OVA production (< 25 pg/ml). Similarly, high levels of mIL-4 (225 pg/ml) were detected in cell supernatant from QT35 cells infected with $FPV_{mIL\text{-}4}$ in comparison to uninfected and FPV M3-infected controls (Figure 3.12).

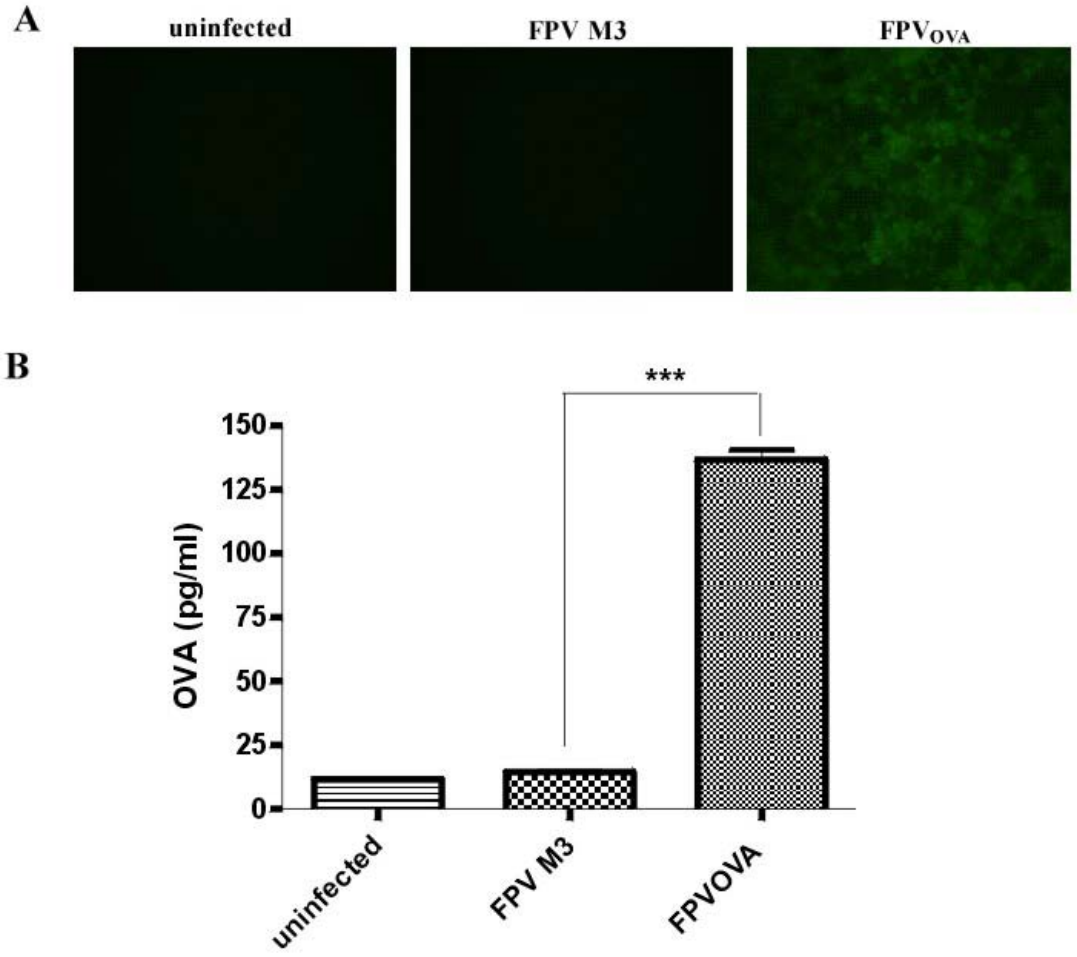


**Figure 3.11 OVA and EGFP expression by QT35 cells infected with FPV$_{OVA}$.**

(**A**) QT35 cells were infected with FPV$_{OVA}$ or wild type FPV M3 at an MOI of 10. 24 hours after infection, cells were examined for signs of infection and EGFP expression via fluorescent microscopy at 20x magnification. (**B**) The presence of OVA was detected in cell supernatants via direct capture ELISA on plates pre-coated with 2 μg/ml of goat anti-OVA polyclonal antibody. Results are expressed as mean $^{+}/_{-}$ SEM of duplicate wells and were considered significant when $p < 0.05$ where p values are denoted thus: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

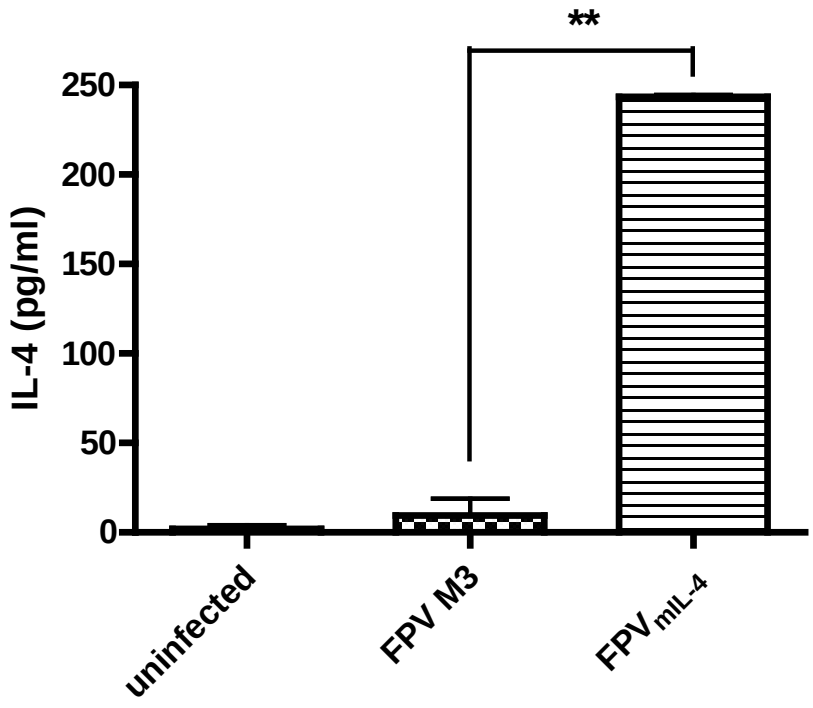


**Figure 3.12 mIL-4 expression by QT35 cells infected with $FPV_{mIL\text{-}4}$.**

mIL-4 expression by the recombinant $FPV_{mIL\text{-}4}$ virus was confirmed by infecting QT35 cells with $FPV_{mIL\text{-}4}$ at an MOI of 10. 24 hours after infection, the presence of mIL-4 was detected in cell supernatants via direct capture ELISA. Results are expressed as mean $^{+}/_{-}$ SEM of duplicate wells and were considered significant when $p < 0.05$ where p values are denoted thus: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Unexpectedly, while $FPV_{OVA}$ and $FPV_{mIL\text{-}4}$ demonstrated high levels of OVA and mIL-4 expression consecutively, $FPV_{OVA+mIL\text{-}4}$ only had detectable levels of mIL-4 but not OVA (Figure 3.13B and C). This was surprising as PCR analysis of $FPV_{OVA+mIL\text{-}4}$ with OVA and mIL-4-specific primers had both yielded clear positive bands of comparative intensity, indicating that the percentage of double-recombinant virus should be high (Figure 3.13A). Although an OVA-positive band was obtained for the uninfected cell sample, it was anticipated due to OVA being a chicken protein and the viral DNA having originated from infected CEF cells. In addition, while $FPV_{OVA+mIL\text{-}4}$ had detectable levels of mIL-4, those of $FPV_{mIL\text{-}4}$ were almost two-fold higher, suggesting that the secondary insertion of the OVA gene had reduced the level of mIL-4 expression.

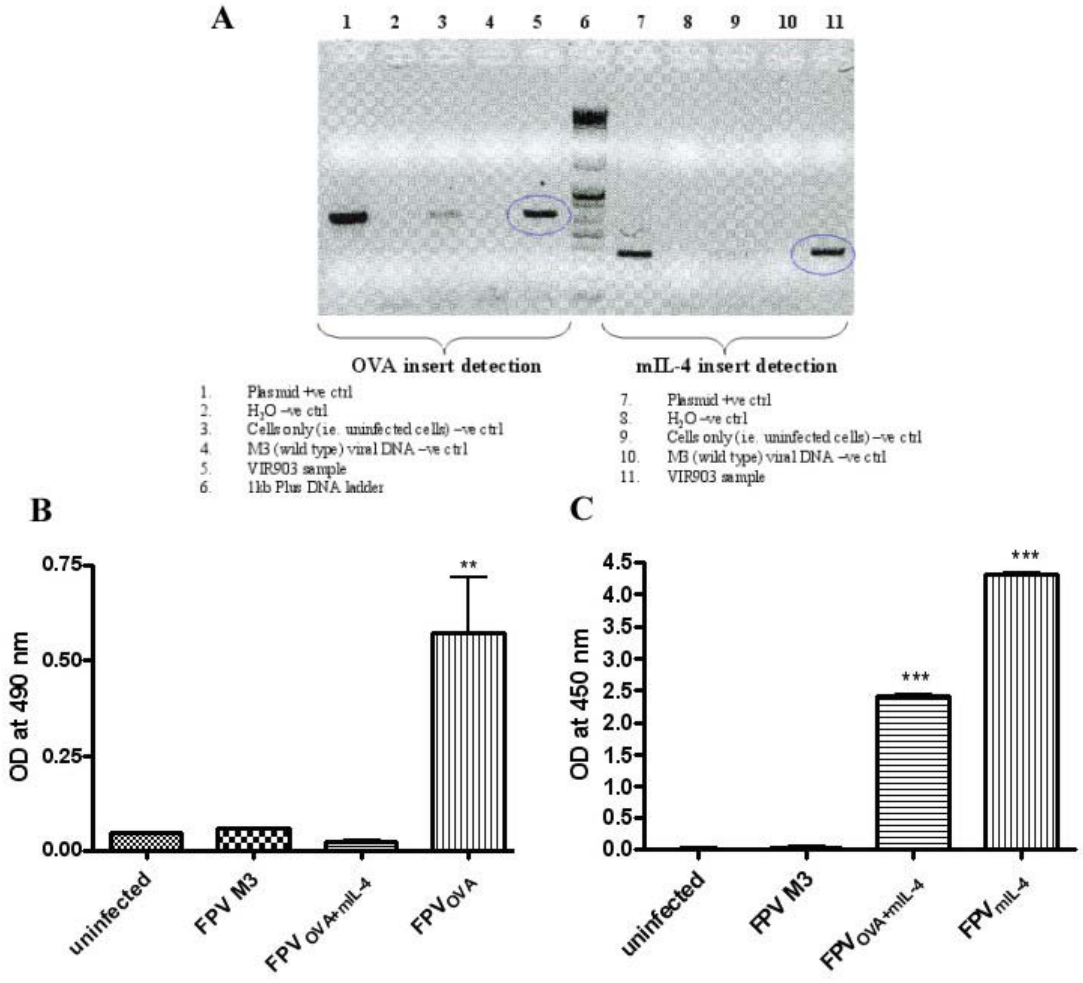


**Figure 3.13 $FPV_{OVA + mIL-4}$ expresses mIL-4 but not OVA.**

(**A**) PCR verification of the presence of OVA and mIL-4 in the viral genome. OVA and mIL-4 expression by the recombinant $FPV_{OVA+mIL-4}$ virus was confirmed by infecting QT35 cells at an MOI of 10. 24 hours after infection, the presence of OVA (**B**) and mIL-4 (**C**) were detected in cell supernatants via direct capture ELISA. Results are expressed as mean $^{+}/^{-}$ SEM of duplicate wells and were considered significant when $p< 0.05$ (as indicated by *) *, P<0.05; **, P<0.01; ***, P<0.001.

### *3.4.2. Kinetics of rFPV-encoded gene expression*

In order to determine the kinetics of rFPV-encoded gene expression, QT35 cells were infected with $FPV_{OVA}$ and examined via fluorescent microscopy for EGFP expression 0-168 hours (7 days) after infection. Figure 3.14 shows direct comparisons of EGFP expression of infected QT35 cells at 0-168 hours (7 days) p.i. where expression was detected within 24 hours of infection and peaked at 48 hours. By 72 hours p.i., infected cells had become apoptotic or died and subsequently EGFP expression declined until 168 hours at which point only a few infected EGFP-expressing cells remained. Uninfected and FPV M3-infected cells were used as negative controls for autofluorescence. As expected, no fluorescence was observed in these groups during the 7 day period (data not shown).

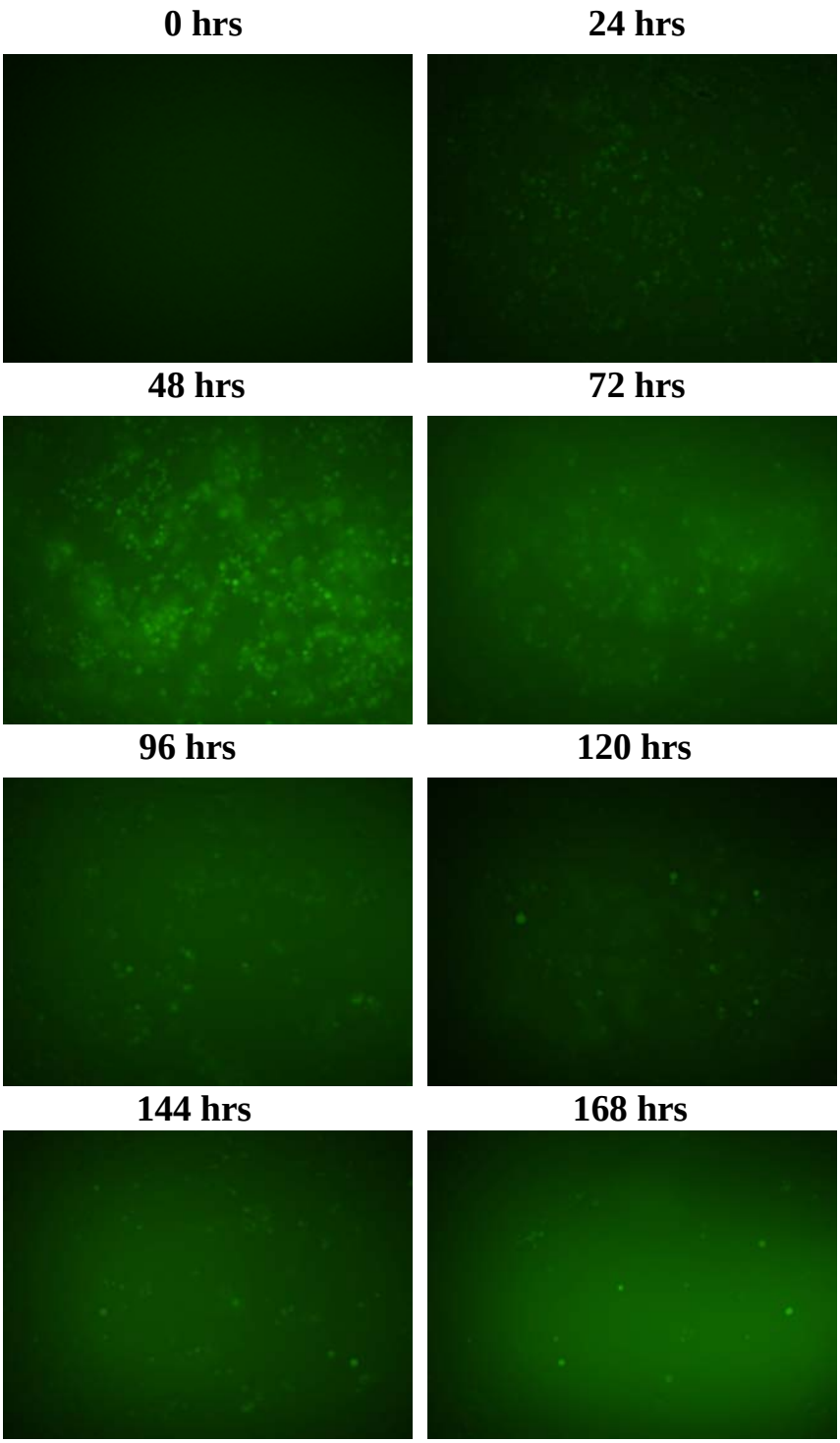


**Figure 3.14 Kinetics of rFPV-encoded gene expression.**

QT35 cells were infected at an MOI of 10 and examined via fluorescent microscopy for signs of EGFP expression 0-168 hours later. Cells were observed at 20X magnification.

### *3.4.3. rFPV infects a wide variety of mammalian cell types*

In order to gain an idea of how well OVA and mIL-4 would be expressed *in vivo* following immunisation with $FPV_{OVA}$ and/or $FPV_{mIL\text{-}4}$, their *in vitro* expression patterns in non-permissive mammalian cell lines were determined. As the cellular tropism of rFPV would not be affected by the identity of the foreign insert (OVA or mIL-4), the $FPV_{OVA}$ virus was used for these studies due to its expression of EGFP where EGFP was used as a fluorescent marker for successful cell infection and effective gene expression.

A range of mammalian cell types were infected including the human embryonic kidney cell line HEK 293, murine macrophage cell line IC21 and murine dendritic cell line JAWS II. All cell types infected had EGFP expression above background and similar profiles resulted from rFPV infection of mammalian cells when compared with those from avian cells (Figure 3.15).

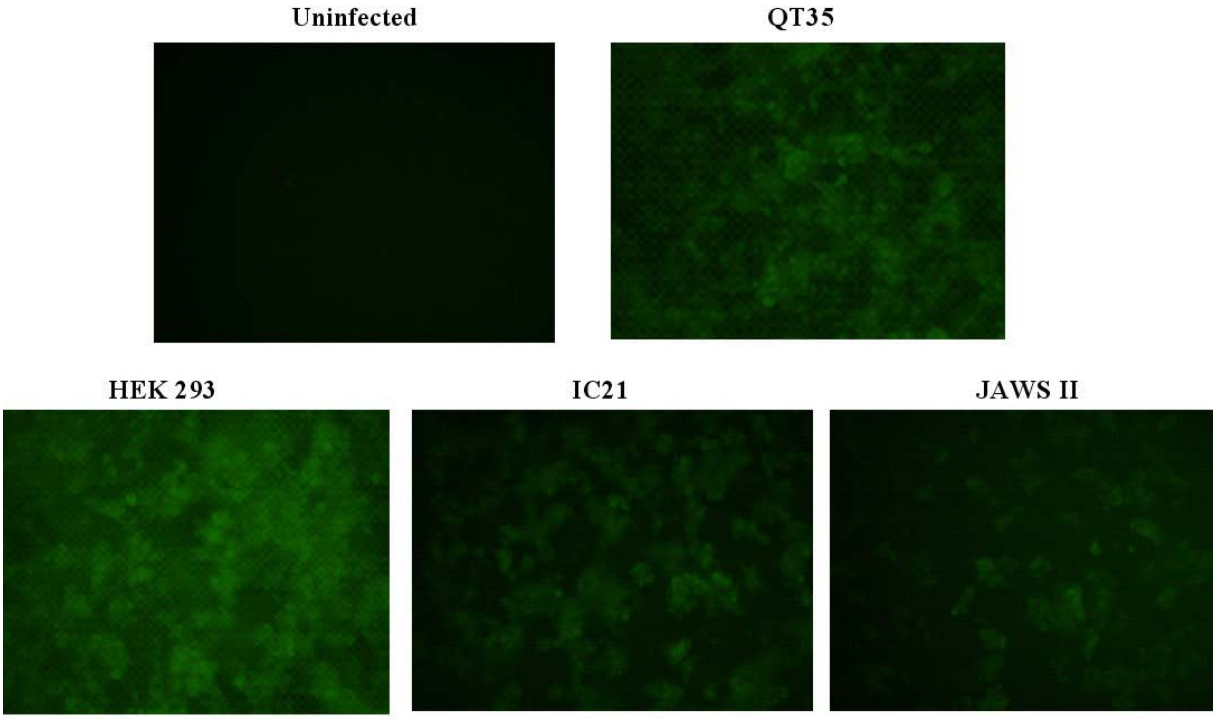


**Figure 3.15 rFPV infects a variety of mammalian cell types.**

Cells were infected at an MOI of 10 and examined via fluorescent microscopy for EGFP expression 48 hours later. Cells were observed at 20X magnification. Data is representative of 3 individual wells per group and 2 independent experiments.

## 3.5 THE INTERACTIONS BETWEEN $FPV_{OVA}$ AND THE MAMMALIAN IMMUNE SYSTEM

Because the $FPV_{OVA+mIL-4}$ virus did not express detectable levels of OVA, the $FPV_{OVA}$ and $FPV_{mIL-4}$ viruses were co-administered in experiments designed to study the effect of mIL-4 on OVA-specific responses (Section 3.6). Therefore, to ensure that the $FPV_{OVA}$ only groups received the same number of viral particles as those immunised with $FPV_{OVA}$ + $FPV_{mIL-4}$, mice were co-immunised with equal doses of $FPV_{OVA}$ and FPV M3.

### *3.5.1 FPV infection induces a strong inflammatory response*

There is accumulating evidence suggesting that generalized poxvirus infections involve an over-production of inflammatory cytokines and soluble mediators (180). These contribute to pathogenesis by inducing sepsis and septic shock. While transmission of FPV is limited to avian species and infection of mammalian cells results in an abortive replication and no evidence of overt disease, infection at high MOIs can cause cytopathic effects in mammalian cells (181). To determine whether FPV infection induces inflammation in a mammalian host, mice were immunised with FPV M3 in the right footpad and the spleen and popliteal and para-aortic lymph nodes were removed 0, 6, 24, 72, 120 or 168 hours later. These were examined macroscopically for visible signs of inflammation. Organs (both draining and non-draining) from FPV-infected mice were larger and inflamed in comparison to uninfected mice 24 hours after immunisation (Figure 3.16) until the termination of the experiment at 168 hours (data not shown).

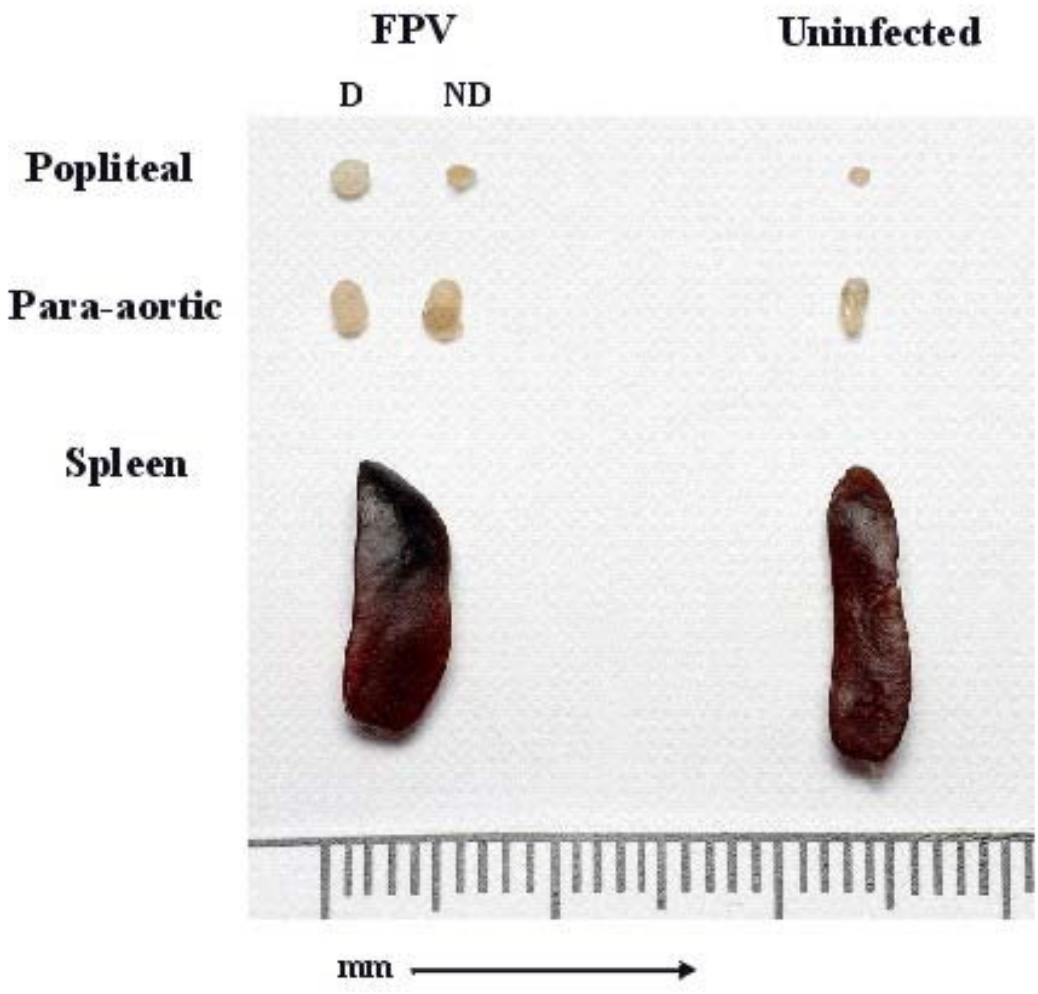


**Figure 3.16 FPV infection induces inflammation.**

C56BL/6 mice were imunised with 5 x $10^6$ pfu FPV M3 in a final volume of 50 µl in the right footpad and 50 µl of PBS in the left. The spleen and popliteal and para-aortic lymph nodes were removed 0, 6, 24, 72, 120 or 168 hours later and examined for macroscopically visible signs of inflammation. Organs appeared larger and inflamed 24 hours after immunisation (shown above) until the termination of the experiment at 168 hours. Data is representative of three mice per time point.

### *3.5.2. Antigen expression pattern and kinetics following $FPV_{OVA}$ infection*

It was clear from macroscopic observation of the organs of infected mice that FPV infection induced systemic inflammation. To identify the location and kinetics of FPV-encoded OVA expression and whether it would be restricted to the local draining lymph nodes (DLN) or be more systemic, CFSE-labelled OT-I T cells were adoptively transferred (AT) into mice 7, 5, 3, 1 or 0 days after immunisation with $FPV_{OVA}$ + FPV M3 in their right footpad and PBS in

their left (Fig 3.17). 2 ¾ days after adoptive transfer (AT), the lymphoid tissues and spleen were examined by flow cytometry for evidence of T cell proliferation. OVA expression was rapid with OT-I T cell proliferation peaking at d0 and being absent by d7. In addition, OT-I T cell proliferation was only observed in the right-hand nodes (draining) of all recipient mice. CFSE-labelled OT-I T cells recovered from the popliteal (POP, PI = 16.89) and para-aortic (PALN, PI = 6.35) lymph nodes having proliferated vigorously in the 3 day period in which they had resided in the recipient mice. There was no evidence of OT-I T cell proliferation in the spleen (SPL, PI = 1), inguinal, brachial, axillary or mesenteric lymph nodes (data not shown). These results indicate that while inflammation is systemic, OVA-expression was rapid and highly localized, being restricted to the draining popliteal and para-aortic lymph nodes.

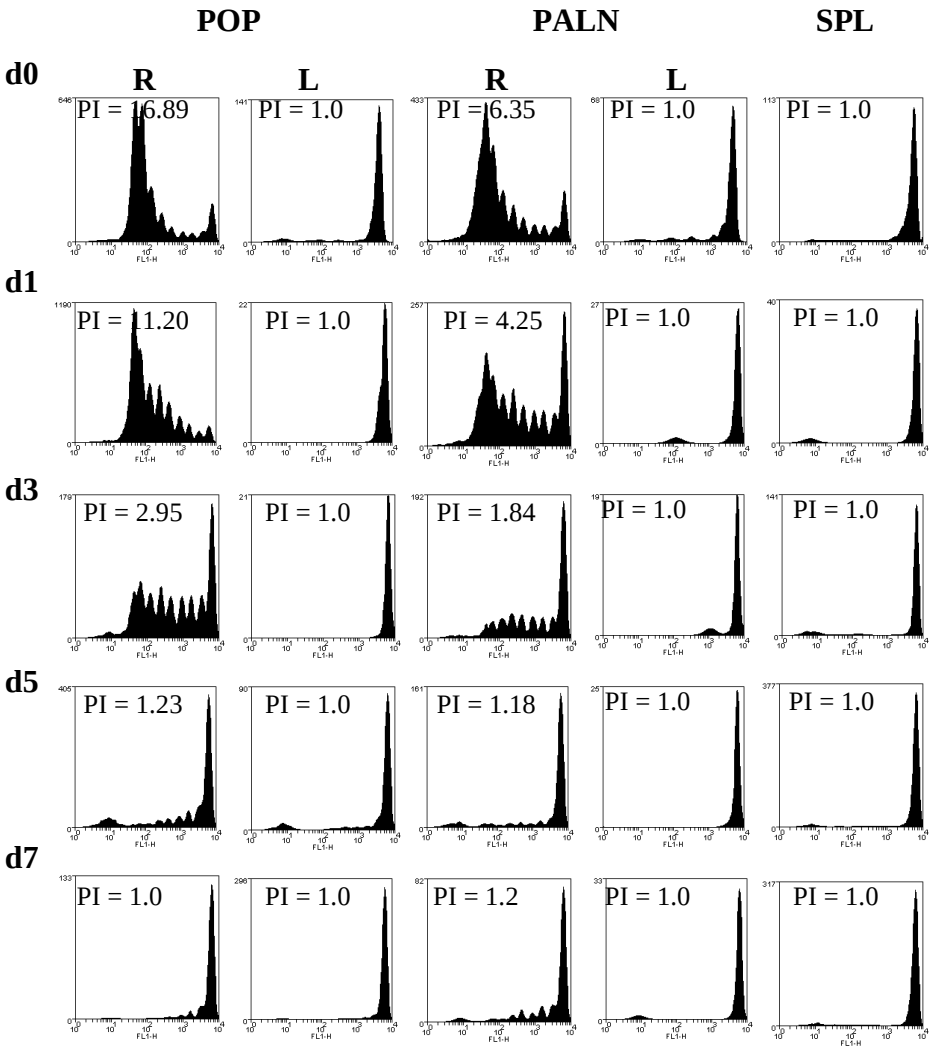


**Figure 3.17 FPV-encoded OVA expression is restricted to the draining lymph nodes and is undetectable by 7 days p.i.**

2.5 x $10^6$ pfu $FPV_{OVA}$ + 2.5x$10^6$ pfu FPV M3 was administered via the footpad to C56BL/6 mice up to 7 days prior to intravenous injection of $10^7$ CFSE-labeled OT-I T cells. 2 ¾ days after adoptive transfer the lymphoid tissues and spleen were examined by flow cytometry for evidence of T cell proliferation. All profiles were gated on the $CD8^+$, $CD45.1^+$, $CFSE^+$ cells and data is representative of 5 mice per group. The extent of division was determined by calculating the proliferation index (PI), which is a ratio of cells recovered: cells transferred. The PI formula is $PI = (n_{PP} + n_{G1} + n_{G2})/(n_{PP}/2^{PP} + n_{PP}/2^{G1} + n_{PP}/2^{G2})$ where *n* = cell number in the peak, *PP* = parent peak, *G1* = generation 1, *G2* = generation 2 etc.

To determine whether FPV-encoded OVA was also presented in the context of MHC class II, CFSE-labelled OT-II cells were adoptively transferred into mice that had been immunised with either $FPV_{OVA}$ + FPV M3 or the MHC class II-restricted OVA peptide $OVA_{323-339}$ in the right footpad and PBS in the left. 2 ¾ days later, these mice were sacrificed and their popliteal (Figure 3.18), para-aortic and inguinal (data not shown) lymph nodes were analysed by flow cytometry. OT-II proliferation was only seen in $OVA_{323-339}$-immunised mice and was restricted to the draining right popliteal and para-aortic lymph nodes. Neither the PBS negative control or $FPV_{OVA}$ + FPV M3 immunised mice showed any OT-II T cell proliferation in any of the lymph nodes analysed (PI =1). It is likely that the lack of OT-II proliferation is as a result of limiting concentrations of OVA that are inadequate to drive OT-II T cell proliferation.

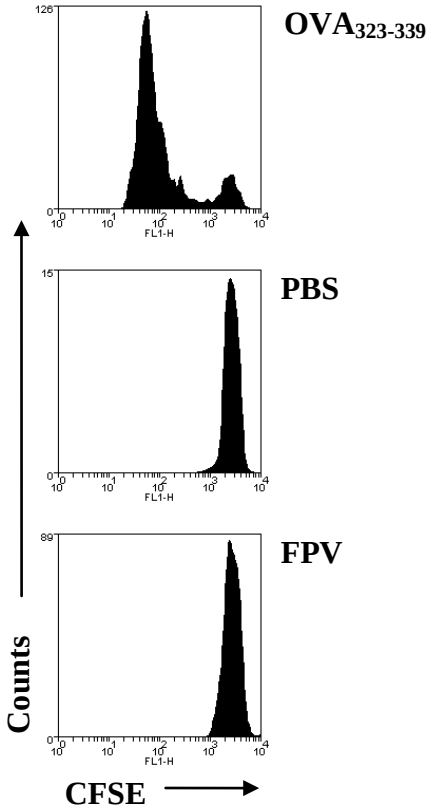


**Figure 3.18 $FPV_{OVA}$ does not induce OT-II proliferation.**

C57BL/6 mice were injected with $10^7$ CFSE-labelled transgenic OT-II T cells i.v. followed by $2.5x10^6$ pfu $FPV_{OVA}$ + $2.5x10^6$ pfu FPV M3 or 20 µg of the MHC class II-restricted OVA peptide $OVA_{323-339}$ in the right footpad and PBS in the left. Three days later, these mice were sacrificed and their popliteal lymph nodes were analysed by flow cytometry. Profiles are gated on $CD8^+$, $CFSE^+$ cells.

While it was clear that FPV-encoded OVA was presented in the context of MHC class I, the identity of the presenting cell was unknown. Therefore, to determine whether bone-marrow (BM)-derived cells were required as antigen presenting cells (APCs), BM chimaeric mice were constructed by lethally irradiating C57BL/6 mice and reconstituting them with BM from $B6^{bm1}$ mice.Both C57BL/6 and $B6^{bm1}$ mice have the $H\text{-}2^b$ MHC class I haplotype, however, $B6^{bm1}$ mice have a single amino acid alteration in the $K^b$ region that prevents them from presenting the $H\text{-}2K^b$-restricted dominant OVA epitope ($OVA_{257\text{-}264}$) to $CD8^+$ T cells (182). All possible combinations were made (where all bone marrow chimaeras will be referred to as bone marrow donor → irradiated recipient) ; B6 reconstituted with B6 (B6 →B6), B6 reconstituted with $B6^{bm1}$ (B6 → $B6^{bm1}$), $B6^{bm1}$ reconstituted with B6 ($B6^{bm1}$ → B6) and $B6^{bm1}$ reconstituted with $B6^{bm1}$ ($B6^{bm1}$ → $B6^{bm1}$). Chimaeric mice were assayed for FPV-derived antigen presentation by the OT-I AT model where mice were immunised with $FPV_{OVA}$ + FPV M3 in the right footpad followed by CFSE-labelled OT-I T cells i.v. (Figure 3.19). Two and ¾ days after AT, the lymphoid tissues and spleen were examined by flow cytometry for evidence of T cell proliferation. As expected, proliferation was only observed in the draining right popliteal (represented in Figure 3.19) and para-aortic lymph nodes. Interestingly, only chimaeric mice reconstituted with B6 BM cells could present FPV-derived OVA to OT-I T cells (Figure 3.19; B6→B6 and B6→$B6^{bm1}$ histograms). A small amount of proliferation was observed in B6 mice reconstituted with $B6^{bm1}$ BM cells; however this likely represents presentation of antigen by radio-resistant B6 host cells ($B6^{bm1}$→B6). No presentation was observed in control $B6^{bm1}$→$B6^{bm1}$ mice, with results taken as a whole clearly indicating that presentation of FPV-derived antigen requires permissive BM-derived APCs. In order to confirm that it was not route-dependent, chimaeric mice were immunised with $FPV_{OVA}$ + FPV M3 i.p. followed by CFSE-labelled OT-I T cells i.v. (data not shown). The same pattern of proliferative responses was observed in B6→B6 and B6→$B6^{bm1}$ mice, suggesting that BM-derived antigen presenting cells were indeed necessary for the presentation of FPV-derived antigen to $CD8^+$ T cells.

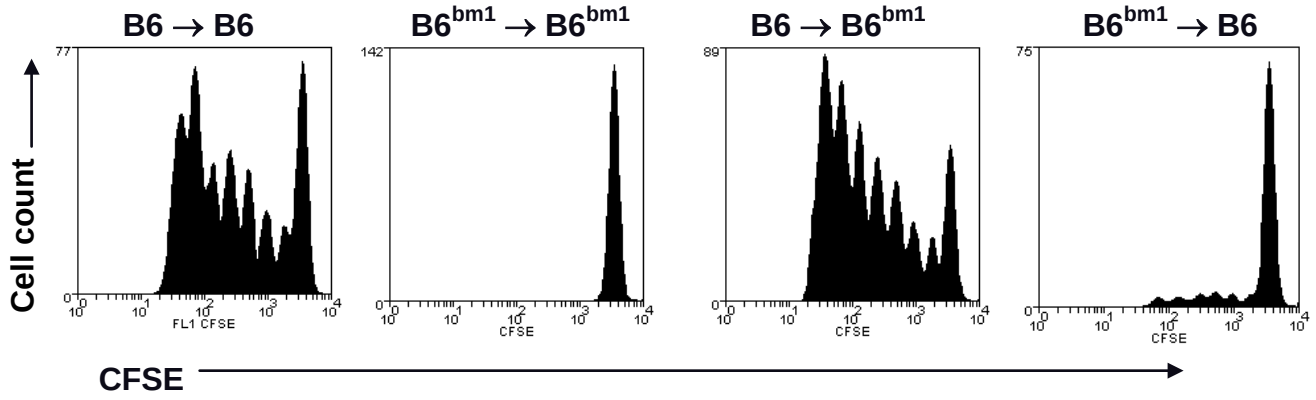


**Figure 3.19 Bone marrow-derived APCs present $FPV_{OVA}$-derived antigen to $CD8^+$ T cells.**

Lethally-irradiated B6 and $B6^{bm1}$ mice reconstituted with BM cells from B6 and $B6^{bm1}$ donor animals were immunised with $FPV_{OVA}$ in the footpad and CFSE-labelled transgenic OT-I T cells i.v. Three days later, these mice were sacrificed and their popliteal lymph nodes were analysed for CFSE-labelled OT-I T cell proliferation by flow cytometry. Profiles are gated on $CD8^+$ $CFSE^+$ cells. Data is representative of three independent experiments with three mice per group.

### *3.5.3. Immunisation with $FPV_{OVA}$ induces a strong anti-OVA cytotoxic T cell response, weak T helper response and moderate humoral response*

To determine whether $FPV_{OVA}$ could generate antigen-specific cell-mediated and humoral immune responses *in vivo*, mice were immunised with $FPV_{OVA}$ or parental vector FPV M3 with serum and splenocytes harvested at the end of the experimental period for analysis. Splenic effector cell cytotoxicity was measured via a direct CTL assay 5 days after $FPV_{OVA}$ immunisation, as this represents the time of maximal antigen-specific CTL responses (determined in previous kinetic experiments). Indeed, *ex vivo* assessment of antigen-specific cytolytic activity indicated the induction of a strong anti-OVA CTL response (55% specific lysis at 200:1 E:T ratio), compared with undetectable responses in mice receiving the parental FPV M3 vector (Figure 3.20A). Helper T cell (Th) responses were determined by assessing the proliferative response of harvested splenocytes incubated *in vitro* with OVA protein or Con A as a non-specific proliferation positive control (Figure 3.20B). Mean levels of OVA-specific proliferation from mice immunised with $FPV_{OVA}$ were 3-4 fold higher than background levels from animals immunised with parental FPV M3 vector (6706 CPM +/- 1020 compared to 1766 CPM +/- 950). In contrast, both groups displayed strong non-specific proliferation (15,000 CPM +/- 2,500) with Con A re-stimulation.

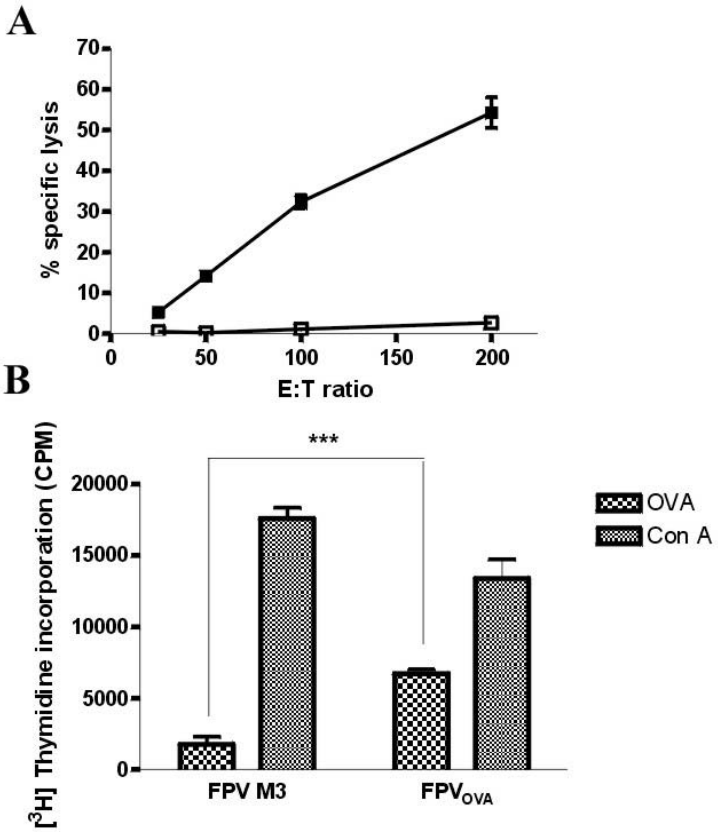


**Figure 3.20 Immunisation with $FPV_{OVA}$ induces a strong anti-OVA CTL response and weak T helper response.**

C57BL/6 mice were immunised with $2x10^7$ pfu FPV M3 (□) or $1x10^7$ pfu $FPV_{OVA}$ + $1x10^7$ pfu FPV (■) i.p. and 5 days later splenocytes were harvested as analysed for cytotoxic T cell responses by direct CTL (**A**) and T helper responses (**B**). (**A**) Cytotoxicity was measured by incubation with EL-4 target cells pulse-labeled with the OVA MHC class I peptide SIINFEKL starting at E:T ratios of 200:1 for 18 hours. The percentage of specific release was determined by the standard equation % specific lysis = (experimental release-spontaneous release)/ (maximum release-spontaneous release) x 100. (**B**) Cells were incubated with 100 μg/ml of OVA protein or 10 μg/ml ConA and incubated at 37°C for 3 days. Proliferation was assessed by the addition of 1 μCi/well of [$^3$H] thymidine for 16 hours. Incorporation of [$^3$H] thymidine was measured by harvesting the cells onto Filtermate glass fibre filters and counting on a Topcount beta counter. Results are expressed as mean $^{+}/_{-}$ SEM of triplicate wells and were considered significant when $p< 0.05$ and p values are denoted thus: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Data is representative of 6 independent experiments containing three mice per group.

In order to measure antibody responses to OVA and determine the isotype of OVA-specific antibody, sera were collected from immunised mice 5, 10, 15 or 30 days later and examined by ELISA with isotype-specific secondary reagents. At 5 days p.i., mice immunised with

$FPV_{OVA}$ developed anti-OVA antibodies that were predominantly of the IgM isotype (Figure 3.21). No indication of an anti-OVA IgG antibody response was observed during the 30 day post-immunisation period and there was no significant difference in IgM and IgG responses by 15 days post-vaccination (data not shown).

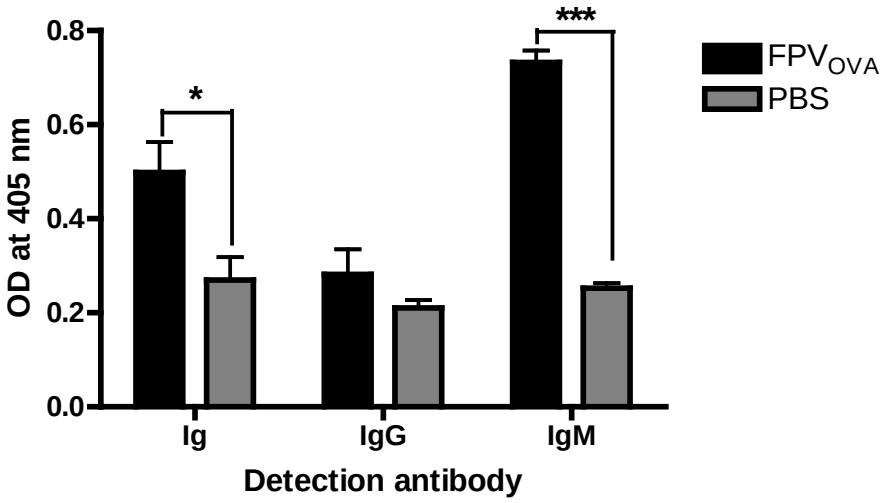


**Figure 3.21 $FPV_{OVA}$ immunisation induces predominantly IgM isotype antibodies.**

6-8 week old C57BL/6 mice were immunised $1x10^7$ pfu $FPV_{OVA}$ + $1x10^7$ pfu FPV M3 or diluent (PBS) i.p. and serum was collected via terminal cardiac puncture 5 days later. Serum samples were analysed for anti-OVA antibodies on ELISA plates precoated with OVA (1μg/ml). Anti-OVA antibodies were detected with anti-mouse Ig, IgM or IgG. Results are expressed as mean $^{+}/_{-}$ SEM of duplicate wells and are representative of 5 experiments. Groups were considered statistically significant as determined by student's t-test, where $p<0.05$ is considered significant and p values are denoted thus: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

### *3.5.4. Bone marrow-derived APCs are required to prime anti-OVA cytotoxic T cell responses*

To determine whether permissive BM-derived APCs were essential for the generation of antigen-specific CTL responses, groups of chimaeric mice were immunised with $FPV_{OVA}$ and compared for the induction of effector function. Effective CTLs were only induced in B6 mice reconstituted with B6 BM (58% specific lysis at 200:1 E:T ratio), with interestingly, no activity observed in either B6→$B6^{bm1}$, $B6^{bm1}$→B6, or in $B6^{bm1}$→$B6^{bm1}$ mice (Figure 3.22A). To distinguish whether the lack of response in $B6^{bm1}$ mice reconstituted with B6 BM was due to the route of immunisation, chimaeric mice were immunised with $FPV_{OVA}$ intravenously. As shown in Figure 3.22B, intravenous immunisation induced a similar profile of CTL activity (80% specific lysis at 200:1 E:T ratio), indicating that either route resulted in the induction of a strong systemic CTL response. The absence of CTL activity in $B6^{bm1}$ mice reconstituted with B6 BM can be interpreted in two ways: one, that both permissive radiation-resistant recipient cells and BM donor cells are essential for the induction of a systemic CTL response, or two, that $FPV_{OVA}$ is cleared rapidly by resident peritoneal or venous cells, resulting in FPV-derived antigens not persisting long enough for BM-derived cells to obtain and present them. To investigate this latter possibility, chimaeric mice were immunised with the maximal concentration of $FPV_{OVA}$ possible to administer, $3x10^8$pfu i.p. As shown in Figure 3.22C, a weak CTL response was indeed generated in B6→$B6^{bm1}$ mice (15% specific lysis at 200:1 E:T), albeit at a 30% lower cytolytic activity than that observed in B6→B6 mice (49% target lysis at 200 E:T ratio). These results suggest that BM-derived antigen presenting cells are indeed necessary for the induction of antigen-specific CTL responses.

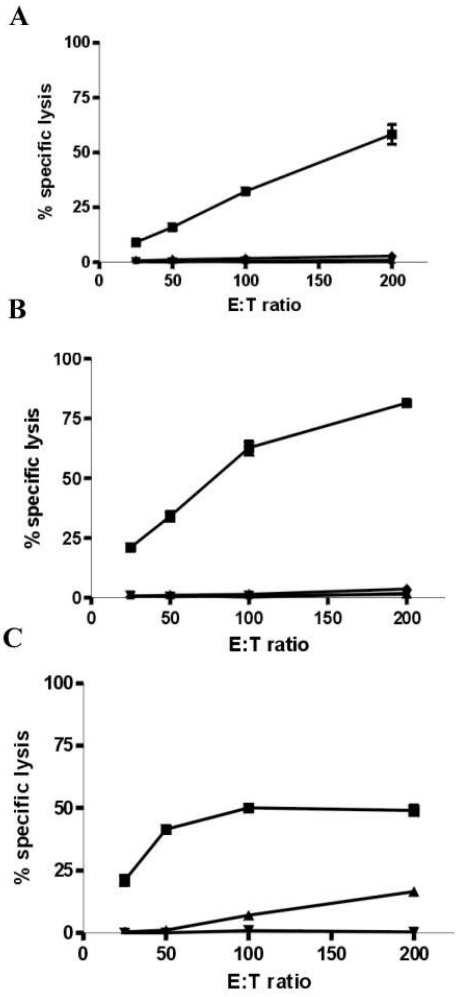


**Figure 3.22 BM-derived APCs are necessary for the induction of antigen-specific CTL responses.**

Lethally-irradiated B6 and $B6^{bm1}$ mice reconstituted with BM cells from B6 and $B6^{bm1}$ donor animals, where B6→B6 (■), B6→$B6^{bm1}$ (▲), $B6^{bm1}$→$B6^{bm1}$ (▼) and $B6^{bm1}$→B6 (♦), were immunised with $1x10^7$ pfu of $FPV_{OVA}$ i.p. (**A**) or i.v. (**B**) or $3x10^8$ pfu of $FPV_{OVA}$ i.p. (**C**). 5 days later OVA-specific lysis was determined via a direct CTL where cytotoxicity was measured by incubation with EL-4 target cells pulse-labeled with the OVA MHC class I peptide SIINFEKL starting at E:T ratios of 200:1. The percentage of specific release was determined by the standard equation % specific lysis = (experimental release-spontaneous release)/ (maximum release-spontaneous release) x 100. Data is representative of two independent experiments containing three mice per group.

### *3.5.5. Correlating OVA responses with vector-specific immunity*

While viral vector vaccine systems are capable of inducing robust immunogen-specific $CD8^+$ T cell immunity, a barrier to their use are the responses directed towards the vector itself (183). To determine the role of vector-specific immunity following immunisation with $FPV_{OVA}$, mice were immunised with $FPV_{OVA}$ and examined for CTL, Th and antibody responses to both OVA and the parental vector FPV M3 5 days later. Direct *ex vivo* assessment of cytolytic activity against HEK 293T-Kb target cells stably transfected to express OVA, infected with FPV M3 or mock-infected with PBS indicated the induction of a strong anti-OVA (56% specific lysis at 200:1 E:T ratio) and moderate anti-FPV (37% specific lysis at 200:1 E:T ratio) CTL response, compared with undetectable responses against mock-infected target cells (Figure 3.23A). Th responses were determined by assessing the proliferative response of harvested splenocytes incubated *in vitro* with OVA protein or UV-inactivated FPV M3. As illustrated in Figure 3.23B, immunisation with $FPV_{OVA}$ generated a significant FPV-specific T cell proliferative response as compared to PBS-immunised animals (79,761 CPM +/- 6,000 vs 4,888 CPM +/- 80). This was in contrast to a weak OVA–specific proliferative T cell response (6706 CPM +/- 1000 vs 1766 CPM +/- 949) and moderate non-specific proliferation observed after Con A re-stimulation (13,366 CPM +/- 2,229 vs 17,575 CPM +/- 665). Analysis by ELISA of serum samples taken from mice immunised with $FPV_{OVA}$, parental vector FPV M3 or diluent PBS at 5 days post-immunisation demonstrated that while no indication of an anti-OVA IgG antibody response was observed, there was a strong anti-FPV IgG response in both the FPVOVA and FPV M3 –immunised mice as compared to PBS-immunised animals (Figure 3.23C).

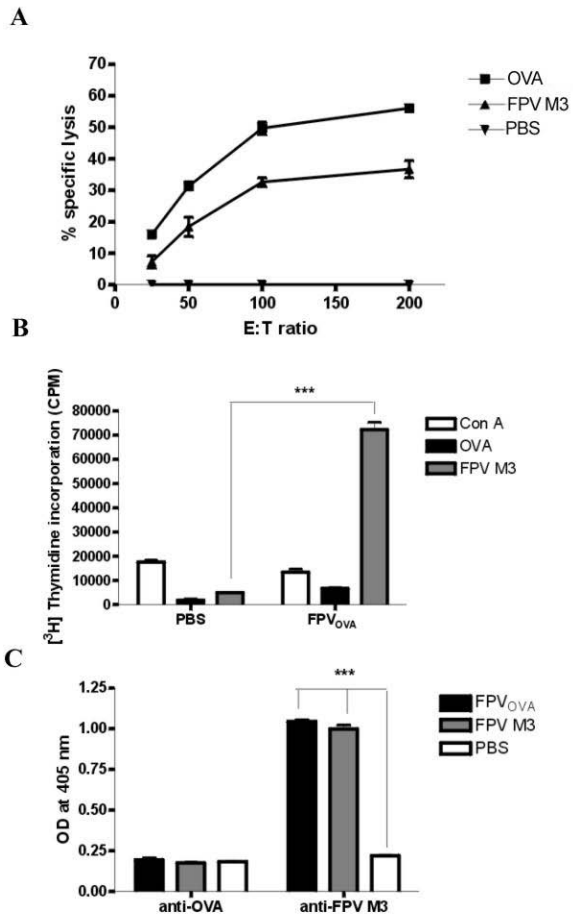


**Figure 3.23 Immunisation with $FPV_{OVA}$ induces strong anti-vector immunity.**

Mice were immunised with $FPV_{OVA}$, parental vector (FPV M3) or diluent (PBS) and anti-OVA and anti-FPV immune responses assessed 5 days later. (**A**) Cytotoxicity was measured by incubation with HEK 293T-Kb target cells stably transfected to express OVA (■), infected with FPV M3 (▲) or mock-infected with PBS (▼) for 18 hours. The percentage of specific release was determined by the standard equation % specific lysis = (experimental release-spontaneous release)/ (maximum release-spontaneous release) x 100. (**B**) Cells were incubated with 100 µg/ml of OVA protein, UV-inactivated FPV M3 or 10 µg/ml ConA and incubated at 37°C for 3 days. Proliferation was assessed by the addition of 1 µCi/well of [$^3$H] thymidine for 16 hours and is expressed as mean +/- SEM of $^3$H-thymidine incorporation in triplicate samples with background cpm subtracted. (**C**) Individual serum samples were assessed for the generation of anti-OVA and anti-FPV IgG antibodies by specific ELISA. All results are expressed as mean $^{+}/_{-}$ SEM of triplicate wells. Groups were considered statistically significant as determined by student's t-test, where $p<0.05$ is considered significant and p values are denoted thus: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Data represented in A and B were kindly contributed by Erin Lousberg (Experimental Therapeutics Laboratory, Hanson Institute, SA, Australia).

In order to define some of the poxvirus determinants recognised by antibody and/or $CD8^+$ T cells and determine their role in protective immunity, four FPV ORFs were selected based on their predicted functions *in vivo*. The selected ORFs were the FPV NTPase DNA replication protein FPV058L, FPV core protein FPV120L, FPV virion envelope protein FPV140L and FPV immunodominant protein FPV168L. The FPV ORFs were aligned with protein sequences derived from poxvirus gene products homologous to VV immunodominant proteins *in silico* in order to gain some insight into potential epitope determinants. Illustrated in Figure 3.24 is the alignment for FPV140L.

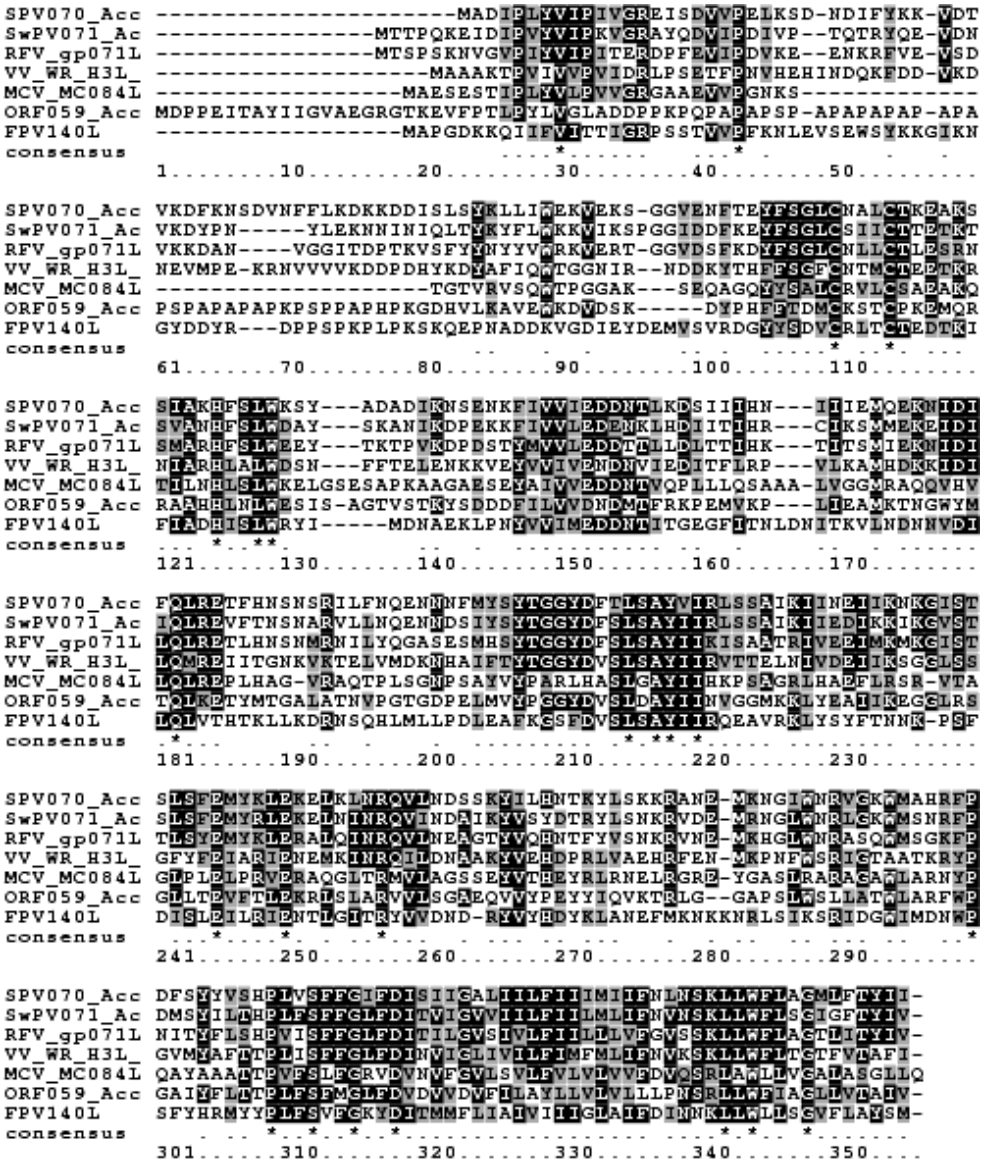


**Figure 3.24 Alignment of protein sequences derived from poxvirus gene products homologous to VV immunodominant proteins.**

Alignment of poxvirus proteins, encoded by the VV H3L homologue with the Angis BioManager program. Poxviruses are as follows: sheeppox virus(SPV070), swinepox virus (SwPV071), rabbit shope fibroma virus (RFV gp071L), vaccinia virus western reserve strain (VV WR H3L), molluscum contagiosum virus (MCV MC084L), orf virus (ORF059) and fowlpox virus (FPV140L). The peptide sequence SLSAYIIRV is highly conserved and has been shown to be a VV HLA-A*0201-restricted CD8+ T cell epitope.

To identify whether the FPV ORFs contained antibody epitopes, mice were immunized with $FPV_{OVA}$ and serum samples were analysed via ELISA against individual FPV proteins as well as OVA (Figure 3.25A). PBS and OVA-CFA-immunised animals were used as the negative and positive anti-OVA controls, respectively. Antibody responses were generated against all of the FPV ORFs, particularly FPV140L and FPV168L although all produced a result superior to that elicited by OVA. To determine whether any of the FPV ORFs contained $CD8^+$ T cell determinants, splenocytes from $FPV_{OVA}$-immunised mice were tested against HEK 293 T-Kb cells stably transfected to express OVA, FPV058L, FPV120L, FPV140L or FPV168L or infected with FPV M3 in the direct cytotoxicity assay (Figure 3.25B). As expected, strong anti-OVA (70% specific lysis at 200:1 E:T ratio) and moderate anti-FPV (30% specific lysis at 200:1 E:T ratio) CTL responses were induced. All of the FPV ORFs failed to induce detectable CTL responses as compared to mock-infected target cells. The absence of CTL activity against the FPV ORFs can be interpreted in two ways: one, that none of the FPV ORFs contain $CD8^+$ T cell determinants, or two, that they do contain $CD8^+$ T cell determinants but are not Kb-restricted. To investigate this latter possibility, the above experiment was repeated but with HEK 293 T cells that had been stably transfected to express the C57BL/6 class I MHC molecule Db and additionally stably transfected to express OVA, FPV058L, FPV120L, FPV140L or FPV168L or infected with FPV M3 (Figure 3.25C). While strong anti-FPV CTL responses were observed (61.5% specific lysis at 200:1 E:T ratio), there was no cytotoxicity against any of the FPV ORFs or OVA when presented in the context of Db target cells.

Taken together, these data suggest that while FPV140L and FPV168L may contain antibody epitopes, none of the selected FPV ORFs contain either Kb or Db-restricted $CD8^+$ T cell determinants.

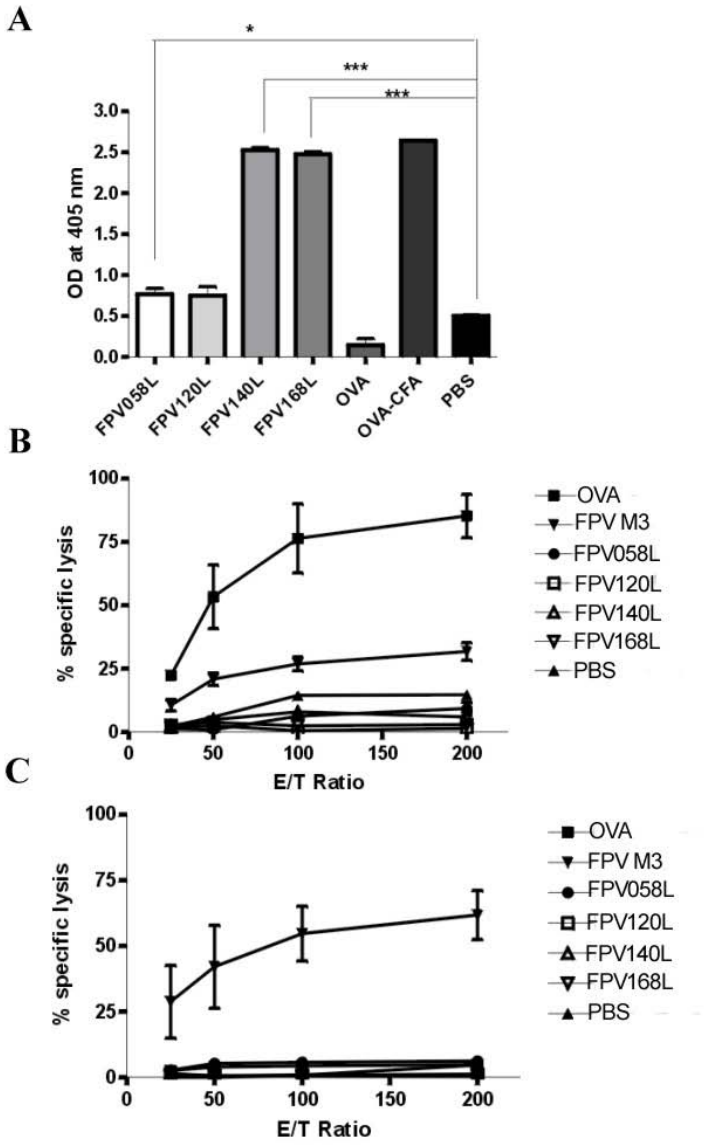


**Figure 3.25 Antibody but not CTL responses are generated against individual FPV proteins.**

Mice were immunised with $FPV_{OVA}$ and anti-OVA and anti-FPV immune responses assessed 5 days later. (**A**) Individual serum samples were assessed for the generation of anti-OVA and anti-FPV ORF IgG antibodies by specific ELISA. All results are expressed as mean $^{+}/_{-}$ SEM of triplicate wells. Cytotoxicity was measured by incubation with HEK 293T- Kb (**B**) or –Db (**C**) target cells stably transfected to express OVA, FPV058L, FPV120L, FPV140L or FPV168L, infected with FPV M3 or mock-infected with PBS for 18 hours. The percentage of specific release was determined by the standard equation % specific lysis = (experimental release-spontaneous release)/ (maximum release-spontaneous release) x 100. Groups were considered statistically significant as determined by student's t-test, where $p<0.05$ is considered significant and p values are denoted thus: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Data represented in B and C were kindly contributed by Erin Lousberg (Experimental Therapeutics Laboratory, Hanson Institute, SA, Australia).

## 3.6 EFFECT OF CO-ADMINISTRATION OF MURINE INTERLEUKIN -4 (mIL-4) ON OVA-SPECIFIC IMMUNITY

Because the $FPV_{OVA+mIL-4}$ virus did not express detectable levels of OVA, the $FPV_{OVA}$ and $FPV_{mIL-4}$ viruses were co-administered in experiments designed to study the effect of mIL-4 on OVA-specific responses.

### *3.6.1. mIL-4 down-regulates primary OVA-specific CTL responses*

It is well established that expression of mIL-4 by replicative viruses such as VV, EV and respiratory syncytial virus (RSV) suppresses cytolytic T cell responses (148, 150, 152, 154, 158). To determine if co-administration of $FPV_{mIL-4}$ with $FPV_{OVA}$ diminished OVA-specific CTL activity, mice were immunised with $FPV_{OVA}$ + FPV M3, $FPV_{OVA}$ + $FPV_{mIL-4}$ or parental vector FPV M3 with splenocytes harvested 5 days p.i.. Direct *ex vivo* assessment of antigen-specific cytolytic activity indicated that mice that received $FPV_{OVA}$ + $FPV_{mIL-4}$ experienced marked attenuation of OVA-specific CTL activity (5.4 LU/$10^7$ effectors) as compared to animals immunized with $FPV_{OVA}$ + FPV M3 (27 LU/$10^7$ effectors) (Figure 3.26).

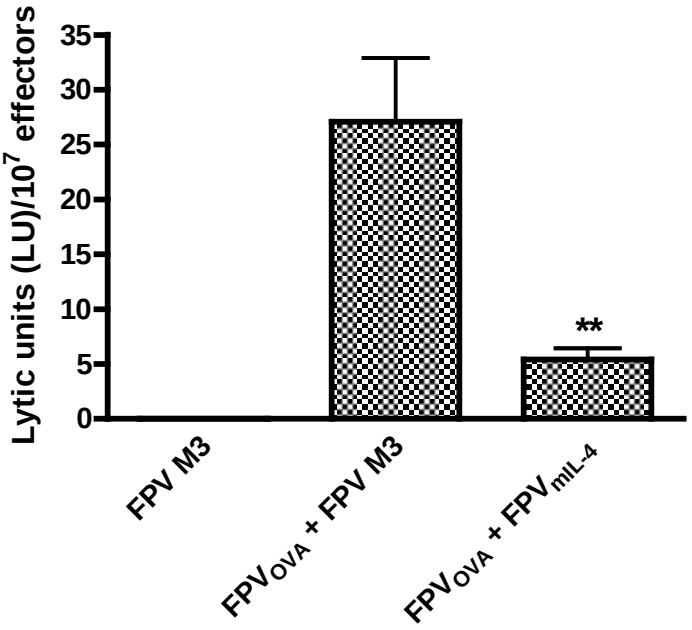


**Figure 3.26 mIL-4 down-regulates primary OVA-specific CTL responses.**

mIL-4 down-regulates primary OVA-specific CTL responses. CTL activity was measured by determination of specific lysis on day 5 p.i. in mice immunised with $2x10^7$ pfu FPV M3 (■), $1x10^7$ pfu $FPV_{OVA}$ + $1x10^7$ pfu FPV M3 (▲) or $1x10^7$ $FPV_{OVA}$ + $1x10^7$ pfu $FPV_{mIL-4}$ by incubation with EL-4 target cells pulse-labeled with the OVA MHC class I peptide SIINFEKL in a direct $^{51}Cr$ release assay. The percentage of specific release was determined by the standard equation % specific lysis = (experimental release-spontaneous release)/ (maximum release-spontaneous release) x 100. Data is shown as lytic units per $10^7$ effectors and is calculated using the formula LU/$10^7$ effectors = $10^7$/ (T-$X_p$) where *T* is the number of target cells ($10^4$), *p* is the reference lysis level (20%) and $X_p$ is the E:T ratio required to lysis p% of the targets. Data is representative of 10 mice per group from 3 independent experiments where values were considered significant when $p < 0.05$ and p values are denoted thus: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

To assess whether mIL-4 simply delays CTL activity rather than diminish it, CTL activity was measured 2, 5, 7, 10 and 14 days p.i. (Figure 3.27). Kinetics for CTL activity in the $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ group was similar to that of the $FPV_{OVA}$ + FPV M3 group, with peak CTL lysis on day 5 p.i.. These data demonstrate that diminished OVA-specific activity observed with co-administration of $FPV_{mIL\text{-}4}$ is not due to a delay in peak CTL activity.

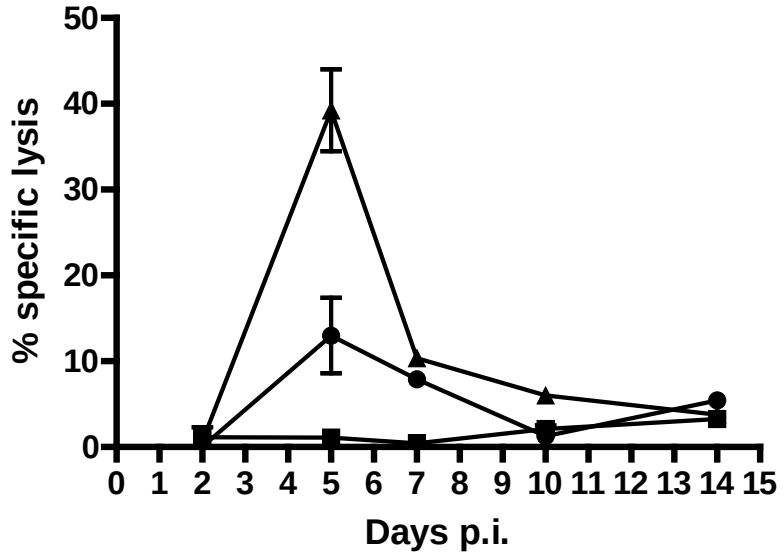


**Figure 3.27 mIL-4 has no effect on the kinetics of the OVA-specific CTL response.**

OVA-specific activity was measured by incubation with EL-4 target cells pulse-labeled with the OVA MHC class I peptide SIINFEKL in a direct $^{51}Cr$ release assay. Kinetics of splenic CTL activity. Mice were injected with FPV M3 (■), $FPV_{OVA}$ + FPV M3 (▲) or $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ (●) and sacrificed on days 2, 5, 7, 10 and 14 p.i.. The percentage of specific release was determined by the standard equation % specific lysis = (experimental release-spontaneous release)/ (maximum release-spontaneous release) x 100. Data represents two independent experiments.

### *3.6.2. mIL-4 has no effect on expression pattern and kinetics of OVA*

Diminished proliferation and clonal expression of antigen-specific $CD8^+$ T cells is one potential mechanism for the reduced CTL activity. Therefore, to determine whether mIL-4 had any effect on the presentation and expression of OVA, CFSE-labelled OT-I T and OT-II cells were adoptively transferred into mice that had been immunised 7, 5, 3, 1 or 0 days before AT in their right footpad with either $FPV_{OVA}$ + FPV M3 or $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$. As

shown in Figure 3.28, mIL-4 had no effect on the proliferation of either OT-I or OT-II cells. Neither $FPV_{OVA}$ + FPV M3 or $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ immunised mice had any OT-II proliferation (PI = 1.0) and this was expected, as previous experiments had demonstrated that FPV-encoded OVA is predominantly expressed in the context of MHC class I. Interestingly, mIL-4 had no effect on the proliferation of OT-I T cells in any of the recipient mice. In both groups FPV-encoded OVA expression was localized rather than systemic, being restricted to the draining right popliteal and para-aortic lymph nodes. No proliferation was seen in the spleen, inguinal, mesenteric or axillary lymph nodes (data not shown). Similarly, mIL-4 had no effects on the kinetics of OVA-expression. Both $FPV_{OVA}$ + FPV M3 and $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ -immunised mice had rapid OT-I proliferation (PI = 12) that was detectable 0 days p.i. and had disappeared 5-7 days later. Together these data demonstrate that immunisation with $FPV_{OVA}$ results in rapid and localized expression of OVA and that co-administration of IL-4 has no effect on this pattern of antigen expression.

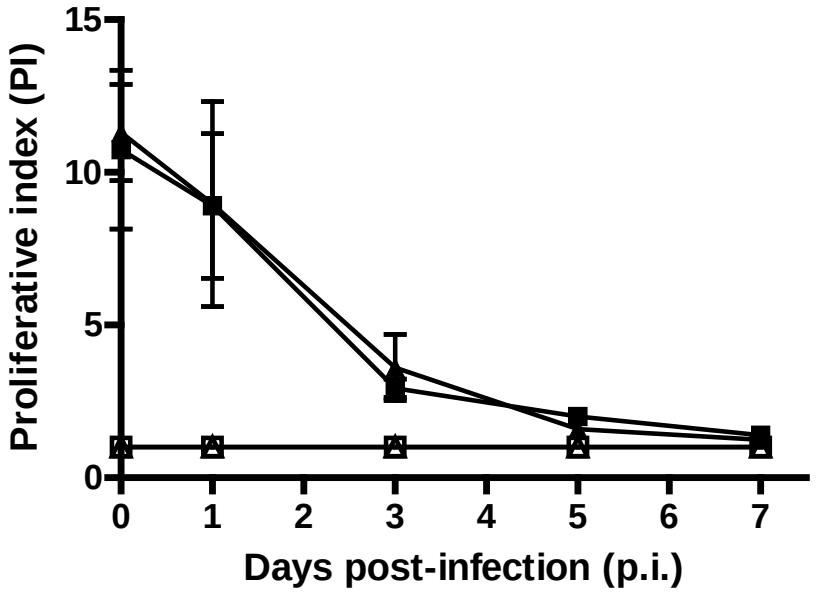


**Figure 3.28 mIL-4 has no effect on the expression pattern and kinetics of OVA.**

C57BL/6 mice were immunized in the right footpad with either $2.5x10^6$ pfu $FPV_{OVA}$ + $2.5x10^6$ pfu FPV M3 (squares) or $2.5x10^6$ pfu $FPV_{OVA}$ + $2.5x10^6$ pfu $FPV_{mIL\text{-}4}$ (triangles) and PBS in the left and 7, 5, 3, 1 or 0 days later they were injected with CFSE-labelled transgenic OT-I (closed symbols) or OT-II (open symbols) T cells i.v.. Three days after adoptive transfer, these mice were sacrificed and their right popliteal lymph nodes removed and analysed by flow cytometry where profiles were gated on the $CD8^+$, $CD45.1^+$, $CFSE^+$ cells. The extent of division was determined by calculating the proliferation index (PI), which is a ratio of cells recovered: cells transferred. The PI formula is $PI = (n_{PP} + n_{G1} + n_{G2})/(n_{PP}/2^{PP} + n_{PP}/2^{G1} + n_{PP}/2^{G2})$ where n = cell number in the peak, PP = parent peak, G1 = generation 1, G2 = generation 2 etc. Data is representative of 5 mice per group.

Although co-administration had no effect on the proliferation of OVA-specific $CD8^+$ T cells, it was possible that the down-regulation in CTL activity was due to a reduction in surface expression of the CD8α molecule itself (161). Therefore, to investigate this, the mean fluorescence intensity of CD8α expression on both OVA-specific (OT-I, CD45.1+) and endogenous (CD45.1-) T cells isolated from the popliteals of mice infected with $FPV_{OVA}$ + FPV M3 or $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ were compared (Figure 3.29). While there was no significant difference in surface CD8α expression on endogenous (CD45.1-) T cells in $FPV_{OVA}$ + FPV M3 or $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ immunised animals, co-administration of mIL-4 resulted in a two-fold reduction in surface CD8α expression on OVA-specific OT-I T cells (895 +/ 146 as compared to 1512.5 +/- 93). Taken together, these data suggest that the mIL-4-induced

down-regulation of primary OVA-specific CTL responses may be in part be due to a reduction in surface CD8α expression on OVA-specific $CD8^+$ T cells.

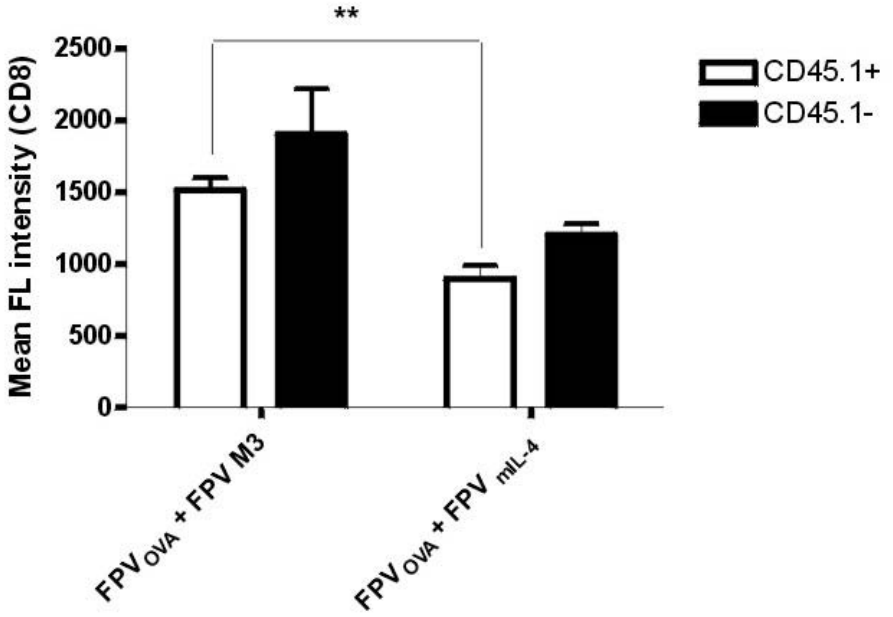


**Figure 3.29 mIL-4 reduces CD8 expression on OVA-specific T cells.**

C57BL/6 mice were injected with CFSE-labelled transgenic OT-I T cells (CD45.1 +ve) i.v. followed by $2.5x10^6$ pfu $FPV_{OVA}$ + $2.5x10^6$ pfu FPV M3 or $2.5x10^6$ pfu $FPV_{OVA}$ + $2.5x10^6$ pfu $FPV_{mIL-4}$ in the right footpad and PBS in the left. Three days later, these mice were sacrificed and their popliteal, para-aortic, inguinal and mesenteric lymph nodes were analysed by flow cytometry. Profiles were gated on either the $CD45.1^+$ (congenic OT-I T cell marker) or $CD45.1^-$ (endogenous T cell population) cells. Graphs are representative of the mean $CD8^+$ fluorescence intensity for each population. Groups were considered statistically significant if $p < 0.05$ and are denoted thus: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

### *3.6.3. Co-administration of mIL-4 increases the Th response but has no effect on antibody responses*

In order to study the effect of co-administered mIL-4 on Th priming, splenocytes isolated from mice immunized with parental vector FPV M3, $FPV_{OVA}$ + FPV M3 or $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ were incubated *in vitro* with OVA protein or Con A as a non-specific proliferation positive control (Figure 3.30A). Mean levels of OVA-specific proliferation from mice immunised with $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ were almost 2 fold higher than animals immunised with $FPV_{OVA}$ + FPV M3 (11,377 CPM +/- 2,638 compared to 6,324 CPM +/- 1,141). All groups displayed strong non-specific proliferation (21,475 CPM +/- 2,500) with Con A re-stimulation. IFN-γ production by the same isolated immune splenocytes following *in vitro* re-stimulation with OVA was also analysed (Figure 3.30B). Interestingly, mIL-4 co-administration markedly suppressed IFN-γ secretion by antigen-specific $CD4^+$ T cells, with a 3 fold reduction in IFN-γ levels as compared to $FPV_{OVA}$ + FPV M3-immunised animals (2.5 pg/ml +/- 1.4 vs 8.8 pg/ml +/- 4.5).

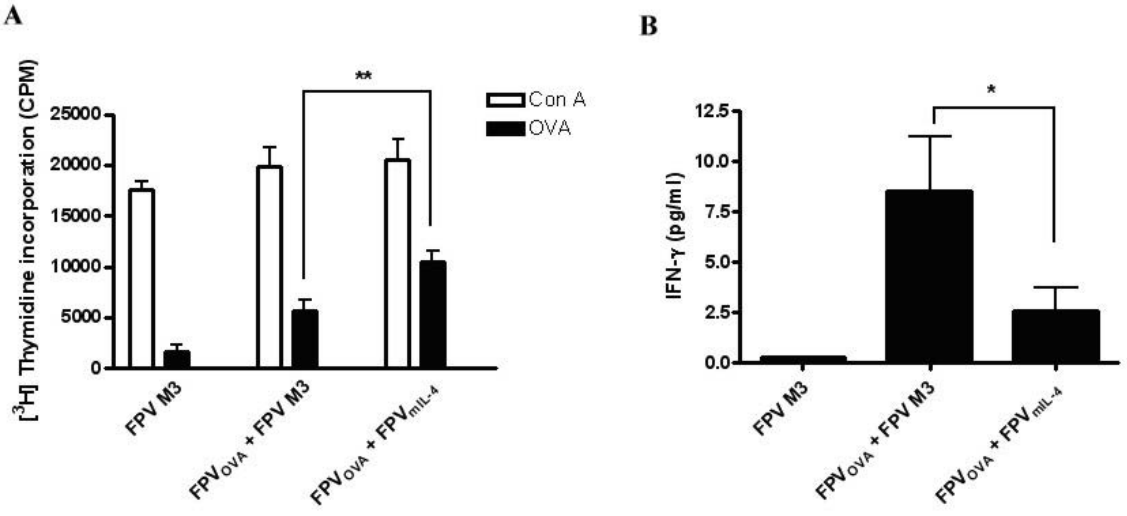


**Figure 3.30 Co-administration of mIL-4 increases the Th response but decreases their IFN-γ production.**

C57BL/6 mice were immunized with $2x10^7$ pfu FPV M3, $1x10^7$ pfu $FPV_{OVA}$ + $1x10^7$ pfu FPV M3 or $1x10^7$ $FPV_{OVA}$ + $1x10^7$ pfu $FPV_{mIL-4}$ and splenocytes were harvested 5 days later and seeded at $4x10^5$cells/well of flat-bottom 96-well plates. (**A**) Cells were incubated with 100 μg/ml of OVA protein or 10 μg/ml ConA and incubated at 37°C for 3 days. Proliferation was assessed by the addition of 1 μCi/well of [$^3$H] thymidine for 16 hours. Incorporation of [$^3$H] thymidine was measured by harvesting the cells onto Filtermate glass fibre filters and counting on a Topcount beta counter. (**B**) Prior to harvesting, 100 μl/well of S/N was removed and the presence of IFN-γ in cell supernatants was detected via ELISA. Results are expressed as mean $^{+}/_{-}$ SEM of triplicate wells and were considered significant when $p < 0.05$ where p values are denoted thus: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

To determine whether co-administration of mIL-4 could influence antibody responses to OVA, sera were collected from immunised mice 5 days p.i. and examined by ELISA with isotype-specific secondary reagents (Figure 3.31). As observed previously, the anti-OVA antibody response generated was primarily of the IgM class, however, there was no significant difference between mice immunized with either $FPV_{OVA}$ + FPV M3 or $FPV_{OVA}$ + $FPV_{mIL-4}$.

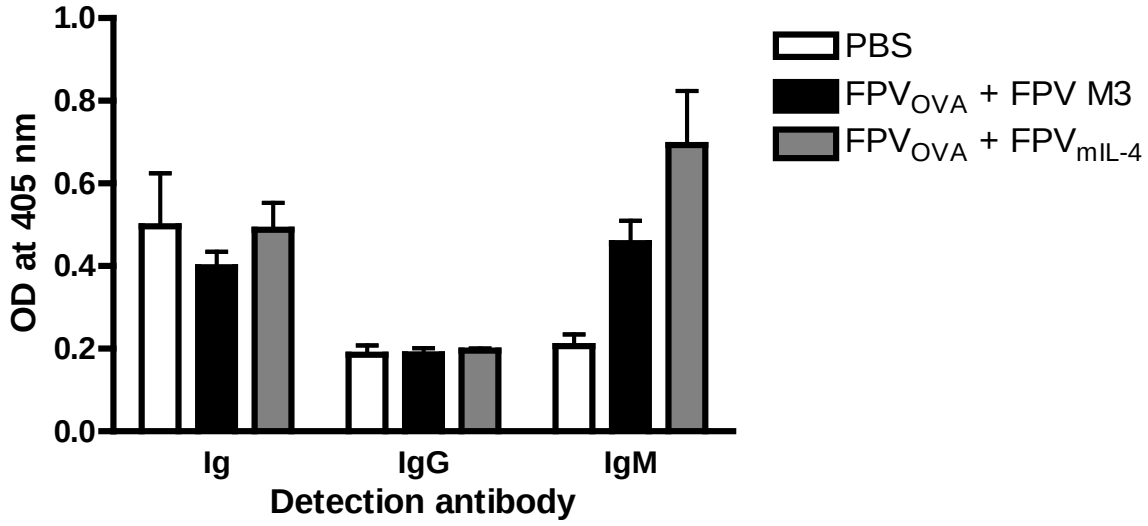


**Figure 3.31 mIL-4 has no effect on antibody isotype.**

6-8 week old C57BL/6 mice were immunised 1x10$^7$ pfu FPV$_{OVA}$ + 1x10$^7$ pfu FPV M3, 1x10$^7$ FPV$_{OVA}$ + 1x10$^7$ pfu FPV$_{mIL-4}$ or diluent (PBS) i.p. and serum was collected via terminal cardiac puncture 5 days later. Serum samples were analysed for anti-OVA antibodies on ELISA plates precoated with OVA (1μg/ml). Anti-OVA antibodies were detected with anti-mouse Ig, IgM or IgG. Results are expressed as mean $^+/^-$ SEM of duplicate wells and are representative of 5 experiments, with 3 animals per group. None of the groups were considered statistically significant as determined by student's t-test, where $p<0.05$ is considered significant.

### *3.6.4. Manipulating the timing of IL-4 induced non-specific immune modulatory effects which inhibited the development of OVA-specific responses*

A study examining the mechanism of immune activation induced by a plasmid encoding GM-CSF (pGM-CSF) administered in combination with a DNA vaccine encoding the envelope of HIV has demonstrated that the timing of pGM-CSF had a significant impact on the resultant T cell response (184). Therefore, although a reduction in primary OVA-specific CTL responses was seen when FPV$_{mIL-4}$ was co-administered at the time of immunisation with FPV$_{OVA}$, it was not clear whether manipulating the timing of IL-4 administration would modulate the resultant T cell responses. To determine this, mice were immunized with parental FPV M3 or FPV$_{mIL-4}$ up to 96 hours before or after immunisation with FPV$_{OVA}$ and CFSE-labelled OT-I T cells were adoptively transferred (Fig 2.32A) or CTL responses were measured *in vitro* in the direct CTL assay (Fig 3.32B). For the AT experiments, all FPVs were injected into the right footpad and PBS in the left. Two and ¾ days after AT, the

lymphoid tissues and spleen were examined by flow cytometry for evidence of T cell proliferation. As observed previously, OT-I T cell proliferation was only seen in the right popliteal (shown in Fig 2.32A) and para-aortic (data not shown) lymph nodes and there was no difference in the amount of proliferation between mice immunized with $FPV_{OVA}$ + FPV M3 (i. , PI = 10.16) or $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ (ii., PI = 10.65). Interestingly, when mice were given either FPV M3 (iii) or $FPV_{mIL\text{-}4}$ 96 hours before $FPV_{OVA}$, OT-I T cell proliferation was significantly reduced (PI = 4.41 and 4.98 consecutively). For cytotoxicity assays, mice were immunised i.p. and splenic effector cell cytotoxicity was measured via a direct CTL assay 5 days after $FPV_{OVA}$ immunization. It was observed that if $FPV_{mIL\text{-}4}$ or FPV M3 was given before presentation of OVA, cytolytic activity was even more dramatically reduced (0-3.5% specific lysis at 200:1 E:T ratio) than when $FPV_{mIL\text{-}4}$ was given in conjunction with $FPV_{OVA}$ (8% ± 7% specific lysis at 200:1 E:T ratio), as compared to mice immunized with $FPV_{OVA}$ + FPV M3 (30% ± 10% specific lysis at 200:1 E:T ratio). In contrast, when mice were immunized with FPV M3 or $FPV_{mIL\text{-}4}$ 96 hours after $FPV_{OVA}$ by which time OVA had already been presented to endogenous T cells, CTL activity was increased (25% ± 10% specific lysis at 200:1 E:T ratio) as compared to mice with $FPV_{OVA}$ + FPV M3 (30% ± 10% specific lysis at 200:1 E:T ratio). There was no significant difference between mice receiving $FPV_{mIL\text{-}4}$ or FPV M3 except for when they were co-administered. Taken together, this data indicates that the effect the timing of IL-4 administration has on OVA-specific responses can not be determined due to non-specific immune modulatory effects of the FPV vector.

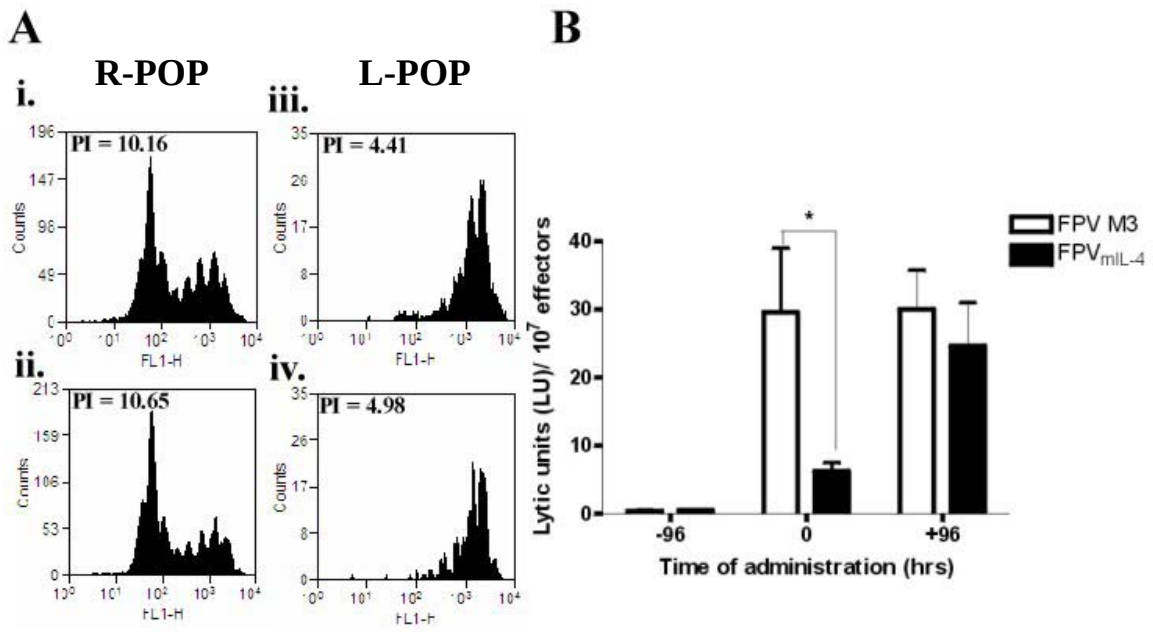


**Figure 3.32 Manipulating the timing of IL-4 induced non-specific immune modulatory effects which inhibited the development of OVA-specific responses.**

(**A**) C57BL/6 mice were immunized in the right footpad with $1x10^7$ pfu $FPV_{mIL-4}$ (ii. and iv.) or FPV M3 (i. and iii.) 96 or 0 hours *before* immunization with $1x10^7$ pfu $FPV_{OVA}$. Mice were then injected with CFSE-labelled transgenic OT-I T cells i.v. and 2 ¾ days later sacrificed and their popliteal lymph nodes were analysed by flow cytometry. Profiles are gated on $CD8^+$, $CD45.1^+$, $CFSE^+$ cells. The extent of division was determined by calculating the proliferation index (PI), which is a ratio of cells recovered: cells transferred. The PI formula is $PI = (n_{PP} + n_{G1} + n_{G2})/(n_{PP}/2^{PP} + n_{PP}/2^{G1} + n_{PP}/2^{G2})$ where n = cell number in the peak, PP = parent peak, G1 = generation 1, G2 = generation 2 etc. (**B**) C57BL/6 mice were immunized with $1x10^7$ pfu $FPV_{mIL-4}$ or FPV M3 96 or 0 hours *before* or *after* immunization with $1x10^7$ pfu $FPV_{OVA}$ and assessed for CTL activity 5 days later in a direct $^{51}Cr$ release assay with SIINFEKL-pulsed EL-4 cells as targets. The percentage of specific release was determined by the standard equation % specific lysis = (experimental release-spontaneous release)/ (maximum release-spontaneous release) x 100. Data is shown as lytic units per $10^7$ effectors and is calculated using the formula LU/$10^7$ effectors = $10^7$/ $(T\text{-}X_p)$ where *T* is the number of target cells ($10^4$), *p* is the reference lysis level (20%) and $X_p$ is the E:T ratio required to lysis p% of the targets. Data represents three independent experiments (where for each experiment $n = 2$) and results were considered significant when $p < 0.05$ (as indicated by *) with p values denoted thus: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

# CHAPTER 4: DISCUSSION

Interest in FPV has increased enormously in recent years due to its potential as a vaccine vector. However, understanding the mechanisms underlying the innate recognition and induction of adaptive immune responses to the viral vector is essential in order to undertake a rational design approach to develop a vaccine capable of providing protective immunity. More complex vaccine design strategies are likely to require the simultaneous expression of different heterologous genes to manipulate the microenvironment to favour the development of appropriate protective immune responses. Cytokines are key regulators of the immune system and their co-expression by recombinant virus vector vaccines has the potential to provide immunomodulatory activity to enhance vaccine potency (185). Poxviruses are attractive candidates for inclusion in such design strategies due to their large genome, which allows for the inclusion of multiple heterologous genes, particularly FPV, as it is also incapable of replicating in mammalian cells. The potential of recombinant FPV vector vaccines is reflected by the number of clinical trials for diseases including HIV, malaria and a number of different types of cancer. Despite their promise, intricate details regarding FPV and how it interacts with the host immune system have not been resolved. This thesis describes the construction of a series of rFPV vectors expressing the nominal antigen chicken ovalbumin (OVA), ($FPV_{OVA}$), and/or murine interleukin-4 (mIL-4). These rFPVs were used for the characterisation of the relationship between FPV and the mammalian immune system and how these responses are altered by the co-administration of mIL-4. This should provide insight into how manipulating the formation of the immune response within the context of poxvirus infection can help tip the balance in the favor of either host or pathogen.

### *4.1 Antigen expression pattern and kinetics following $FPV_{OVA}$ infection*

While avian poxviruses such as FPV are apathogenic and replication deficient in mammals due to their natural host range restriction, they still have the ability to enter mammalian cells, reach an early stage of morphogenesis, and express exogenous genes (181, 186). *In vitro*, EGFP expression by $FPV_{OVA}$ was detectable within 24 hours of infection in both permissive and non-permissive cell lines including QT35, HEK 293, IC21 and JAWS II. While infection of mammalian cells does not result in productive viral replication, cytopathic effects were

still observed and by 72 hours p.i., infected cells had become apoptotic or died. The observed *in vitro* expression profile for $FPV_{OVA}$ was reflected *in vivo* using the OT-I T cell AT model where OVA expression was rapid and restricted to the DLNs. This was despite the fact that a strong systemic inflammatory response had been observed, as evidenced by marked splenomegaly and lymphadenopathy in immunised mice. The use of adoptively-transferred $K^b$/OVA-specific OT-I T cells allows the determination of the time course for $CD8^+$ T cells to respond to infection with $FPV_{OVA}$ with respect to sites of proliferation and clonal expansion. OVA expression was highly localized, with the primary site of infection being the right footpad and OT-I T cell proliferation being observed only in the DLNs (right politeal and para-aortic). No proliferation was seen in the spleen, mesenteric, inguinal, brachial, cerviacal or axillary LNs (data not shown except for spleen). OT-I T cell proliferation was rapid, with cell division already occurring on the day of infection and being absent by day 7. Until day 3 CFSE was fully diluted, indicating that the cells had undergone at least seven to eight rounds of division and significant clonal expansion. These findings are consistent with those of $VV_{OVA}$ where several rounds of OT-I T cell division have occurred by day 2 after infection, peaking at day 5 and then declining (187). While no OT-II T cells proliferation was observed following immunisation with $FPV_{OVA}$, it is most likely that this as a result of inadequate concentrations of OVA required to drive OT-II proliferation as opposed to a lack of presentation in the context of MHC class II. Indeed, it has been demonstrated by others that OT-I T cells are approximately 500-fold more responsive to peptide stimulation than OT-II cells (188).

### *4.2 Immunisation with $FPV_{OVA}$ induces a strong anti-OVA CTL response and weak T helper response and moderate humoral response*

Effective vaccines should generate humoral immunity and produce protective antibodies, as well as establish lasting cell-mediated T cell responses (45) and it has been shown that recombinant poxviruses are capable of inducing helper T cells, CTL and high levels of antibodies concurrently (46, 47). However, while immunisation of mice with $FPV_{OVA}$ induced a strong $CD8^+$ CTL response, only weak $CD4^+$ T helper and antibody responses were observed. It is hardly surprising that $FPV_{OVA}$ induced weak anti-OVA $CD4^+$ T helper proliferative responses. In contrast to $CD8^+$ T cells, for which a relatively short antigen pulse

seems sufficient for APCs to drive clonal expansion and differentiation, antigen persistence is required for $CD4^+$ T cells throughout their expansion phase (189). As seen using the OT-I AT model, FPV-encoded OVA expression was rapid and transient, with OVA expression being undetectable 5 days post-infection. This perhaps provided an insufficient antigen pulse for the stimulation of more robust $CD4^+$ T helper responses. While the $CD4^+$ T helper response was modest, $CD4^+$ T helper responses still played a crucial role in the provision of help for CTL function. Indeed, findings published by other members of our laboratory have shown that mice lacking $CD4^+$ T cells or MHC class II molecules exhibit reduced anti-OVA CTL responses following immunisation with $FPV_{OVA}$ indicating that T cell help is required for the induction of an effective antigen-specific CTL response (46). Similar findings have been published for MVA where naive, CD8-enriched, CFSE-labelled F5 cells transferred into MVA-immunised, CD4+ cell-depleted mice displayed an overall smaller expansion and significantly decreased frequency of CD8+ T cell memory after contraction (190).

Antibody responses detected in the serum of $FPV_{OVA}$-immunised mice were predominantly weak IgM and there was little evidence of class switch or improvement in titre over time. The rapid kinetics of antibody production is the decisive factor for survival following infection with acutely cytopathic viruses such as poxviruses (191). As the priming of T helper responses takes several days, the rapid induction of early neutralizing IgM antibodies, independently of T cell help, might overcome this delay. Indeed, it has been shown that most cytopathic viruses can induce T cell-independent, neutralizing IgM antibody responses (192, 193). However, the weak IgM response and lack of an IgG antibody response in this study suggest that the primary OVA-specific immune response to $FPV_{OVA}$ immunisation may be suboptimal. The induction of $CD8^+$ T cell responses but not antibody responses to recombinant antigens expressed from FPV vectors has been observed in other studies utilizing both single FPV and prime-boost regimes incorporating FPV, DNA and MVA (68, 72, 87, 194-196). In the case of the DNA-prime-FPV-boost experiments, upon FPV boosting, a decline in antigen-specific antibody titres and a coincident marked enhancement of CTL and Th responses occurred (194). Because many of the studies examining the immunogenicity and efficacy of recombinant poxvirus vector vaccines are HIV vaccine candidates, the lack of robust antibody responses has been largely discounted as the induction

of CTL and T helper responses and not antibody are widely seen as critical to the success of an HIV-1 vaccine. However, there is now mounting evidence that antibody plays a crucial role in recovery from poxvirus infection in several animal models (197-200). Indeed, B cell deficient mice are susceptible to EV infection, demonstrating 100% mortality despite mounting a normal CTL response (200). It is therefore becoming increasingly apparent that antibodies along with T cells and other arms of the innate immune system need to be considered as targets of new generation vaccines for poxviruses.

One interpretation for the lack of antibody responses in $FPV_{OVA}$-immunised mice is that the lower level of antigen production by non-replicating poxvirus vectors such as FPV may preferentially stimulate T cell rather than antibody responses. However, while the level of antigen production does impact the magnitude of subsequent immune responses (201), a recent study characterizing antigen-specific immune responses induced by recombinant canarypox virus (ALVAC) vectors has demonstrated that the capacity of ALVAC vaccines to elicit CTL immunity against transgene-encoded antigens critically depends on the nature of the antigen itself (202). Indeed, immunisation with ALVAC-CEA elicited a mixed Th1/Th2 profile that was accompanied by a potent CEA-specific humoral response but no measurable CEA-specific CTL immunity. Interestingly, when the identity of the encoded antigen was changed from CEA to OVA strong CTL responses were induced, indicating that the ability of ALVAC-based vaccines to elicit $CD8^+$ T cell immunity is dependent on the presence of highly immunogenic CTL epitopes in the antigen. The identity of the vaccine vector itself also plays a role in the type of immune response induced and its magnitude (203). Whereas rFPV encoding HIV-1 antigens fail to boost antibody responses in DNA-immunised mice, recombinant protein boosts of the same proteins dramatically enhanced antibody responses (194, 204, 205). Virus strain also affects immunogenicity of the vaccine and this is likely due to the different immune-modulatory effects that they have on host cells (206). Thus, while avipoxvirus vectors such as FPV show promise as vaccine vectors, considerable potential exists for novel strategies designed to enhance their T cell and antibody immunogenicity and efficacy. Modulating the immune response to generate Th1 or Th2 responses by co-expressing selected immunomodulatory molecules is one prospect in overcoming this barrier.

### *4.3 Bone marrow-derived APCs are required to prime anti-OVA cytotoxic T cell responses*

Substantial progress has been made in the characterization of the antigen presentation pathways for MHC class I-restricted determinants. However, for many vectors, including FPV, it is still unknown which pathways contribute to the primary induction of $CD8^+$ T cell responses. Analysis of OT-I T cell proliferation profiles in BM chimaeric mice (B6 →B6, B6 → $B6^{bm1}$, $B6^{bm1}$ → B6 and $B6^{bm1}$ → $B6^{bm1}$) demonstrated that only chimaeric mice reconstituted with B6 BM cells could present FPV-derived OVA to OT-I T cells. No presentation was observed in control $B6^{bm1}$→$B6^{bm1}$ mice, with results taken as a whole clearly indicating that presentation of FPV-derived antigen requires permissive BM-derived APCs. In addition, the requirement for a BM-derived APC was not only essential for the presentation of FPV-derived antigen to $CD8^+$ T cells but also for the priming of antigen-specific CTL responses. Indeed, effective CTLs were only induced in B6 mice reconstituted with B6 BM. While studies examining the contributions of direct and cross-presentation in the induction of $CD8^+$ CTL responses following exposure to VV have found that it varies depending on the route of immunisation (119), this was not the case here. Both footpad and i.p. immunised mice demonstrated the same pattern of proliferative responses in the different chimaeric mice and CTL responses were only observed in the B6→ B6 animals for i.p. and i.v. routes of immunisation. The importance of a BM-derived APC in the induction of CTL responses is supported by studies with $VV_{OVA}$ in $TAP^{0/0}$ mice where virally infected non-haematopoietic cells were unable to stimulate CTL-mediated immunity directly. Instead, BM-derived cells were required as APCs to initiate anti-viral CTL responses (118). These findings have subsequently been extended to include other antigens including those from LCMV, VSV, influenza, tumours, DNA vaccines and bacteria (207-211). This indicates that BM-derived APCs are a general requirement in the initiation of most primary CTL responses. Antigen presentation by cells of non-haemopoietic origin shouldn't be entirely discounted, as they can contribute markedly to the clonal expansion of effector $CD8^+$ T cells, but the extent to which they can amplify it is determined by the pathogen (207).

It is surprising that no CTL activity was observed in $B6^{bm1}$ mice reconstituted with B6 BM and it can be interpreted in two ways: one, that both permissive radiation-resistant recipient cells and BM donor cells are essential for the induction of a systemic CTL response, or two,

that $FPV_{OVA}$ is cleared rapidly by resident peritoneal or venous cells, resulting in FPV-derived antigens not persisting long enough for BM-derived cells to obtain and cross-present them. Immunisation of chimaeric mice with a maximal dose of $FPV_{OVA}$ did generate a weak CTL response in B6→$B6^{bm1}$ mice suggesting that the standard concentration administered is indeed limiting and rapidly cleared. Irrespective of this, the CTL response generated in B6→$B6^{bm1}$ mice was still significantly lower than that observed in B6→B6 mice, suggesting that BM-derived APCs are necessary for the induction of FPV-encoded, antigen-specific CTL responses. While these experiments have demonstrated the necessity for BM-derived APCs in the presentation of FPV-encoded antigen to $CD8^+$ T cells and induction of CTL responses, it is still unclear whether the APCs themselves are being infected or whether they are obtaining antigen exogenously via the cross-presentation pathway. It is well established that poxviruses such as VV, MVA and ALVAC are able to infect both murine and human DCs, although the timing and magnitude of antigen expression varies depending on the identity of the virus (120, 201, 212, 213). *In vitro,* FPV has been shown to infect human DCs that have been cultured *ex vivo* from peripheral blood monocytes (19). While infection of DCs is a potential mechanism for direct presentation particularly when immunizing into APC-rich sites such as i.p. or i.v., they are also capable of cross-presenting poxvirus-derived antigens including those encoded by VV and MVA (214, 215). It is probable that both mechanisms are involved, possibly with one predominating over the other depending on the level of antigen expression, cell type specificity of the vector and other defined factors relating to the nature of the antigen. Regardless, since the efficiency of antigen presentation influences the number of memory $CD8^+$ T cells following immunisation with viruses, identifying which presentation pathway dominates is clearly essential for successful vaccine design.

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### *4.4 Correlating OVA responses with vector-specific immunity*

While viral vector vaccine systems are capable of inducing robust immunogen-specific $CD8^+$ T cell immunity, a barrier to their use are the responses directed towards the vector itself (183). This is of particular importance as often the strong $CD8^+$ T cell response targeted to determinants expressed naturally by the vector reduces or prevents a $CD8^+$ T cell response to the recombinantly encoded foreign antigen (50, 55). Therefore, a better understanding of the

relationship between the immune response directed towards the heterologous gene products, and that directed towards the viral vector itself is critically important. Interestingly, preliminary results examining CTL, Th and antibody responses to both OVA and the parental vector FPV M3 following immunisation with $FPV_{OVA}$ indicate that a strong CTL T cell response is induced against the target antigen with this vector, in spite of the presence of strong anti-vector CTL, Th and antibody responses (unpublished data in collaboration with Erin Lousberg). In an attempt to define some of the poxvirus determinants recognised by antibody and/or $CD8^+$ T cells and determine their role in protective immunity, four FPV ORFs were selected based on their predicted functions *in vivo* for use in CTL. T helper and antibody assays (216, 217). The selected ORFs were the FPV NTPase DNA replication protein FPV058L, FPV core protein FPV120L, FPV virion envelope protein FPV140L and FPV immunodominant protein FPV168L. Antibody responses were generated against all of the FPV ORFs, particularly FPV140L and FPV168L although all produced a result superior to that elicited by OVA (data not shown). In addition, hyperimmune serum from rabbits immunised with FPV expressing HIV Clade B gag-pol protein in conjunction with human interferon gamma (huIFN-γ) that was part of a Food and Drug Administration (FDA) Investigational New Drug Application (IND) was obtained by our collaborators in this project (Virax Holdings Ltd) to test whether a similar response profile as seen in the mouse would be observed in another animal species. Results showed that a similar pattern of antibody responses were produced in the rabbits as that in mice. To determine whether any of the FPV ORFs contained $CD8^+$ T cell determinants, splenocytes from $FPV_{OVA}$-immunised mice were tested against HEK 293 T-Kb and -Db cells stably transfected to express OVA, FPV058L, FPV120L, FPV140L or FPV168L or infected with FPV M3 in the direct cytotoxicity assay (unpublished data in collaboration with Erin Lousberg). As expected, strong anti-OVA and moderate anti-FPV CTL responses were induced. However, all of the FPV ORFs failed to induce detectable CTL responses as compared to mock-infected target cells. Taken together, these data suggest that strong anti-vector responses are induced although while FPV140L and FPV168L may contain antibody epitopes, none of the selected FPV ORFs contain either Kb or Db-restricted $CD8^+$ T cell determinants. One approach to the reduction of vector gene expression is UV inactivation. While UV/psoralen treatment ablates viral replication of VV and enhances in vivo CD8+ T cells responses to the encoded

minigenes whilst inhibiting responses to native VV epitopes (55), we have found that UV inactivated $FPV_{OVA}$ loses its infectivity of mammalian cells (data not shown). As a result, there is a lack of OVA expression and hence induction of OVA-specific humoral or cell-mediated responses. Further investigation is required to elucidate the role of the FPV proteins in generating a cell mediated response, which may provide a better understanding of the success of these viruses. Assessment of the nature and extent of anti-vector immune responses will provide a benchmark against which responses generated against the heterologous gene product(s) can be compared, thus enabling vaccines to be reverse-engineered to facilitate the induction of immune responses of the optimal type and magnitude required for prevention and/or therapy for a particular disease state.

### *4.5 Effect of co-administration of mIL-4 on OVA-specific immunity*

The generation of an appropriate type of immune response against a particular pathogen is important since the dominance of an inappropriate response can exacerbate disease and lead to the inability to eradicate the infecting organism (218). The inclusion of cytokines or other immunomodulatory molecules in vaccine formulations has been attempted as a way to customize vaccine-induced immune responses, with the aim of eliciting protective immune-effector mechanisms avoiding pathological immune responses (219-223). Many diseases awaiting prophylactic or therapeutic vaccines require the induction of cellular immunity, particularly $CD8^+$ T cell-mediated responses. A number of cytokines regulate the induction and effector function of CTLs including IL-2, IL-6, IL-7, IL-12 and IFN-γ (151). The effect of IL-4 on the CTL response has been of particular interest in regard to viral infections and viral vaccines due to CTL being important anti-viral effectors and for long term immunity (153). While it is well established that IL-4 is a pivotal mediator of $CD4^+$ Th type 2 T cell differentiation and IFN-γ-producing $CD4^+$ Th1 T cell suppression (224), its role in the regulation of CTL activity and acquisition of cytolytic function is conflicting. IL-4 has been ectopically expressed using a number of recombinant viruses with little effect on virus infectivity and pathogenesis in the host, including in retroviruses (225), herpes simplex virus (HSV) (226, 227) and vesicular stomatitis virus (VSV) (228). In contrast, the expression of IL-4 by influenza virus (149, 157, 229), respiratory syncytial virus (RSV) (152-155) and poxviruses such as VV (148, 150), EV (158) and myxoma (230) diminishes the cytolytic

activity of primary and memory $CD8^+$ virus-specific CTL responses, tipping the balance in favour of the virus. While the effect of ectopically-expressed IL-4 on the development of Th1 immunity and viral pathogenesis by replicative viruses has been extensively investigated, its effect on these processes to a host-restricted virus such as FPV has not. In this study, primary infections of mice with $FPV_{OVA}$ + $FPV_{mIL\text{-}4}$ were associated with a significantly diminished CTL response that was not due to a delay in peak CTL activity as it remained largely non-functional throughout the infection. These results are consistent with findings from the studies mentioned above with replicative viruses.

As expected, co-administration of $FPV_{mIL\text{-}4}$ skewed the T helper response towards Th2 with a reduction in IFN-γ production by OVA-specific Th cells. While IL-4 appeared to have some effect on antibody responses, with an increase in IgM, these were not considered to be statistically significant. This may be considered surprising when the role of IL-4 in the stimulation and differentiation of activated B cells and class switching (231) is taken into account, however it is consistent with findings with VV-expressing HA and IL-4 where no enhancement in the antibody response to either VV or antigen HA was observed (148, 232).

Interestingly, IL-4 had no effect on the expression pattern and kinetics of OVA as visualized by the OT-I T cell proliferation profile. As for $FPV_{OVA}$-immunised animals, OVA expression was highly localized, with proliferation being observed only in the DLNs (right politeal and para-aortic). There was no reduction in viral clearance; with cell division already occurring on the day of infection and being absent by day 7. This is in direct contrast to the IL-4-expressing replicative viruses including IL-4-expressing VV (150), wt RSV infections of transgenic mice engineered to over-express IL-4 (152) and wt influenza infections of mice treated with IL-4 (149).

The down-regulation of CTL activity by IL-4-expressing viruses and elucidating the mechanism(s) involved has received significant attention as a result of its potential to create "killer viruses" with biological warfare applications. It appears that IL-4 reduces CTL activity in a number of ways including via a reduction in proliferation of antigen-specific $CD8^+$ T cells (151), a switch in the cytolytic mechanism of CTL from the $Ca^{2+}$-dependent

perforin/granzyme-mediated pathway to the Fas /Fas ligand mediated pathway (147, 162, 233), the development of a non-cytolytic population of T cells with low CD8 ($CD8^{low}$) surface expression (159-161, 234) and in the case of poxviruses, the production of a soluble IFN-γ binding protein (44, 158). In addition, other studies suggest that IL-4 expression can exacerbate disease in a manner that is independent of T cells. For example, a group examining VV as a method of gene delivery found that expression of IL-4 by VV exacerbates disease through a decrease in macrophage cytotoxic activity, potentially through the suppression of IFN-γ production by NK cells (235). All of this suggests that an excess of IL-4 could severely impair the ability of the host immune system to mount a successful antiviral CTL response and it is likely that ectopic IL-4 expression acts to increase the pathogenesis of poxviruses by a combination of T cell-dependent and T cell-independent effects.

### *4.6 Manipulating the timing of IL-4 induced non-specific immune modulatory effects which inhibited the development of OVA-specific responses*

Rational manipulation of the cytokine environment is not a simple task because cytokine functions are complex and the final effects on the immune response will depend on timing and length of exposure, cell(s) targeted and other cytokines present in the same microenvironment (185). Indeed, a study examining the mechanism of immune activation induced by a plasmid encoding GM-CSF (pGM-CSF) administered in combination with a DNA vaccine encoding the envelope of HIV has demonstrated that the timing of pGM-CSF had a significant impact on the resultant T helper response and that this was mediated through BM-derived DCs (184). Most T cells recognize antigen as a complex of an antigenic peptide bound to an MHC molecule present at the surface of an APC and engagement of the T cell receptor (TCR) by peptide/MHC complexes is insufficient to trigger a productive T cell response unless co-stimulatory ligand-receptor pairs are also ligated (236). The most-studied co-stimulatory molecules are B7-1 (CD80) and B7-2 (CD86) and these are expressed by professional APCs (pAPCs); B7-2 in resting APCs and B7-1 during APC activation (237). The importance of B7 co-stimulation in the generation of CTL responses has been demonstrated via antibody-mediated blockade experiments where blockade of B7-1 and B7-2 prevented CTL responses to both exogenous and viral antigens (236). Interestingly, it has

been shown in a mouse model of autoimmune diabetes that IL-4 acts at the locus of the antigen-presenting DC to down-regulate CTL responses (238). In this model, transgenic mice express IL-4 in pancreatic islets under the control of the human insulin promoter and do not develop diabetes following infection with LCMV because IL-4 inhibits the generation of diabetogenic CTLs. The inhibitory effect of IL-4 on autoreactive $CD8^+$ T cell responses may be likened to results obtained IL-4 deficient mice and with recombinant IL-4-expressing viruses where inhibition of CTL activity was associated with a reduction the number of IFN-γ-producing $CD8^+$ T cells and their content of perforin and granzymes. The most striking finding in this study was that IL-4-mediated failure of diabetogenic CTL generation resulted from IL-4 modulation of DCs that prevented effective activation of antigen-specific CTLs *in vivo* (238). Indeed, IL-4 increased B7-2 and decreased B7-1 expression on pancreatic DC, which supported the expansion of antigen-specific CTL but inhibited their acquisition of cytolytic function (238). Moreover, B7-2 blockade overcame IL-4-induced suppression of diabetes however, this only occurred when anti-B7-2 treatment began after LCMV infection. Treatment beginning before LCMV infection decreased CTL activity, suggesting IL-4-influenced DC produce distinct effects on $CD8^+$ T cells depending on their state of activation. This is consistent with *in vitro* findings of activated single T cell clones, where the addition of IL-4 only led to the development of poorly cytolytic T cells with low CD8 expression when it was added prior to or early on during activation (161).

The purpose of the final experiments was to investigate the effect of IL-4 on primary OVA-specific responses when administered at different times either before or after antigen presentation and its subsequent impact on CTL activity. Unlike previous studies with IL-4-expressing viruses where the antigens of interest and IL-4 were co-expressed in the same viral vector, recombinant FPV vectors singly expressing OVA or IL-4 were constructed in order to be able to manipulate the timing of IL-4 administration. It was hypothesized that administration of $FPV_{mIL\text{-}4}$ prior to or at the same time as $FPV_{OVA}$ would down-regulate CTL activity whereas administration afterwards would have no effect. However, while this was indeed the case, it was due to non-specific immune modulatory effects of the FPV vector. Indeed, animals that had been immunised with parental FPV M3 displayed the same CTL responses as for $FPV_{mIL\text{-}4}$ when administered before or after immunisation with $FPV_{OVA}$.

This phenomenon, the failure to mount effective immunity to a virus variant in a previously virus-infected host fails, is known as original antigenic sin (OAS) (239, 240). Hence, when the initial immunisation is with FPV M3 or $FPV_{mIL\text{-}4}$, pre-existing $CD8^+$ T cell responses against viral epitopes reduced subsequent $CD8^+$ T cell responses against another, in this case OVA's dominant MHC class I epitope SIINFEKL. Alternatively, when animals are first immunised with $FPV_{OVA}$, boosting with FPV M3 or $FPV_{mIL\text{-}4}$ enhanced OVA-specific $CD8^+$ T cell responses.

Another important factor to consider is the role of type I IFNs. Type 1 IFNs induce potent defense mechanisms against viruses (92) as well as exerting regulatory effects on cellular and humoral immune responses including the stimulation of Th functions and survival of activated T cells (241). Furthermore, it has recently been demonstrated that the massive expansion of antigen-specific $CD8^+$ T cells that occurs in response to viral infection is critically dependent on the direct action of type I IFNs on $CD8^+$ T cells (242). Indeed, our laboratory has recently demonstrated that $FPV_{OVA}$ induces rapid type I IFN responses in immunised mice, mediated primarily by plasmacytoid dendritic cells (pDCs), and that these cells are necessary for the development of $CD8^+$ T cell responses (46). Interestingly, recent evidence suggests that after an acute primary viral episode the host undergoes a transient period of partial immune unresponsiveness for between 5 and 9 days which is associated with exhausted type I IFN responses (243). This is in common with our findings where immunisation with $FPV_{OVA}$ 96 hours after FPV M3 or $FPV_{mIL\text{-}4}$ failed to induce OVA-specific immunity. While studying the timing of IL-4 administration and whether it influences the nature of the resultant antigen-specific response is worthwhile, it can not be done using a homologous prime-boost strategy, but perhaps with the use of recombinant IL-4.

### *4.7 Conclusion*

In conclusion, this thesis describes the construction of a series of rFPV vectors that express the nominal antigen OVA or mIL-4 and their *in vivo* characterisation. Immunisation with $FPV_{OVA}$ resulted in rapid and highly localized OVA expression which induced strong $CD8^+$ CTL activity but only weak $CD4^+$ T helper and antibody responses. In addition, presentation of FPV-derived antigen and the priming of antigen-specific CTL responses required a

permissive BM-derived cell as the APC. Co-administration with $FPV_{mIL-4}$ resulted in a dramatic reduction in CTL activity that remained largely non-functional throughout the infection and a skewing of the T helper response towards Th2 with a reduction in IFN-γ production by OVA-specific Th cells. However, the effect on T cells may not be sufficient to explain the exacerbated lethality of IL-4-expressing viruses and there needs to be a closer examination of other immune cells whose function could be drastically altered by IL-4 including those of the monocyte/macrophage lineage (180). How the timing of IL-4 administration influences the nature of resultant antigen-specific response is also an area of fertile exploration with significant implications in rational vaccine design. Future studies will be directed at further characterization of how FPV interacts with the innate and adaptive arms of the immune system, how these can be manipulated via the co-administration of cytokines, and discovering if future rationally designed modifications result in FPV-vectored vaccines that induce durable cellular and humoral immunity.

# CHAPTER 5: APPENDIX

## 5.1 APPENDIX 1: BUFFERS AND SOLUTIONS

**Agar**
2X YT medium recipe
15g bacto-agar

Make up to 1L with $dH_2O$ and autoclave

**Alkaline lysis buffer**
8.29 g $NH_4Cl$
1 g $KHCO_3$
37.2 mg $Na_2EDTA$

Make up to 1L with $dH_2O$ and filter sterile, pH should be between 7.2-7.4

**CEF cell growth medium (GM)**
MEM medium
5% FCS
2 mM glutamine
10mM HEPES
0.05 mg/ml gentamicin

**CEF cell maintenance medium (MM)**
same as for GM but 2% FCS

**Complete RPMI cell culture medium**
RPMI 1640
5% FCS
20 ml/L penicillin/streptomycin
2 mM glutamine
10 mM HEPES
$5x10^{-5}$ M 2-mercaptoethanol

**FACS buffer**
PBS
0.1% BSA
0.05% sodium azide

**FACS Fix**
20 g glucose
2 ml 10% sodium azide
40 ml 10% formalin

Make up to 1L with PBS

**Incomplete RPMI cell culture medium**
RPMI 1640
2 mM glutamine
10 mM HEPES
$5x10^{-5}$ M 2-mercaptoethanol

**Laemmli sample buffer (2 ml)**
0.5 ml 0.5M Tris base pH 6.8
0.8 ml 10% ultrapure SDS
0.2 ml β-mercaptoethanol
0.35 ml glyercol
0.15 ml 0.1% bromophenol blue

**MM plus MXHAT**
MM
25 μg/ml MPA
250 μg/ml xanthine
1X HAT

**NP40 lysis buffer**
1% NP40
50 mM Tris-HCl, pH 8.0
150 mM NaCl
100 μg/ml PMSF

**10X Orange G DNA loading buffer**
20 g sucrose
100 mg orange G
50 ml $dH_2O$

**10X SDS running buffer**
30.3 g Tris
144 g glycine
10 g SDS

Make up to a final volume of 1L with $dH_2O$

**SDS transfer buffer**
200 ml MeOH
6 g Tris
28.8 g glycine

Make up to a final volume of 2L with $dH_2O$

**50X TAE electrophoresis buffer**
242 g Tris base dissolved in 750 ml $dH_2O$ dissolved in 750 mL deionized water
57.1 ml glacial acetic acid
100 mL of 0.5 M EDTA (pH 8.0)

Make up to a final volume of 1L with $dH_2O$

**TENS buffer**
TrisEDTA pH 8.0
0.1M NaOH
0.5% SDS

**10X TBS**
111 g NaCl
39.4 g Tris HCl

Make up to a final volume of 1L with $dH_2O$

**TBS 1X/ Tween (TTBS)**
100 ml 10X TBS
1 ml Tween 20 (0.1%)
0.65 ml 10M NaOH

Make up to a final volume of 1L with $dH_2O$, pH should be between 7.8-8.0.

**YENB medium**
7.5 g bacto yeast extract
8 g bacto nutrient broth

Make up to 1L with $dH_2O$ and autoclave

**2X YT medium**
16 g bacto tryptone
10 g bacto yeast extract
5 g NaCl

Make up to 1L with $dH_2O$ and autoclave

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