

GENERATION OF MULTIVALENT RECOMBINANT MVA VACCINES FOR MALARIA

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Abstract

Modified vaccinia virus Ankara (MVA) has been used extensively as a recombinant vector for delivery of antigens from diverse pathogens. Its ability to generate strong antigen specific $CD8^+$ T cell responses in humans has been shown in clinical trials of novel vaccines against malaria, tuberculosis, HIV/AIDS, influenza and cancer. The work in this thesis describes the use of BAC recombineering technology to harness the endogenous regulatory signal (promoter) that drives the expression of non-essential open reading frames (ORFs) in MVA for immunogenic expression of a recombinant antigen. Replacement of the ORFs of four non-essential genes in MVA; *C11R, F11L, A44L* and *B8R* with an epitope tagged luciferase positioned to use the same endogenous promoter showed early transgene expression equal to or slightly higher than traditional p7.5 and SSP constructs. The frequency of antigen-specific $CD8^+$ T cell induced in mice by single dose MVA or adenovirus-prime, rMVA-boost vaccination showed equivalent or slightly higher responses by the endogenous promoters compared to the traditional p7.5 and SSP constructs. Assessment of the growth rate of these viruses showed they were unimpaired and the insertions were genetically stable. The insertion driven loci of *B8R* and *C11R* were used for the construction of a bivalent MVA expressing an epitope tagged luciferase (rLucPb9) and a *Photinus pyralis* (pLuc) luciferase. The frequency of antigen-specific $CD8^+$ T cells induced in mice by bivalent MVA was equivalent to single-pLuc and single-Pb9 recombinants co-administered as a mixture, at separate sites or administered alone following single dose MVA vaccination but slightly lower for Pb9-specific $CD8^+$ T cell following adenovirus-prime, rMVA-boost.

Dedication

This thesis is dedicated to the memory of my father

Mayomi Baali Orubu

22:07:1941 – 13:02:2011

"I am deeply saddened that you never saw this dad"

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Abbreviations

aa	Amino acid
Ad-MVA	Adenovirus-prime, MVA-boost
Ag(s)	Antigen(s)
AMA-1	Apical membrane antigen 1
APC	Antigen Presenting Cells
BHK	Baby hampster kidney cells
CEF	Chicken embryo fibroblast
ChAd63	Chimpanzee adenovirus serotype 63
CMI	Cell mediated immunity
CSP	Circumsporozoite protein
DMSO	Dimethyl Sulfoxide
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EBA	Erythrocyte binding protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
ELISpot	Enzyme-linked Immunosorbent spot assay
FP9	Fowlpox strain virus 9
GFP	Green fluorescent protein
GSK	Glaxo Smith Kline
HAdV5	Human adenovirus serotype 5
HBsAg	Hepatitis B surface antigen
HIV	Human immunodeficiency virus
hpi	Hour post-infection
h	Hour
ICS	Intracytokine staining
iDC	Immature dendritic cells
IFN	Interferon
IL	Interleukin
i.m	Intra-muscular
i.u	Infectious units
kDa	Kilodalton
LIPS	Luciferase Immunoprecipitation system
LSA	Liver stage antigen
Luc	Luciferase
mDC	Mature dendritic cells
ME	Multiple epitopes
MEM	Minimal eagle medium
MHC	Major histocompatibility complex
min	Minutes
MSP-1	Merozoite surface protein
mRNA	Messenger ribonucleic acid
MVA	Modified vaccinia virus Ankara
NYVAC	New York city vaccinia
OD	Optical density

OVA	Ovalbumin
Pb9	$CD8^+$ T cell epitope of circumsporozoite protein of *Plasmodium berghei*
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS/T	0.05% Tween 2D in phosphate buffer saline
PCR	Polymerase chain reaction
Pfu	Pox forming units
pLuc	*Photinus pyralis* luciferase
RBC	Reb blood cells
rLuc	*Renilla* luciferase
RT	Room temperature
RPM	Revolutions per minute
RTS'S	CSP based malaria vaccine co-administered with HBsAg polypeptide from GSK
SFC	Spot forming cell
SFU	Spot forming unit
TCR	T cell receptor
TIP	Multiple $CD8^+$ T cell epitope containing Pb9
tPA	Tissue plasminogen activator
TRAP	Thrombospodin related adhesion protein
VACV	Vaccinia virus
WT	Wild Type

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Vaccines

In the world today, vaccines and the provision of clean drinking water are the two most important interventions for the prevention and control of many infectious diseases. Vaccines save lives and give children a healthy start and remain the most cost-effective investment in public health. For just a few dollars per child, vaccines can prevent disability and disease for a lifetime – the cost benefits hugely outweighs the potential health risks. The protective effects are immediate, and the long-term impacts have great value in the health, educational, social and economic status of children and adults.

The year 1796 witnessed the first scientific evidence that humans can be protected from infectious pathogens as demonstrated by Edward Jenner, that intradermal inoculation with cowpox could confer protection against smallpox (1). Following this discovery, Louis Pasteur later demonstrated in the late nineteenth century that pathogens that cause infectious diseases could be attenuated and inactivated for the purpose of vaccination (2). These two scientific observations pioneered vaccine research and development and to this day vaccines remains a fundamental branch of medicine and considered the most cost-effective health care intervention for reducing morbidity and mortality caused by infectious pathogens in animals and humans. These discoveries paved the way for routine vaccination against smallpox and in the year 1980, the World Health Assembly declared the world free of smallpox after an Intensified Smallpox Eradication Programme. Smallpox remains the remains the only disease in humans to have been eradicated through vaccination apart from rinderpest in animals (3-6).

The efficacy or performance of a vaccine is dependent on a number of factors: the disease itself, the strain of the pathogen, individual status (ethnicity, age, genetic predisposition) and vaccine delivery vehicle. While all factors are fundamental in vaccine development, the composition of the pathogen and the vaccine delivery vehicle are considered the most important, and advances made in science and engineering in recent years such as genome sequencing as well as new vaccine delivery platforms have greatly influenced vaccine development. For example, over 5,300 putative genes have been identified in the *Plasmodium* genome and the availability of this information now forms the basis of most malaria vaccines under development.

As mentioned above, advances in science and engineering have had significant impact on the design and development of vaccines with over twenty licensed vaccines in the Expanded Programme on Immunisation (EPI) aimed at reducing morbidity and mortality in infants and children.

Vaccine platform	Vaccine/disease
Killed	▪ Influenza vaccine ▪ Cholera ▪ Polio ▪ Hepatitis A vaccine ▪ Rabies
Attenuated	▪ Yellow fever ▪ Measles ▪ Rubella ▪ Mumps ▪ Typoid ▪ BCG ▪ Influenza
Toxoid	▪ Tetanus ▪ Diptheria
Sub-unit	▪ Hepatitis B ▪ HPV
Conjugate	▪ Haemophilus influenza type B

Table 1.1: Licensed vaccines for different infectious diseases.

Vaccines are traditionally designed to generate antibodies but because of disappointing efforts to induce neutralizing antibodies through vaccination newer vaccine platforms such viral vectored vaccines are now been explored for generating $CD8^+$ T cells against infectious pathogens. Recombinant adenoviruses and modified vaccinia virus Ankara (MVA) are the most studied viral vectors for the expression of foreign antigens because they are capable of inducing potent antigen-specific $CD8^+$ T cells against many infectious diseases such as HIV, TB, Influenza (7-9).

In recent years, MVA, the focus of this thesis, have been shown to induce protective T cells responses against HIV (10), TB (8), and influenza (11) and this protective response has been explored against malaria infection (12). The work carried out in this thesis aims to (i) induce stronger T cell responses using MVA vector (ii) explore induction of potent antigen-specific $CD8^+$ T cells against multiple antigens which

could be of use in multiple diseases where there are currently no vaccines. To investigate these possibilities, the work carried out in this thesis explores improving MVA for the expression of foreign antigens and malaria has been chosen as one of the potential application for the work carried out. The etiologies, mechanism of infection and strategies for making recombinant MVA vaccines are discussed later in this chapter.

1.2 Malaria

Malaria is an infection caused by a protozoan parasite of the genus *Plasmodium* and is transmitted by an infective bite of a female *Anopheles* mosquito. Malaria has been reported as one of the most devastating parasitic diseases in human history, infecting over 500 million people each year, mainly children under the age of five years and pregnant women (13). Global estimates suggest that malaria causes approximately 1 to 3 million deaths each year and about 90% of these deaths occur in sub-Saharan Africa (13).

The devastation caused by malaria has been shown to have serious burden on the economic growth of countries affected by the disease. Cost attributable to treatment and prevention of malaria has been reported to exceed US $12 billion each year in Africa alone (14). The high cost in treatment, prevention and control strategies has been shown to significantly affect individual household income due to loss of workdays and productivity at work. Childhood under-development, school absenteeism, premature delivery and deaths have been found to have strong correlation with malaria morbidity and mortality (15-18).

1.2.1 Control Strategies

In addition to development of vaccines against malaria, the subject of this thesis, there are two other main strategies that have been employed for prevention and control of malaria (i) vector control – environmental management, residual spraying (IRS) and insecticide treated bednets (ITNs) (ii) chemoprophylaxis

1.2.1.1 Vector Control

The most effective preventive strategy against malaria infection has been achieved by avoiding contact with the vector – prevention of mosquito bites through the treatment of potential mosquito breeding sites with insecticides, indoor residual spraying and ITNs. In the early 1950 the World Health Organisation (WHO) through its National Malaria Control program was able to eradicate malaria from the United States and southern Europe. This was achieved by the deployment of widespread use of the insecticide dichloro-diphenyl-trichloroethane (DDT) in combination with monitoring and treatment of infected persons. This strategy eradicated malaria from many industrialized nations but because of political and environmental conditions in developing countries mainly in Africa and Asia, the strategy was abandoned in 1969. Since then and in addition to other factors such as civil war, climate change, adulterated insecticides and pesticides, there has been a resurgence of insecticide resistant mosquitoes and drug resistant parasites in these regions (19).

Before the deployment of DDT, malaria was successfully controlled or eliminated in some endemic countries by removing stagnant water or poisoning breeding grounds with diesel oil. The introduction of fish to feed on the larva of mosquitoes also achieved some success, but these methods were found unsuitable for large-scale

deployment. The application of arthropod vector engineering to develop genetically modified (sterile) mosquitoes (20) has also been studied as an alternative measure for the control and elimination of malaria, but due to ethical considerations the strategy is yet to prove feasible in the field.

The use of indoor residual spraying (IRS) has been widely used for the control of mosquitoes and studies have shown significant reduction in clinical episodes of malaria due to the application of IRS (21). Despite the success achieved with IRS, large-scale deployment have not been feasible due to adulterated products which have made the mosquito develop resistance to insecticides such as DDT, pyrethroid, permethrin and deltamethrin (22).

Studies have shown that the use of ITNs reduces morbidity and mortality in young children and pregnant women in Africa (22). These encouraging results have renewed hope in the control of malaria. Despite these successes, efforts to scale-up this intervention have been hampered due to poor compliance, cost, mosquito resistance to insecticides and lack of political motivation on the part of governments in countries where malaria is endemic.

1.2.1.2 Chemoprophylaxis

The use of chloroquine (CQ) as first line treatment for malaria infection has been in use for decades due to its efficacy and cost-effectiveness. However, a new menace in malaria treatment is the parasite's resistance to this drug. In addition, there have also been reports of parasite's resistance to newer drugs such as sulphadoxine-pyrimethanime (SP) and quinine that were developed to replace CQ in most malaria endemic countries.

In recent years, the application of combination therapies has been explored as an alternative strategy for malaria treatment due to multidrug resistance of *Plasmodium falciparum* to traditional low-cost first line drugs – CQ and SP. Combination therapy with different antimalarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite. Combination therapies have been shown to increase efficacy, shorten duration of treatment (hence increase compliance), and decrease risk of resistant parasites arising through mutation during therapy (23).

In the last two decades, the emergence of artemisinin drugs such as artesunate and artemether, derived from the Chinese herb *Artemisia annua*, have renewed hope for malaria treatment. Artemisinin derivatives in combination (ACTs) with other antimalaria drugs have shown encouraging results in large-scale field trials in Africa. For example, combining artesunate and SP in comparison to SP alone was shown to significantly reduce uncomplicated malaria in children between the age of 6 and 59 months in Benin (24) and Equatorial Guinea (25). Another study carried out in Nigeria also showed that combining artesunate and amodiaquine in comparison to CQ and SP, reduced uncomplicated malaria among children less than 12 years of age (26). However, the emergence of artemisinin-resistant *P. falciparum* in Cambodia (27, 28) and on the western border of Thailand (29) now poses serious challenge to the use ACTs.

The use of intermittent preventative therapy (IPT) has also been explored as an alternative malaria treatment for pregnant women and children. Studies have shown that administering SP at specific times during pregnancy (IPTp), protects against maternal anaemia and low birthweight in Mozambique (30) and Mali (31). Studies

have also shown that administration of IPT in the first year of life (IPTi) reduced incidence of malaria in Tanzania (32, 33).

It has been suggested that ITNs together with other malaria interventions such as malaria chemotherapy and a malaria vaccine with 50% efficacy would eliminate malaria from the globe (34). However, due to the high cost of ACTs and IPTs, emergence of multidrug resistance strains of *P. falciparum* and the mosquito's resistance to insecticides, the development of a malaria vaccine that would be cost-effective remains the final strategy for the prevention, control and possible eradication of malaria.

1.2.2 Malaria Vaccines

There are five *Plasmodium* species that infect humans namely: *P. falciparum, P. ovale, P. vivax, P. malariae* and *P. knowlesi*. *P. falciparum* cause the most severe disease in man, while the other three except *P. knowlesi* cause milder infections. *P. knowlesi* is a zoonosis that infects macaques but can also infect humans.

The malaria parasite undergoes three stages of life cycle development with two stages occurring in man and one in the mosquito vector. These stages of the parasite's life cycle are antigen specific and have formed the framework for the development of a malaria vaccine; the pre-erythrocytic, blood stage and transmission blocking vaccines.

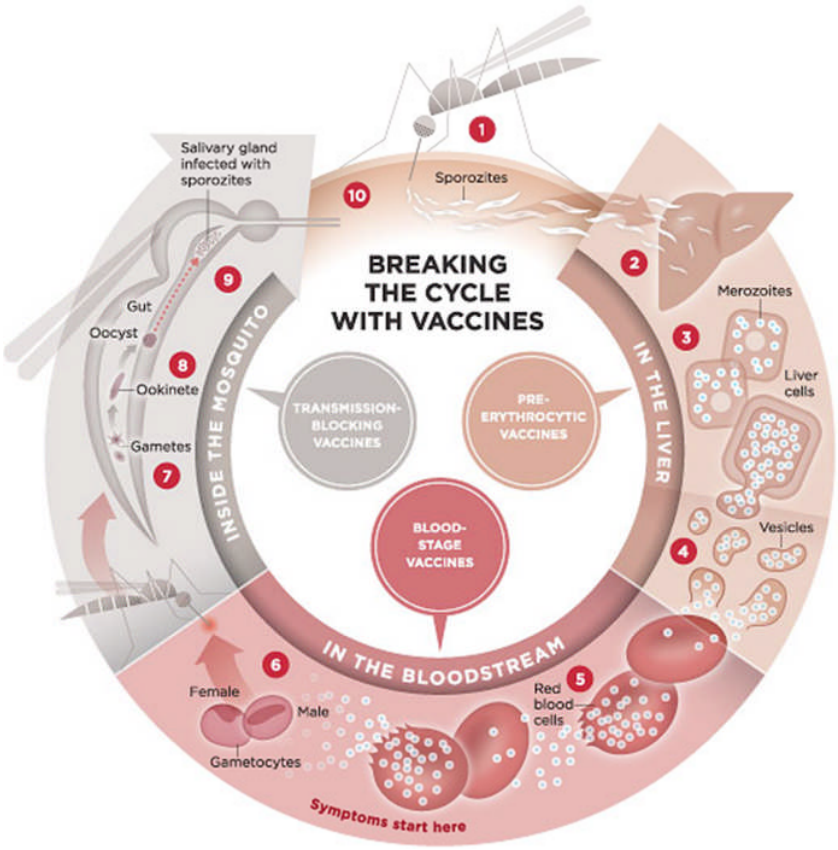


Figure 1.1: Life cycle of malaria parasite. (Adapted from (35))

Step 1. Malaria infection begins when an infected female *Anopheles* mosquito bites a person, injecting *Plasmodium* parasite in the form of sporozoites, into the blood stream alongside the saliva of the mosquito. **Step 2.** Within 60 minutes of infection the sporozoites enter liver cells where they multiply for 7 – 10 days. **Step 3.** In the liver cells, thousands of merozoites are formed (liver schizont), causing no symptoms of malaria. **Step 4.** In a typical animal model, merozoites are released from the liver cells in vesicles and they journey through the heart, and arrive in the lungs, where they settle within lung capillaries. The vesicles eventually disintegrate, freeing the merozoites to enter the blood phase of their development. **Step 5.** The blood stage development of the parasites takes about 2 days to occur. In the bloodstream, the merozoites invade red blood cells (erythrocytes) and multiply again until the cells burst, releasing merozoites, which infect more erythrocytes. This cycle (intra-

erythrocytic stage) is repeated causing fever each time merozoites are released and invade new erythrocytes. **Step 6.** Some of the infected red blood cells leave the cycle of asexual multiplication. Instead of replicating, the merozoites in these cells develop into sexual forms of the parasite, called gametocytes. These gametocytes circulate in the bloodstream (sexual stages of the parasite). **Step 7.** When a mosquito bites an infected human, the gametocytes are ingested, which develop into mature sex cells called gametes. **Step 8.** The fertilized female gametes develop into motile ookinetes that burrow through the mosquito's midgut wall and form oocysts on the exterior surface. **Step 9.** Inside the oocyst, thousands of active sporozoites develop. It takes about 5 -7 days before the oocyst eventually bursts, releasing sporozoites into the body cavity that travel to the mosquito's salivary glands, ready to infect another human. **Step 10.** The cycle of human infection begins again when the mosquito bites another person.

The year 1941 witnessed the first evidence that a vaccine can be developed against malaria infection. The work by Mulligan and colleagues (36) demonstrated in fowls that immunisation with irradiated sporozoites conferred protection against *P. gallinaceum*. Following this, a series of experimental works were carried out with irradiated sporozoites in non-human primates, but by the year 1973, Clyde and colleagues were the first to demonstrate in human subjects that immunisation with irradiated sporozoites could protect against *P. falciparum* (37).

Since the work carried out by Clyde and colleagues, the application of irradiated sporozoites has continued to show promise as a malaria vaccine model, but the approach remains unfeasible for mass vaccination. This is because over 1000 infectious bites are required to confer sterile protection in one human subject, which

poses serious constraints for large-scale production (38, 39).

In the last three decades, several attempts have been made to develop a malaria vaccine that could confer sterile and long lasting immunity against malaria infection, but these attempts have been stymied by the parasite's complex life cycle, large genome, practical difficulties, poor immune memory and unknown correlates of protection. Constraints in financial requirements required for taking a vaccine from pre-clinical development to clinical trials have also contributed to the delay in the quest for a malaria vaccine. However, the completion of the *P. falciparum* genome project, which identified about 5,300 putative genes (40) that make up the parasite's genome has paved the way for the discovery of many sub-unit malaria vaccines. There are currently over 90 vaccine candidates under development with RTS,S developed by GlaxoSmithKline (GSK) the most promising (41-43).

1.2.2.1 Malaria immunity and vaccine strategies

Current knowledge of protective immunity to malaria has been derived, in part, from studies of malaria in animals and from human trials of experimental vaccines. Animal studies have great value for elucidating immune mechanisms and for developing concepts and new approaches for clinically relevant vaccines, but the complex and multiple stages of the malaria parasite's life cycle have restricted vaccine development to single-antigen, single-stage vaccines, which have been disappointing. It has been suggested that because *Plasmodium* is a complex, multistage organism, a multi-antigen, multi-stage vaccine that will induce protective antibodies, $CD4^+$ and Cytotoxic $CD8^+$ T cell responses will be an ideal vaccine candidate.

1.2.2.1.1 Pre-erythrocytic Stage Vaccines

The life cycle of the malaria parasite begins with an infectious bite, at this stage the mosquito release sporozoites from its salivary glands into the host; the sporozoites then circulate in the bloodstream for about 60 minutes before invading hepatocytes (liver cells). Vaccines targeting the pre-erythrocytic stage of the parasite have focused on circumsporozoite protein (CSP), thrombospodin related adhesive protein (TRAP) and the liver stage antigen (LSA is identified in the liver stage of the parasite's life cycle). A vaccine that would achieve optimal efficacy at this stage is expected to (i) induce high antibodies by preventing sporozoites from invading hepatocytes (ii) eliminate all infected hepatocytes by cell mediated immunity (CMI). The parasite lives in the liver for 1 – 2 weeks (intra-hepatocytic) before forming merozoites, cytotoxic T cells (CTL) and natural killer (NK) T-cells (44) have been implicated in the killing of infected hepatocytes. $CD4^+$ T cells have also been shown to contribute to protection in a *P. yoelli* model (45, 46). Because MVA is capable of inducing potent antigen-specific T cell responses it is considered as a promising vector for pre-erythrocytic candidate vaccines.

Presently, the most advanced malaria vaccine is the RTS,S developed by GSK. It is a sub-unit vaccine based on the CSP protein of *P. falciparum* strain NF54, clone 3D and consist of 19 copies of central NANP repeats and the entire c-terminal of the protein containing T cell epitopes. This is fused at the N-terminal regi to hepatitis B surface antigen (HBsAg), which forms virus-like particles (VLPs) when co-expressed with additional copies of S. The RTS, S have been tested in clinical trials in combination with different GSK proprietary adjuvants; AS01B/E, AS02A/D, AS03 and AS04, but AS01B/E was reported as the most potent adjuvant (47) and conferred

protection against malaria in three African countries – Ghana, Tanzania, and Gabon. RTS,S was primarily designed to induced strong antibodies against malaria infection, but studies have begun to emerge than the vaccine also induce potent CS-specific $CD4^+$ T cells producing IFN-γ, TNF-a and IL-2 (41).

The RTS,S/AS02A vaccine have been assessed over a 45 months surveillance period in a large phase 2b in children aged 1 – 4 years in Mozambique. The vaccine efficacy was found to be 30% after six months (48) and induced long-lasting anti-circumsporozoite antibodies, persisting at least 42 months after immunisation (49). Presently, there is an ongoing phase 3 study of the efficacy, safety, and immunogenicity of candidate malaria vaccine RTS,S/AS01 being conducted in seven African countries. Initial results have shown 50% protection against both clinical and severe malaria in African children (50). More reports are expected from this trial by the end of 2012 and there are suggestions that the vaccine might be licensed by the year 2015.

Following, RTS,S, the ME-TRAP developed by Adrian Hill *et al*. (51) is another promising pre-erythrocytic stage vaccine. The ME-TRAP vaccine encodes multiple B and T cell epitopes of different malaria antigens fused to the liver-stage antigen, thrombospodin related adhesive protein (TRAP). The ME-TRAP was expressed in three vectors, DNA (D), FP9 (F) and MVA (M). Initial results expressing ME-TRAP in three vectors, DNA (D), FP9 (F) and MVA (M) showed that showed that the DDMF and FFM immunisation regimes were partially protective in malaria naïve adults that resulted in delay in time to patent parasitaemia after sporozoite challenge (51). Further testing of the efficacy of the vaccine in semi-immune Gambian men showed 10% protection efficacy with the FM regime (52). A more effective

immunisation regime employing a chimpanzee adenovirus (ChAd63) as the priming vector followed by boosting with MVA showed 21% vaccine efficacy against *P. falciparum* sporozoite challenge in malaria naïve adults (O'Hara *et al*., submitted). In addition, there are suggestions that combining RTS,S and ME-TRAP might favour a malaria vaccine towards greater efficacy (53). Based on these observations, could we improve the ME-TRAP by improving its expression in MVA? As there are many candidate promoters in MVA, can we harness these promoters to improve ME-TRAP expression? The work carried out in this thesis explores these possibilities by harnessing different candidate promoters in MVA for the expression of foreign antigens.

1.2.2.1.2 Erythrocytic or Blood stage Vaccines

The next stage of the parasite's life cycle is the blood stage. At this stage, the sporozoites mature and develop into liver schizonts after 1-2 weeks in the hepatocytes. Liver schizonts then release merozoites into the bloodstream where they invade red blood cells (RBCs). The merozoites reside in the bloodstream for 1-2 minutes before invading RBCs. In the erythrocytes (RBCs) the merozoites undergoes ring, trophozoites, and schizonts stages (intra-erythrocytic stage). The erythrocyte then ruptures releasing about 36 merozoites into the blood stream. This intra-erythrocytic cycle takes about 36-72 hours to complete (repeated cycles) and it is at this stage of rupturing and release of merozoites that malaria morbidity and mortality occurs. A malaria vaccine developed against this stage of the parasite's life cycle would not prevent the disease but reduce morbidity. Two plausible blood stage vaccine models have been proposed; anti-invasion and anti-complication (54). Anti-invasion would prevent merozoites from invading RBCs preventing disease

occurrence while anti-complication would kill merozoites, thereby reducing severity of malaria disease.

The apical membrane proteins 1 (AMA-1) and merozoites surface proteins (MSP) found on the surface of merozoites have been the focus of blood stage malaria vaccines. There are three distinct MSP proteins; MSP-1, MSP-2 and MSP-3. The MSP-3 was recently indentified and has been explored as a promising vaccine candidate in human subjects (55, 56).

The Combination B malaria vaccine is another blood stage vaccine that has shown promising results (57). The vaccine construct encodes the ring-infected erythrocyte surface antigen (RESA), MSP-1 and the 3D7 allele of MSP2 formulated in Montanide®ISA720. The study was carried out in a malaria endemic region, New Papua Guinea, in 120 children between 5-9 years. This age range was considered empirical for this study because the children were semi-immune and immunisation may boost responses by natural exposure. Vaccine efficacy was found to be 62% and shown to reduce parasite density over 4.5 months follow-up period (57).

Pre-clinical development of MSP-1 candidate vaccine employing heterologous prime-boost vaccination with ChAd63 and MVA have been shown to induce protective MSP-1-specific $CD4^+$ T cells which provided help for protective B cell responses, and $CD8^+$ T cells in mice (58) and macaques (59). Because of these encouraging results, MSP-1 was taken forward into phaseI/IIa clinical trials employing ChAd63 and MVA heterologous prime-boost vaccination strategy. Vaccine efficacy showed induction of potent antibodies as well as $CD8^+$ T-cells that resulted in delay in onset of patent parasitaemia after sporozoite challenge (12). AMA-1 has also shown promising results in mice (60) and is currently been explored in a phaseI/IIa clinical trial at the

Jenner Institute, University of Oxford. These observations clearly shows that MVA based vaccines have the potential to act at the blood as well as the liver stage, via antibodies as well as T cells. One of the important questions in this thesis is how these approaches (liver and blood stage antigens) can best be combined, since complete protection against malaria has not been achieved with the monovalent MVAs currently in use.

1.2.2.1.3 Transmission Blocking Vaccines

This is the vaccine that has received the least attention in the league of malaria vaccine candidates because there are suggestions that it would not prevent the disease (altruistic vaccine - vaccine that is taken after falling sick. It renders the blood poisonous to the mosquitoes after a blood meal and the mosquito becomes unable to transmit the malaria parasite) but possibly used to control epidemics or to prevent escape mutant when used as part of a combination vaccine. The last stage of the parasite's life cycle takes place in the mosquito gut. During the repeated intra-erythrocytic cycle, some merozoites differentiate into male and female gametocytes, which are then taken up during a mosquito blood meal. Experimental models have shown that a vaccine developed at this stage would function in the mosquito gut as the mosquito ingest both antibodies and complement proteins with the parasite during a blood meal (61, 62)

A DNA-based vaccine construct that encoded two antigens identified on different sexual stages of *P falciparum*, Pf27 in gametocytes and Pf25 in zygotes (61) induced high antibody titres in vitro. The study also reported 97% reduction of oocyst numbers in midguts of the mosquito and 75% decrease in the rate of infection. Another *P falciparum* antigen, Pf230 has also shown transmission blocking activities

(62). It was reported that this candidate antigen elicited antibodies that blocked development of gametocytes in the mosquito.

Pre-clinical development of ChAd63 and MVA vectors expressing Pf25, Pf27 and APN1 candidate antigens are currently ongoing at the Jenner Laboratories. Results from this work have been promising and all three-candidate antigens have been reported to induce neutralising antibodies with transmission blocking activities on gametocytes (Kapulu *et al*. submitted).

1.2.2.2 Cytotoxic T Lymphocyte ($CD8^+$) vaccines for Malaria

The rational for an effective $CD8^+$ T cell-based malaria vaccine is the induction of broad $CD8^+$ T cell responses to reduce the chance of liver schizont from escaping from hepatocytes (liver cells) to form merozoites in the blood. The possibility that generation of $CD8^+$ T cells against liver stage antigens could confer complete protection against malaria infection comes from studies that involved murine models (63). The study identified a T cell epitope from the circumsporozoite protein of *P. berghei* that were adoptively transferred to naïve Balb/c mice and reported a high degree of protection against challenge with sporozoite. Subsequent challenge with infected red blood cells did not confer protection confirming the protective effect was stage specific.

Indirect evidence on the role of protective $CD8^+$ T cell against malaria has also been reported in West African children expressing the HLA B53 allele, implying an important role for HLA class-I-restricted T cells and is suggestive that vaccines designed to induce antigen-specific $CD8^+$ T cell could provide equivalent protective responses (64). As mentioned before, current knowledge of the protective role of T

cells in malaria comes from murine models and studies have shown that the intrahepatocytic stage of the malaria parasite is where the protection is being mediated through (i) direct recognition of infected liver cells by naïve CD8 T cell (ii) effector $CD8^+$ T cell secrets IFN-γ that directly kills infected hepatocytes (iii) Type 1 helper T cells facilitate production of nitric oxide which have inhibitory effect on the development of liver-stage parasites (65, 66).

Following these observations, A. V. S Hill and colleagues have in the last two decades developed recombinant adenovirus and MVA vectored vaccines that induced protective T cell immunity (51, 67-72). As mentioned earlier (section 1.2.2.1.1), the most advanced of these vaccines is the ME-TRAP and has shown 21% sterile efficacy in malaria naïve adults after *P. falciparum* sporozoite challenge (O'Hara *et al*., submitted). Success achieved with this approach is partly due to MVA as the boosting vector following prime with adenovirus. The application of heterologous prime with adenovirus followed by boosting with MVA for malaria and other infectious diseases is discussed in details in section 1.2.4.

1.2.3 T-cell inducing vectored vaccines

As discussed earlier (section 1.2.2.1.2), T-cell inducing vectored vaccines are beginning to emerge as suitable vaccine delivery platforms for induction of protective T cell responses as well as antibodies. These vectored vaccines have been shown to induce cellular immune responses against intracellular pathogens and have therefore pioneered the development of vaccines against malaria (12), HIV (73), TB (8), influenza (9) and cancer (74).

The following vectored vaccines have been reported to induce protective T cell responses:

- DNA vaccine such as pTH (75) and pSG2 (76)
- Recombinant BCG (77-79)
- Recombinant salmonella (63)
- Virus like particles (VLS) (80)
- Baculovirus (81)
- Replication deficient adenoviruses (12)
- Attenuated orthopoxviruses, such as MVA (82) and NYVAC (83-86)
- Attenuated avipoxviruses, such as fowlpox virus strain FP9 (87-89) and ALVAC (90)

The viruses employed in this thesis are recombinant MVA and adenovirus vectors which are discussed in details in sections 1.3 and 1.4. Pre-clinical and clinical trials on malaria vaccines carried out by A. V. S. Hill and colleagues has revealed the central role of MVA as the boosting agent following priming with adenovirus prime.

1.2.4 Heterologous Prime-Boost Regimes

Despite the availability of the different vaccine delivery vectors mentioned above, repeated immunisations with the same vaccine vector has been a challenge for the induction of effector T cells against the target antigen. Although repeated immunisations is a well-recognised practice for the induction of humoral immunity, but its application has failed at boosting cell-mediated immunity which is important for protection against diseases caused by intracellular pathogens. The development of

heterologous prime-boost immunisation regime was aimed at directing the immune responses to the recombinant target antigen instead of the vector. This is because for some vaccine vectors such as recombinant adenoviruses and poxviruses, pre-existing immunity or immune responses generated against viral antigen dampens the quality of immune responses generated against the target antigen.

In the case of malaria, it has been reported in previous challenge studies employing DNA and MVA vectors expressing CSP or ME-TRAP, that DNA-MVA regime was better at inducing protective antigen-specific T cells in mice (91) and humans (92) in comparison to homologous DNA or MVA regimes. Following these observations, the next step was to explore heterologous prime-boost immunisations with different vaccine delivery vectors expressing the same foreign antigen. While attempts were made to explore different, unrelated vaccine platforms it was found that despite the development of anti-vector immunity, immunisation with two poxviruses – priming with FP9 (avipoxvirus) followed by boosting MVA (orthopoxvirus) induced potent antigen-specific effector and memory T cell responses (52, 71). This is because minimal cross-reactivity has been reported between two poxviruses (89).

Some important observations have been made following these immunisation regimes: (i) recombinant chimpanzee adenovirus ChAd63 primes as effective as the human HAdV5 serotype (ii) the timing between priming and boosting is critical for inducing potent $CD8^+$ T cells to the target antigen boosting (ii) recombinant adenovirus vectors are better at priming and MVA good at boost. Priming with ChAd63 followed by boosting eight (8) weeks apart with MVA expressing the same antigen, ME-TRAP, was capable of inducing protective antigen-specific $CD8^+$ T cells after sporozoite challenge in mice (93) macaques (94) and humans (12). This adenovirus prime and

MVA boost immunisation regime strategy have also been employed for inducing high antibodies against MSP-1 as well as potent MSP-1-specific $CD8^+$ T cells in mice (58) and macaques (59). Similar heterologous prime boost strategy employing BCG-MVA have been developed for TB (8) and HIV (95).

1.3 Adenovirus Vaccine Vectors

Adenoviruses are members of the *Adenoviridae* family and are double-stranded DNA (dsDNA) viruses of 34 – 44kb. The virus was first isolated in 1953 in the adenoid tissue, hence the name (96). The virion is non-enveloped, and has an icosahedral capsid about 60 – 90 nm in size. The genome encodes about 30 proteins and both strands of the adenovirus DNA and encodes genes that are transcribed in three stages, immediate early, early and late. There are approximately fifty-two human adenovirus (HAdV) serotypes identified so far, which have been further classified into eight species (A – F) within the *Mastadenovirus* genus. HAdV serotypes cause approximately 5 – 10% of upper respiratory tract infections in children as well as many infections in adults including conjunctivitis and tonsillitis (97).

The genome of adenovirus is not as complex as poxvirus and could be easily manipulated for the expression of foreign antigens. To attenuate the virus as an expression vector the E1 region that is essential for the replication of the virus has been deleted. This deletion renders the virus replication incompetent in mammalian cells. In order to increase the insertion capacity of the virus, a further deletion of the E3 region that encodes proteins involved in immunomodulations as is it non-essential *in vitro* was made (98, 99). The E1-E3 deficient adenovirus can be propagated to high titres in cell cultures, using cell lines that provide the missing gene products *in trans*.

Recombinant adenoviruses were initially developed as vehicles for gene therapy. For example, adenoviruses have been trialled as transfer vectors for correction of human genetic defects such as cystic fibrosis but the technology was unsuccessful in animals and humans due to immune responses induced by viral antigens (100). However, observations made during application of adenoviruses for gene therapy pioneered its use as vaccine delivery vehicle for infectious pathogens owing in part to transgene-specific T cells as well as antibodies induced by the virus.

The most common adenoviruses employed for the delivery of foreign antigen are the human type 5 (HAdV-5), HAdV-11 and HAdV-35, which have been shown to induce antigen-specific $CD8^+$ T and $CD4^+$ T cells against different pathogens. Vaccines based on HAdV-5 expressing disease specific antigens have been reported to induce antigen-specific $CD8^+$ T and $CD4^+$ T cells for malaria AMA-1 antigen (60), HIV (101) and TB (102). The HAdV-35 vector expressing the TB antigen85A (Atg85A) have also shown promising results for inducing specific $CD8^+$ T and $CD4^+$ T cells in mice (103) and humans (104).

Despite these success stories, there is high prevalence of neutralizing antibodies in the human population to HAdV-5 and HAdV-35, which make them unsuitable for clinical use. Alternative adenoviruses from the chimpanzee serotype 63 (ChAd63) have emerged as potential replacement for human serotypes because they lack pre-existing immunity in humans (105). ChAd-63 based vectors have been explored for the induction of antigen-specific cellular and humoral immune responses and were reported to induce similar responses to HAdV-5 in mice (106). In a recent phase I/IIa study, ChAd-63 encoding the malaria ME-TRAP antigen followed by boosting with MVA-ME-TRAP, showed ChAd-63 to be safe, well tolerated, immunogenic and

capable of inducing unprecedented magnitude of $CD8^+$ T cells that were protective against sporozoite challenge (O'Hara *et al*., submitted)

1.4 Poxvirus

In all the Jenner trials, MVA has featured as a potent boosting agent. MVA is a poxvirus and here is some background on poxviruses. The *Poxviridae* are a family of complex double-stranded, large (180 – 300kb) DNA viruses. The viruses carry their own transcriptional enzymes and replicate in the cytoplasm of vertebrate and invertebrate cells. Poxviruses are divided into two subfamilies; *Chordopoxvirinae* that infect vertebrates and *Entomopoxvirinae*, which infect insects. The *Chordopoxvirinae* subfamily consists of eight genera including *Avipoxvirus* (infects birds) and the *Orthopoxvirus*, which is the most studied. Four viruses of the genus *Orthopoxvirus,* are known to infect humans – vaccinia virus (VACV), cowpox virus (CPXV), monkeypox virus (MPXV) and variola virus (VARV – now eradicated), although others can be transmitted from animals to humans such as monkey poxvirus, orf virus (ORFV), pseudocowpoxvirus (PCPV) and bovine papular stomatitis virus (BPSV) (107-109). Several members of the orthopoxviruses have been sequenced with greater than 90% common genes seen for VACV, VARV and ectromelia virus (ECTV).

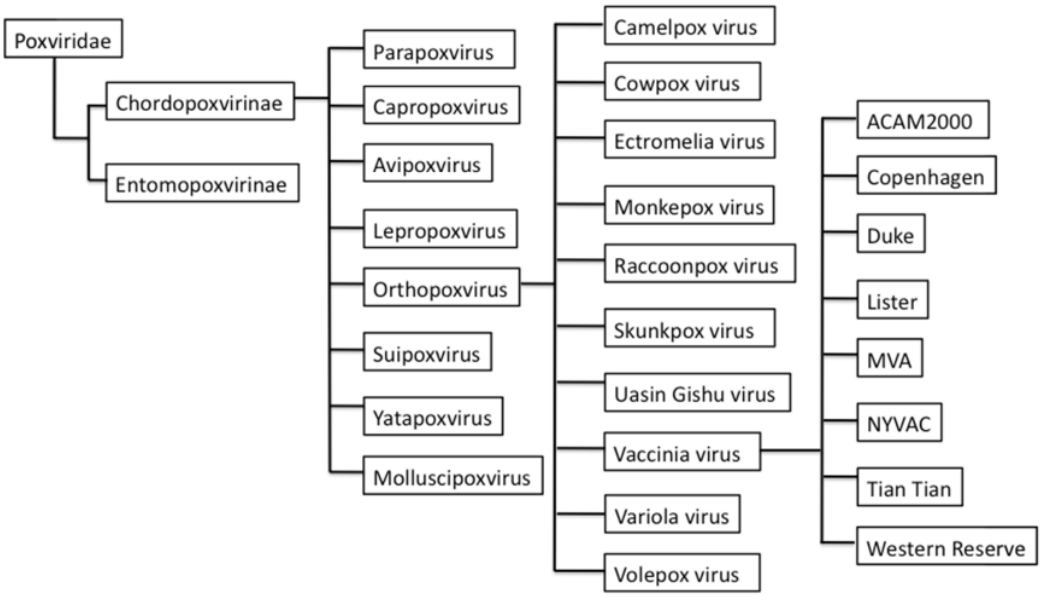


Figure 1.2: Phylogenetic tree of Poxviridae. (Text adapted from (110)

Variola virus is the causative agent of smallpox, a disease described to have profoundly influenced human history. Because of the cross-reactivity between orthopoxviruses, Edward Jenner demonstrated in 1796 that intradermal inoculation with cowpox could confer protection against smallpox (1). This cross-reactivity was further explored with another closely related strain, vaccinia virus (VACV), which is the vaccination agent that was used to eradicate smallpox. In the year 1980, the World Health Assembly declared the world free of smallpox after an Intensified Smallpox Eradication Programme and it remains the only disease in humans to have been eradicated through vaccination apart from rinderpest in animals (3-6).

The natural host reservoir of VACV remains unknown to this day (111), and there are suggestions that because VACV was extensively propagated for many years as a smallpox vaccine, the virus may no longer have a natural host (111). However, there have been mild to fatal complications reported after immunisation with the replication competent VACV in immuno-compromised adults. In the 1960, there were serious

side effects reported in the United States in 1:800 primary vaccinees, and death in one per million after vaccination with Dryvax (NYCBH-Wyeth strain) (112-114), and even with ACAM2000 (current cell-culture derived smallpox vaccine licensed in the United that replaced Dryvax).

The first description that vaccinia virus could express foreign genes to elicit immune responses was demonstrated in 1983 by Geoffrey Smith and colleagues (115). In these inaugural studies, the cDNA of Hepatitis B surface antigen (HepB SAg) was inserted into VACV via two different methods (1) fused to the coding sequence of a VACV early promoter and inserted into the thymidine kinase (TK) gene locus (ii) inserted upstream of the TK gene promoter. Because of the safety concern of VACV, other highly attenuated strains such as NYVAC and modified vaccinia virus Ankara (MVA) has been developed as alternative vaccination agents against smallpox (116). The attenuated NYVAC strain was derived from the Copenhagen strain of VAVC (VACV-COP), by the precise deletion of 18 genes involved in pathogenesis and host range (117). MVA, was derived from chorioallantois vaccinia virus Ankara (CVA), which was a smallpox vaccine strain used in Turkey that was serially passaged more than 570 times in chicken embryo fibroblast (CEF) (118). The resulting NYVAC and MVA vectors were proven to be replication deficient in most mammalian cells whilst maintaining their ability to express foreign genes in animals and humans (119-122).

The subject of this thesis is MVA, and the following section discusses the derivation, properties and applicability of MVA as a vaccine vector in more detail.

1.4.1 Modified Vaccinia Virus Ankara

Modified Vaccinia virus Ankara (MVA) is a replication deficient poxvirus that was derived from vaccinia virus through more than 570 passages in chick embryo fibroblast cells (CEF). During its attenuation MVA lost about 30kb or 15% of its genome and it is replication deficient in most mammalian cells (119). It was developed towards the end of the campaign for the eradication of small pox. The genome of MVA is 178kb in length and comprises 193 open reading frames (ORF) of which 25 are have suffered mutations resulting in truncated proteins (123). The genome of all poxviruses including MVA is divided into major regions, left terminal regions (LTR), right terminal regions (RTR) and the conserved region (CR). In MVA, the LTR contains four large deletions and one large insertion compared to the Copenhagen strain. Many ORFs in this region are fragmented, having only eight structurally intact genes. The RTR carries three large deletions, which consists of poxviral immune evasion and ankyrin-like genes. For example, the *B8R* and *B18R* in vaccinia virus Western Reserve (VACV WR) are responsible for secretion of IFN-γ and IFN-α/β soluble receptors respectively, but are fragmented in MVA (124-126). The conserved genomic region in MVA also carries genes that are fragmented such as *F5L*, a major membrane protein and *F11L* that encodes a protein involved in cell motility.

Despite these findings, MVA is still as immunogenic as the conventional replication competent VACV and presently considered as the VACV strain of choice for clinical investigation because of its safety profile in humans (127, 128). In recent years, significant progress has been made in the development of MVA as a vaccine delivery vehicle because of (i) large packaging capacity for recombinant DNA (ii) ease of

vector and vaccine production (iii) lack of persistence or genomic integration in host (iv) precision of virus-specific control of target gene expression (v) high immunogenicity as vaccine (vi) genetic stability (vii) thermostability in high temperature (viii) ability to express heterologous antigens.

As mentioned earlier, recombinant MVA has been used extensively in combination with other vaccine vectors such as DNA and adenoviruses for eliciting antigen specific $CD8^+$ T cells for infectious diseases such as malaria, influenza, HIV, and tuberculosis (TB) (9, 71, 73, 89, 129, 130).

MVA expressing the *Mycobacterium tuberculosis* antigen 85A, known as MVA85A, is one of the most advanced recombinant MVA vaccines in clinical trials. Data from recent Phase I/II clinical trials have shown MVA85A to be safe and effective in boosting responses induced by BCG vaccine (8, 127). MVA expressing influenza NP and M1 antigens (MVA-NP+M1) have also been found to be safe and immunogenic in a Phase I clinical trial (9). Preliminary data from a Phase II study in healthy adults volunteers showed MVA-NP+M1 also induced antigen-specific $CD8^+$ T cells that were protective against challenge with influenza A virus (11). MVA encoding multi-epitope string of different malaria antigens (ME) fused to the liver-stage antigen – thrombospodin-related adhesion protein (TRAP) have also been shown to protect adult malaria-naïve volunteers when used in heterologous prime-boost immunisation regime with an attenuated avipox virus –fowlpox strain FP9 (51) and adenovirus vector ChAd-63 (O'Hara *et al*., submitted)

1.4.2 Mechanism of infection and gene expression in vaccinia virus

The life cycle of all poxvirus viruses with vaccinia virus the most studied is comprised of three distinct phases – early, intermediate and late. The life cycle is tightly controlled by expression of the three classes of genes with the transcription of each gene class being dependent upon expression of the genes of the previous class (131).

There are two infectious forms of the virus named intracellular mature virus (IMV) and extracellular enveloped virus (EEV) and they both can initiate the virus replication cycle via different mechanisms (132). The life cycle begins by virus attachment (IMV or EEV) to glycosaminoglycans (GAGs) on the surface of the target cell or by components of the extracellular matrix, triggering membrane fusion and release of the virus core into the cytoplasm.

In the cytoplasm, viral RNA polymerase transcribes early genes within 30 minutes of infection. The early gene class represent approximately half of the virus genome and encode proteins that are responsible for virus virulence, immunomodulators and proteins the encode enzymes required for DNA replication such as DNA polymerase. At the end of early gene expression, the core is completely uncoated enabling the viral genome to move freely in the cytoplasm.

The intermediate phase begins approximately 100 minutes post-infection, prior to initiation of genomic DNA replication. Once the virus core is uncoated, the expression of intermediate genes begins. The intermediate genes encode several proteins required for the expression of late genes such as vaccinia late transcription factor (VLTF).

Late phase of VACV life cycle occurs between 140 minutes lasting up to 48 hours post-infection, producing all structural proteins and proteins packaged inside progeny virions required for the next cycle of infection. The assembly of progeny virions starts in association with internal membranes of the infected cell, which then produce a spherical immature virus (IV) particle that is not infectious. The non-infectious IV particle matures into brick-shaped intracellular mature virion (IMV) during a process that involves condensation of virus core and proteolytic cleavage of several capsid proteins (133).

IMV can be released upon cell lysis, or transported near the microtubule organising centre (MTOC) where it acquires a second double membrane from *trans*-Golgi or endosomes to form intracellular enveloped virus (IEV). The IEV is thought to contain two additional membranes compared to IMV, which also has only one lipid bilayer. The IEV is transported away from the MTOC to the cell periphery where it fuses with the plasma membrane to expose an enveloped virion on the cell surface. This virion is called cell-associated enveloped virus (CEV), if it is retained on the cell surface or extracellular enveloped virus (EEV), if released into the extracellular matrix {Smith, 2004 #2387;Smith, 2002 #3323

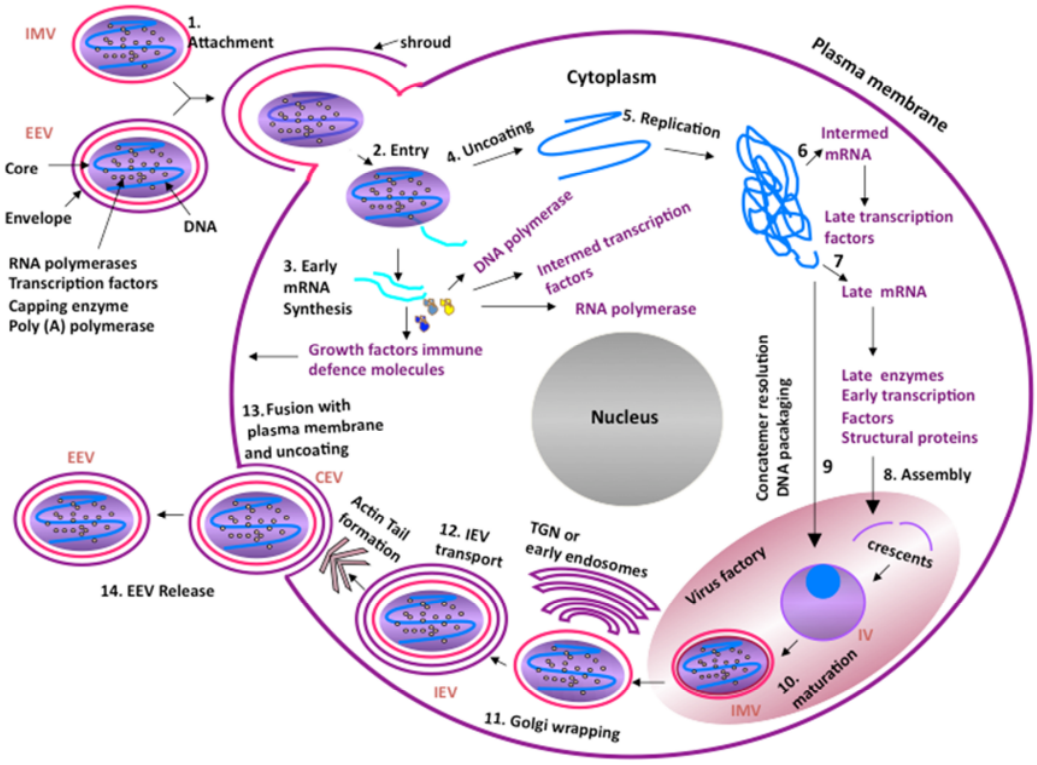


Figure 1.3: Life Cycle of Vaccinia Virus (or MVA in CEF or BHK cells).

(Adapted from Saroj Saurya)

Step 1: An infectious form of the virus (IMV or EEV), containing a double-stranded DNA genome, enzymes and vaccinia early transcription factors (VETF), attaches to a cell, and fuses with the cell membrane. **Step 2:** Virion attachment is followed by entry and release of a core into the cytoplasm. **Step 3:** The core synthesises early mRNA that is translated into different proteins such as viral growth factor (VGF), immune defence molecules, enzymes for DNA replication, and vaccinia intermediate transcription factors (VITF). **Step 4:** The core is uncoated and the DNA moves freely in the cytoplasm. **Step 5:** The DNA polymerase facilitates DNA replication to form concatemeric molecules. **Step 6:** Transcription of intermediate genes contained in the progeny DNA occurs, and the mRNA is translated into vaccinia late transcription factors (VLTF). **Step 7:** Transcription of late genes begins and the mRNA is

translated to form virion structural proteins, enzymes and vaccinia early transcriptional factors (VETF). **Step 8:** Assembly of progeny virion begins with formation of discrete membrane structures. **Step 9:** The concatemeric DNA intermediates are transformed into unit genomes and packaged in non-infectious immature virions (IV). **Step 10:** The non-infectious IV particle matures into brick-shaped intracellular mature virion (IMV). **Step 11:** The IMV can be release via cell lysis or wrapped by modified trans-Golgi and endosomal cisternae to form intracellular enveloped virions (IEV). **Step 12:** The IEV is transported to the cell periphery by microtubules. **Step 13:** On the cell surface, the IEV fuses with the plasma membrane to uncoat an extracellular enveloped virus (CEV). The actin tails polymerizes in the cytoplamic beneath the CEV to form actin rockets, or the CEV detaches to form EEV. Adapted from (110)

1.4.3 Gene classification and promoters in vaccinia virus

Vaccinia virus gene classification is based on the timing of individual gene expression (early, intermediate and late). Gene expression is tightly controlled and the transcription of each gene class is dependent on the expression of the preceding gene class (133, 134). In VACV, gene classification and promoter functions are tightly conserved and the promoters have been classified on the basis of the class of genes they transcribe. A promoter can also exert its transcription activities in more than one gene class and an example is the vaccinia virus p7.5 which has both early and late promoter functions (135) and commonly used in making recombinants. In addition to the three classes of VACV genes, an immediate-early (IE) class of genes was recently identified (136). The distinction of this class of gene from traditional early genes is not well understood, but it was defined based on its kinetic description of preferential

expression before the early genes. Unlike other vaccinia virus promoters, no known promoter consensus sequence has been reported for this class of gene.

Transcriptional analysis of vaccinia virus early promoters are characterized by an A-T rich region of approximately 45 nucleotides in length preceding the RNA start site with a specific 3'-terminal sequence of 28 nucleotides (137). The intermediate promoters share similar transcription start site (TAAATG) with the late promoters. Their mechanism of transcription is poorly understood but sequence analysis reveals an A-T rich upstream element (137) and an example is the I1L promoter (138), which was initially considered as a late promoter. Late promoters are known to share similar transcription start sites with intermediate promoters and have A-T rich upstream element.

There are many promoters used for making recombinant vaccinia viruses but three are commonly used (p7.5, modified H5 (mH5) and short synthetic promoter (ssp)) all having early and late promoter elements. The SSP has strong early/late promoter core that elicits robust antigen specific $CD8^+$ T-cells and antibodies. It was developed based on consensus sequences of vaccinia early and late promoters (139). However, studies have shown that the promoter transcribes transgenes in excess that could affect the growth and stability of the recombinant virus (140). The p7.5 is classified as transcribing moderate to high levels of transgenes, while studies have shown that the mH5 has the strongest early promoter core compared to SSP and p7.5 (95, 140).

1.4.4 Generation of $CD8^+$ T cells against poxviral early gene products

The ability of different strains of VACV to induce virus-specific $CD8^+$ T cells has provided useful information for rational design of vaccine candidates against pathogens that require $CD8^+$ T cells for protective immunity. A comprehensive analysis of the VACV genome and the genes it encodes has been conducted to better understand how VACV elicits $CD8^+$ T cells and data from different VACV strains that have been studied shows that the cellular and humoral target different classes of VACV genes (141-145). For example, previous studies have shown that when vaccinia viruses such as MVA infects DCs only viral early genes, the main target of $CD8^+$ T cells are expressed and the viral life cycle is arrested before late gene expression occurs (146, 147).

Sette and colleagues (142) have demonstrated that the different classes of genes control the mechanism of mounting immune responses to VACV infection. The study employed the use of the type of protein encoding mRNA for predicting the type of immune responses targeted by different viral proteins and reported that mRNA expressed 4 hours post-infection (hpi) correlated with viral proteins that target $CD8^+$ T cells. They further reported that $CD4^+$ T cells or antibodies were not targets of mRNA expressed 4hpi but preferentially targeted by viral proteins with late mRNA expression and that $CD4^+$ T cells frequently recognised antigens prominently recognised by antibodies and vice versa. The study concluded that $CD4^+$ T cells and $CD8^+$ T cells preferentially recognise different set of antigens, suggesting a strong correlation between early gene products and $CD8^+$ T cells, and a high association between late gene products, $CD4^+$ T cells and antibodies.

Arssasson and colleagues (136) have also used kinetic mRNA analysis to define the VACV transcriptome and identified four broad classes of mRNA defined on the basis of their expression kinetics namely immediate early (IE), early (E), early/late (E/L) and late (L). The study also found a strong correlation between genes in the IE and E clusters and antigens that target $CD8^+$ T cells. For example, *B8R* was categorized into the E cluster and reported as the immunodominant determinant (IDD) accounting for >25% of the total VACV response in C57BL/6 mice and up to 40% when combined with the other four sub-dominant determinants (SDD – A19L, A47L, A42R and K3L)(144). *K3L* is another VACV gene that is expressed early and has been shown to correlate with potent $CD8^+$ T cells in C57BL/6 mice (141, 144). F11L and A44L are also early VACV genes that have been found to induce strong $CD8^+$ T cells in mice (143).

The work described in this thesis aimed to turn the $CD8^+$ T cell immunodominance of early viral antigens to our advantage by replacing them with a recombinant antigen regulated by the endogenous promoters of these genes (henceforth referred to "poxviral endogenous promoter driven insertion loci").

1.4.4.1 MVA and antigen presentation by dendritic cells

In vitro studies have shown that MVA preferentially infects professional antigen presenting cells (APCs) such as dendritic cells (DCs) and that when MVA infects DCs only viral early genes are expressed and that viral life cycle is arrested before late gene expression occurs (146, 147). Since late poxviral genes are not expressed on DCs, several studies have been carried out to determine the type of immune responses they generate and it has been reported that late viral proteins preferentially target antibodies (148, 149).

In vivo studies on poxviruses have shown that induction of $CD8^+$ T cells by DC is mediated by peptides derived from viral proteins bound to major histocompatibility complex (MHC) class I molecules (150). Another study involving expression of the human Her-2/neu antigen driven by the p7.5 promoter also showed that MVA infection of human DC is restricted to the production of viral early proteins. The study reported that analysis of early and late viral proteins on immature DC (iDC) and mature DC (mDC) showed that only early viral polypeptides were made and that late viral proteins were not detectable on either iDC or mDC. DCs generally display preferential presentation of viral antigens on MHC class I molecules via (i) direct presentation or (ii) cross-presentation pathways.

The direct presentation pathway involves presentation of viral peptides on MHC class I molecules that have been derived from proteins that have undergone proteolytic degradation in the proteasome. These peptides are then delivered from the cytoplasm to the endoplasmic reticulum (ER) by an ATP- dependent transporter associated with antigen processing (TAP). In addition, the newly synthesized MHC class I molecules in the ER are attached to the TAP complex and receive peptides transported into the ER. Stable complexes of class I MHC molecules with bound peptides are then transported out of the ER, through the Golgi complex, to the cell surface as peptide-MHC complexes.

In contrast, the cross presentation pathway involves uptake of exogenous antigen transferred from virus-infected cells. The underlying mechanism of this pathway is that antigens that are not available to the direct presentation pathway are processed and presented on MCH class I molecules for the induction of $CD8^+$ T cells. In addition to other mechanisms, infected cells undergo apoptosis, and the apoptotic

blebs are taken up by uninfected DCs and cross-presented on MHC class I molecules which then induces $CD8^+$ T cells. So because poxviral early genes are preferentially expressed on DCs, we aim to replace the ORF of poxviral early genes with the ORF of a transgene for the induction potent $CD8^+$ T cells.

1.4.5 Generation of recombinant MVA

The conventional method for generating recombinant MVA (rMVA) vaccine vector is straightforward and requires a single insertion mediated by genomic homologous recombination between the virus genome and DNA from a plasmid that carries one or two transgenes being placed under a promoter inserted at the TK locus (151) or other insertion loci such HA (152) or deletion I to IV. The TK locus is commonly used for insertion of foreign genes and it is the insertion locus used for generating rMVAs in Jenner Laboratories, partly because it was first used in vaccinia virus and found to be genetically stable for transgene expression.

The conventional method for generating rMVAs relies on inefficient endogenous recombination in poxvirus-infected cells, requiring extensive plaque picking to get rid of unrecombined MVA, but the use of a recombinant mediated engineering (recombineering) of a bacterial artificial clone of MVA (MVA-BAC) is a faster and efficient method for manipulating the genome of MVA when deleting and adding foreign genes into the genome of MVA.

During the attenuation of MVA, some of its genes became fragmented or truncated rendering them non-functional or essential for the survival of the virus. For example the ORF of *B8R* is fragmented in MVA so we aim to use the BAC recombineering technology to replace the ORFs of these genes with the ORF of a transgene positioned

to use the same endogenous poxviral promoter. This means that the insertion site would have its own promoter and the promoter would have its own insertion site enabling transcription of the transgenes from the natural locus of the deleted gene.

1.4.5.1 Recombineering

Cottingham *et al*. (153) have previously explained the construction of the parent MVA-BAC used for the generation of rMVA viruses in this thesis. A summary of the recombineering technology is explained below but specific details of each BAC derived MVA generated is explained in material and methods and in subsequent chapters.

Recombineering (recombinant mediated genetic engineering) is a fast and efficient method for construction of vectors for subsequent manipulating in cell cultures. The method allows the DNA of bacterial artificial chromosomes (BACs) to be modified by homologous recombination in *Escherichia coli* (*E. coli*). BACs are stable and have a large insert capacity of about 100 – 300kb. (154, 155).

The *SW102* is one of the *E. coli* strains conventionally used for recombineering. It has a defective λ prophage in its genome and also has a *λPL* promoter that transcribes three phage genes, *exo, beta* and *gam*. *Exo* is a 5'-3' exonuclease that creates single stranded overhangs when nuclear DNA is introduced. *Beta* protects these overhangs and assists in the subsequent recombination process and *gam* prevents degradation of linear DNA by inhibiting *E. coli Rec*BCD proteins.

At 32^{o}C the *λPL* promoter is repressed by a temperature sensitive repressor protein *cI*857 and derepressed at 42^{o}C. When bacteria containing this phage are cultured at

32°C, they do not produce these recombination proteins but when the temperature is raised to 42°C for 15 minutes a sufficient amount of these proteins are produced.

The *E. coli* galactose operon is made up of four genes that are essential for growth and utilization of galactose as it only carbon source (*GalK, GalE, GalT and GalM)*. For recombination to occur the galactose operon has been modified such that the *E. Coli SW102* strain is defective of *GalK* and when *GalK* is added *in trans* the bacterium regains its ability to grow in the presence of galactose as its only carbon source (155). The small size of the *GalK* gene, about 1200bp allows its use in the recombineering system for both positive (to select for *GalK* in the presence of galactose) and negative selection (removal of *GalK* in the presence of de-oxygalatose), thus its advantage for addition and deletion of genes in MVA.

Recombineering is achieved by electroporating a PCR product (insert) with *SW102* competent cells followed by a recovery period in liquid broth (LB) at 32°C, before washing with M9 salts to remove enriched media followed by plating on galactose minimal plates. To rescue the MVA-BAC clones (DNA) back to infectious viruses, infection must be initiated with the help of a helper virus that provides early functions because infection cannot be initiated by viral DNA alone (MVA-BAC), To achieve this, the MVA-BAC DNA is transfected in BHK cells infected with an attenuated Fowlpox virus. The Fowlpox virus acts as a helper virus by providing transcription machinery for the MVA-BAC. Cells are then monitored for GFP expression by epiflourescence microscopy, and viruses harvested by freeze-thaw then used to infect fresh BHK cells. Rescued viruses are plaque purified before propagation in CEFs.

1.5 Thesis outline

This thesis describes the development of recombinant MVAs designed to transcribe a recombinant antigen (epitope tagged luciferase) from poxviral endogenous promoter driven insertion loci. Because MVA carries many genes that are non-functional and non-essential for the survival of the virus, seven strong early or immediate early genes from the literature were selected. This is because we hypothesized that the $CD8^+$ T cell immunodominance of these genes could be turned to our advantage by replacing them with the recombinant antigen under the control of the natural promoters of these genes. To do this, a recombineering technology to delete the ORF of these genes for replacement with the model antigen epitope tagged luciferase was employed.

As earlier mentioned, there is the urgent public health need to develop T cell based vaccines against infectious diseases such as malaria, HIV, TB and influenza where antibodies have failed to confer sterile immunity. For some pathogens like malaria, two or three antigens from different stages of the parasite's life cycle might be required to achieve sterile immunity, whereas most clinical trials have been conducted with a single antigen expressed from a recombinant viral vector. To address this question, the work described in this thesis covers the design, construction and generation of a multivalent MVA for the expression of two recombinant antigens from different poxviral endogenous promoter driven insertion loci. It further explores whether the multivalent MVA is capable of eliciting as potent antigen-specific $CD8^+$ T cells as monovalent rMVAs administered alone, combined as a mixture or administered at physically separate sites.

Following these observations, the work presented in this thesis explores whether two foreign antigens could be inserted into two insertion driven loci in MVA for the induction of potent antigen-specific responses. It is hoped that the findings from this

work would provide useful insight into generation of a multivalent MVA that wolud be clinically relevant for other infectious diseases such as HIV, TB and influenza.

1.5.1 Aims

- To identify non-essential or non-functional genes in MVA as antigenic insertion driven loci.
- To explore endogenous poxviral promoters in their natural position for the regulation of transgenes.
- To assess the cellular and humoral responses of these endogenous promoters compared with traditional p7.5 and SSP promoters
- To improve MVA as a vectored vaccine for expressing multiple antigens
- To test the genetic stability of these insertion driven loci for expressing transgenes.
- To assess deletion of non-essential or non-functional genes with concomitant insertion of the transgene impairs viral growth.

1.5.2 Chapters outline

Chapter 2: Describes the materials and methods used in this study. It also gives a detailed description of the design and construction of the MVA vaccines used.

Chapter 3: Describes the design, construction and *in vitro* characterization of the *B8R* endogenous promoters.

Chapter 4: Gives in depth *in vitro* analysis of the endogenous promoters and insertion loci. The transcriptional pattern of each promoter was analyzed at different times (time course) for 24 hours. It also describes the growth rate of recombinant viruses in comparison to their parent derivative. The stability of each insertion loci is also described in this chapter.

Chapter 5: Describes the cellular and humoral responses generated by the model antigens (tPA-Pb9-rLuc8PV) expressed under the control of the poxviral endogenous promoters. The cellular responses generated are reported in a single dose MVA and Adeno-prime and MVA-boost immunisation regime.

Chapter 6: Gives an insight into the rational design, construction and immunogenicity of multivalent MVA expressing two transgenes.

Chapter 7: Gives a general discussion of work carried out in the thesis. It also highlights future direction to improve work that has been carried out.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

2.1.1 Materials

0.2μm Syringe Filter	Sartorius, UK
0.2mL PCR Tubes	Thermo Scientific, UK
1.5mL Eppendorf Tubes	Treff Laboratories, Switzerland
12mL Culture Tubes	BD Biosciences, UK
15mL Centrifuge Tubes	Fisher, Inc., UK
50mL Centrifuge Tubes	Greiner, UK
60mm Petri Dishes	Corning Inc., USA
0.1cm Cuvette	Bio-Rad, UK
6-Well Tissue Culture Plates	Corning Inc., USA
96-Well Tissue Culture Plates	Corning Inc., USA
96-Well U Bottom Plates	Jencons, UK
96-Well V Bottom Plates	Fischer, Inc., UK
Pipettes	Corning Inc., USA
T25 Flask	Corning Inc., USA
T75 Flask	Corning Inc., USA
T150 Flask	Corning Inc., USA
Cell Scrapper	Corning Inc., USA
Agarose	Sigma Aldrich, UK
Ampicillin	Sigma Aldrich, UK
Kanamycin	Sigma Aldrich, UK
Chloramphenicol	Sigma Aldrich, UK
Primers	Eurofins MWG Operon (London, UK)
One Shot Max Efficiency DH5" *E. Coli* Competent Cells	Invitrogen, UK
DH5α Sub-Cloning Efficiency Cells	Invitrogen, UK
Restriction Enzymes	New England Biolabs, UK
Rapid DNA Ligation Kit	Roche Diagnostics, Germany
Qiagen Plasmid Midi Kit	Qiagen, UK
Qiaprep Spin Miniprep Kit	Qiagen, UK
GeneElute™ Mammalian Genomic DNA Kit	Sigma Aldrich, UK
Proteinase K	Qiagen, UK
S.O.C. Medium	Invitrogen, UK
Smart Ladder DNA Marker	Eurogentec, UK
SYBR Safe in DMSO	Sigma Aldrich, UK
Dimethyl Sulfoxide	Sigma Aldrich, UK
Sodium Azide	Sigma Aldrich, UK
Terrific Broth	Sigma Aldrich, UK
Sodium Dodecyl Sulfate (SDS)	Sigma Aldrich, UK
Trizma® HCL Buffer Solution	Sigma Aldrich, UK
GST Bind Purification Kit	Novagen, UK
BCA Protein Assay Kit	Pierce, UK

12% Tris-Hcl Precast Gel	Bio-Rad, UK
Protein Molecular Weight Markers	Amersham Biosciences, UK
Phosphate Buffer Saline (PBS)	Sigma Aldrich, UK
PBS/Tween Sachets	Sigma Aldrich, UK
Pen/Strep [100U Penicillin 100μg Strep]	Sigma Aldrich, UK
L-Glutamine [4mm]	Sigma Aldrich, UK
ECL Western Blotting Kit	Amersham Biosciences, UK
CASYton	Roche Diagnostics, Scotland
Anti-PK Mab	Serotec, UK
Superfect Transfection Reagent	Qiagen, UK
Amplitaq Gold	Invitrogen, UK
Klentaq Polymerase	Invitrogen, UK
293A Cell Line	Invitrogen, UK
Virapower Adenoviral Expression Vectors	Invitrogen, UK
Pdonor-221 Vector	Invitrogen, UK
Pentr4 Vector	Invitrogen, UK
BP And LR Clonase Enzymes	Invitrogen, UK
OPTIMEM	Invitrogen, UK
Lipofectamine 2K	Invitrogen, UK
Ketaset™	Fort Dodge Animal Health, UK
Domitor™	Pfizer Animal Health, Germany
Antisedan™	Pfizer Animal Health, Germany
MAIP Elisot Plates	Millipore, UK
Mab For IFN-Γ Elispot Assay	Mabtech, Sweden
LIVE/DEAD® Aqua	Invitrogen, UK
Low Phosphate PBS- Gibco	Invitrogen, UK
MAIP Elispot Plates	Millipore, USA
MEM □modification	Sigma-Aldrich, UK
Methanol	Fischer Scientific, UK
Ethanol	Sigma-Aldrich, UK
Isopropanol	Fischer Scientific, UK
70"M Cell Strainer	VWR, UK
LS Columns	Mitenyi Biotech
ELISPOT Color Development Kit	Bio-Rad, UK
Peptides	Invitrogen, UK
Peptides	Protein Peptide Research
Peptides	Sigma Aldrich, UK
Microvette Serum Collection Tubes	Sarstedt, UK
Nunc-Immuno Maxisorp Plates	Fisher Scientific, Germany
Goat Anti-Mouse Whole Igg Alkaline Phosphatase Conjugate	Sigma, UK
Extravidin Alkaline Phosphatase Conjugate	Sigma, UK
Brefeldin A (Golgiplug)	BD Biosciences, UK
Cytofix/Cytoperm Solution	BD Biosciences, UK
P-Nitrophenyl Phosphate Disodium Hexahydrate	Sigma-Aldrich, UK
Anti-Mouse CD11b Alexa Flour ®	Ebioscience, USA

Anti-Mouse Cd11C PE	Ebioscience, USA
Anti-Mouse CD3ε APC	Ebioscience, USA
Anti-Mouse CD11Cε FITC	Ebioscience, USA
Anti-Mouse CD4 Pacific Blue® Clone RM4-5	Ebioscience, USA
Anti-Mouse CD4 PE Clone RM4-4	Ebioscience, USA
Anti-Mouse CD4 Qdot 605	Ebioscience, USA
Anti-Mouse CD8α Percp-Cy5.5	Ebioscience, USA
Anti-Mouse CD44	Ebioscience, USA
Anti-Mouse CD65	Ebioscience, USA
Anti-Mouse IFN-Γ APC	Ebioscience, USA
Anti-Mouse Igg Γ-Chain Specific	Ebioscience, USA
Anti-Mouse Interleukin-2 (IL-2) PE	Ebioscience, USA
Anti-Mouse Ly-6C APC	Ebioscience, USA
Anti-Mouse Ly-6G Eflour® 450	Ebioscience, USA
Anti-Mouse TNF-FITC	Ebioscience, USA
Micro-fine Insulin Syringe with needle 1mL 29gx12.7mm	Nu-care Products
Plastipak Syringe 1mL Luer Slip	Nu-Care Products
Microlance needle 26gx5/8 inch	Nu-Care Products
MiniElute™ Extraction Kit	Qiagen, UK
Plasmodipur Filter	Euro-Diagnostica, Netherlands
Rnase-Free Dnase	Qiagen, UK
Omniscript Kit	Qiagen, UK
Rnasin Plus Inhibitor	Promega, UK
Quantitect SYBR Green RT-PCR Master Mix	Qiagen, UK
Luciferase Assay Kit	Promega, UK
Bacto™ Agar	BD Biosciences, UK
Difco™ McConkey Agar	BD Biosciences, UK
Dried Skimmed milk powder	Tesco, UK
Dulbecco PBS	Sigma Aldrich, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, UK
Expand PCR High Fidelity Kit	Roche, UK
Foetal Calf Serum	Sigma Aldrich, UK
Hydochloric Acid (HCL) [1M]	Sigma Aldrich, UK
$KHC0_3$ [1mM]	Sigma Aldrich, UK
Na_2EDTA	Sigma Aldrich, UK
NaSCN – Sodium Thiocyanate	Sigma Aldrich, UK
NH_4Cl [0.15M]	Sigma Aldrich, UK
KH_2PO_4	Sigma Aldrich, UK
K_2HPO_4	Sigma Aldrich, UK
$(NH_4)_2\,SO_4$	Sigma Aldrich, UK
$FeSO_4$	Sigma Aldrich, UK
Galactose	Sigma Aldrich, UK
Sucrose	Sigma Aldrich, UK
Glycerol	Sigma Aldrich, UK

Leucine	Sigma Aldrich, UK
Biotin	Sigma Aldrich, UK
Difco™ M9 Minimal Salt 5x	BD Biosciences, UK
M63 Medium	US Biologicals, USA

2.1.2 Solutions

The composition of various buffers and cell culture media are described below, and are referred to accordingly in the Methods section.

Coating Buffer: 15mM sodium carbonate and 35mM sodium bicarbonate capsules were dissolved in dH_2O and autoclaved.

Ammonium Chloride Potassium (ACK) Lysis Buffer: 8.29g NH_4Cl (0.15M), 1g $KHCO_3$ (1mM), and 37.2mg Na_2EDTA, made up to 0.8L in water.

Phosphate Buffer Saline (PBS) – Tablet – 0.01M**:** Nacl 0.138M, KCl 0.0027M, pH 7.4: made by dissolving tablets in dH_2O.

PBS/Tween (PBS/T) 0.01M**:** Nacl 0.138M, KCl 0.0027M, pH 7.4 Tween-20 0.05%**.** Sachets dissolved in 1L dH_2O.

Tris-acetate-EDTA (TAE) buffer: Made up from 50x concentrate with dH_2O.

Tris buffer: a stock solution of 1M Tris was diluted in dH_2O to a working concentration of 20mM. The pH was adjusted to 7.5 with HCl (1M) and the solution was filter sterilized for use *in vivo*.

Terrific Broth: 12g bacto-tryptone, 24g bacto-yeast extract, 4mL glycerol made up to 900mL with distilled water and autoclaved. 100mL sterile solution of 0.17M KH_2PO_4, 0.72M K_2HPO_4 added before use.

6x SDS-PAGE Reducing Sample Buffer: 7mL 0.5M Tris pH 6.8 containing 0.4% SDS, 3mL glycerol, 1g SDS, 0.93g dithiothreitol (DTT), and 1.2mg bromophenol blue, made up to 10mL in water.

Loading Dye: 0.4% orange G, 15% Ficoll® 400, 10mM Tris-HCl (pH 8.0), 50mM EDTA (pH 8.0) made up with dH_2O.

10% DMEM: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, and 1% penicillin/streptomycin.

2% DMEM: As 10% DMEM, except percentage of FCS lowered to 2%.

CMC- Overlay: 1:3 mix of 1 part CMC and 2 parts Dubelco Eagle Medium (with 2% FCS)

Agarose-Overlay: 1:1 mix of i) 2% low-melting point agarose in water and ii) MEM (2x) supplemented with 4% FCS and 2% penicillin/streptomycin.

Complete Medium: Modified Eagle's Medium alpha-modification, supplemented with 10% FCS (heat-inactivated at 56ºC, 30min), 2mM L-glutamine, 1% penicillin/streptomycin and 50µM 2-mercaptoethanol.

M9 Medium: 6g Na_2HPO_4, 3g KH_2PO_4, 1g NH_4Cl, 0.5g NaCl dissolved in 1L dH_2O and autoclaved.

M63 Medium: 10g $(NH_4)_2SO_4$, 68g KH_2PO_4, 1mL (2.5mg/mL) $FeSO_4$, adjust to pH7 with KOH and autoclaved.

Bacto Agar Plates: 15g agar in 800mL dH_2O and autoclaved. Add 200mL 5X M63 medium, 1mL 1M $MgSO_4$, 10mL galactose, 5mL Biotin, 5mL Leucine, 500μl (final conc. 12.5μg/mL).

MacConkey Indicator Plates: 16g agar in 400mL dH_2O and autoclaved. Add 20mL galactose and 400μl Chloramphenicol (final conc. 12.5μg/mL).

2.1.3 Cloning and Molecular Biology

Routine molecular biology protocols are outline below.

2.1.3.1 Restriction Endonuclease Digests

1μl	per restriction enzyme
2.5μl	10x reaction buffer
2.5μl	Bovine serum albumin or as required
5μl	DNA template or as required depending on DNA concentration
13μl	H_2O or as required
Total: **20μl**	

Box 2.1: Restriction digest mix

Digests were incubated at 37^{o}C for ≥ 1 hour (h). To minimize vector religation from complementary sticky ends, digests were incubated further by addition of 1μl Antarctic Phosphatase incubated at 37^{o}C for 15 min or Calf Intestinal Alkaline Phosphatase (CIP) incubated at 37^{o}C for 20 min.

2.1.3.2 Agarose Gel Electrophoresis

DNA reactions products or PCR reactions were separated by 1% (or as required) agarose gel electrophoresis (100V, 40min) in 1x Tris-acetate-EDTA (TAE) buffer, ethidium bromide, and visualised on a UV-transilluminator (UVP Biospectrum AC Imaging System, USA). The sizes of bands were estimated by comparison to the DNA marker.

2.1.3.3 Ligation Reactions

Digested DNA fragments were excised from agarose gels, excised and purified using Qiagen MinElute Gel Extraction Kit before ligation using the protocol below. Ligation reactions were incubated at 16°C for 2 h or overnight.

4μl DNA 1μl T4 DNA ligase 2μl T4 DNA ligase buffer 13μl H_2O
Total: **20μl**

Box 2.2: Ligation reaction mix.

2.1.3.4 Bacterial Transformation

50µl Maximum efficiency or Sub-cloning efficiency DH5α *E. coli* were incubated on ice for 30min with 10μl ligation reaction. Following this, cells were heat-shock at 42ºC for 45sec and for another 2 min on ice. Bacteria were plated onto LB agar containing selection antibiotic(s) ± screening indicators as required. Bacteria were grown with shaking (220 rpm) in 300μl S.O.C. medium at 37ºC for 1h before plating. Plates were incubated at 37ºC over-night before storage at 4ºC.

2.1.3.5 Plasmid DNA Preparations

Day 1: Once colonies were visible on selection plates, single colonies were cultured individually over-night in 2mL LB medium plus selection antibiotic(s). **Day 2:** Plasmid constructs were purified by Qiagen QIAprep Spin Miniprep Kits, and screened using diagnostic restriction enzyme digests. Bacteria containing a correct version of each construct were amplified over-night in 150mL LB culture as for day 1. **Day 3:** Plasmid constructs were purified by Qiagen Plasmid Midi Kits and stored

at -20ºC. 500μl cultures were mixed with 500μl 50% glycerol in water to form bacterial stabilates that were stored at -80ºC.

2.1.3.6 GalK-recombineering PCR (long oligos) with Phusion HF

GalK recombineering primers were dissolved at 100μM stock (100pmol/μL) and use at 1:100 dilution. Inserts were generated with specific primers as in Table 2.1 using Phusion High Fedility enzyme with the protocol below.

10μl	Phusion HF buffer
0.5μl	Phusion HF enzyme
1.25μl	dNTPs
2.5μl	Primer F (1:100)
2.5μl	Primer R (1:100)
1.5μl	DMSO
29.25μl	H_2O
Total: **50μl**	

Box 2.3: PCR recipe for recombineering

PCR reactions were sealed in a 0.2mL PCR tube and performed on a G-Storm using a PHUSIGAL programme. *Initial denaturation: 98°C for 30 , Denaturation: 98°C for 5s, Annealing: 68.4°C for 10s, Elongation: 72°C for 30s, x 35 cycles, Final Elongation: 72°C for 5mins and 4°C on Hold*.

On completion of the PCR programme, PCR reactions were further digested with 2μl *DpnI* at 37°C for 1hr, followed by DNA separation on 1.2% agarose gel. Insert DNA were excised, purified by Qiagen QIAprep Spin Miniprep Kits and stored at -20°C until use for recombineering.

2.1.4 Methods - Construction of Model Antigens (insert)

2.1.4.1 Pb9-rLuc

The Pb9-rLuc model antigen contains Pb9 (H2-K^d restricted murine $CD8^+$ T cell epitope SYIPSAEKI (Pb9) from the *Plasmodium berghei* circumsporozoite protein) and a recombinant luciferase protein of Sea Pansy *Renilla reniformis*.

A synthetic cassette containing ORF encoding tPA fused to Pb9 was ordered from Geneart. The cassette contained various restriction sites designed for (i) insertion of luciferase (rLuc) at an in-frame *BStB*I site (ii) subcloning of fusion protein ORF into other vectors (iii) removal of tPA, which was flanked by NcoI sites. Wild type rLuc was cloned into this cassette using *BstB*I and *Xba*I, making an in-frame fusion to tPA-Pb9. *Nco*I digestion and religation was used to remove the tPA, giving two versions with and without tPA.

The fusion protein ORFs were excised with *Bam*HI and NotI and inserted into p*GalK* (a kind gift from Soren Warming (155)) upstream of the *GalK* to make a cassette for recombineering. The cassette comprising Pb9-rLuc and *GalK* was amplified with Roche enzyme with the primers designated “no tPA” as shown in Table 2.1.

The cassette without tPA was used for the proof of principle experiment (Chapter 3) which gave a proof of concept for the approach but the reporter gene was not ideal because (i) it had two transcription terminating signals (T5NT), which were overlooked and not ideal for studies of early gene expression (ii) the reporter gene was not secreted when tPA was added because rLuc activity was not detected in culture supernatant which makes it not ideal for a timecourse study (iii) most antigens studied in the Jenner laboratory are secreted. For example, the malaria apical

membrane antigen (AMA) was shown so be secreted when tagged with a tPA, so we wanted rLuc to be comparable with other antigens such as AMA (60).

To enable measurement of rLuc activity in the supernatant a variant (rLuc8) optimized for secretion was obtained from Andreas Leoning (156), and was used for the construction of the model antigen employed in chapter 4, 5 and 6.

2.1.4.2 tPA-Pb9-rLuc8PV

The tPA-Pb9-rLuc8PV model antigen contains a tPA (the leader sequence of the human tissue plasminogen activator) and Pb9 (described above) and rLuc8PV (an optimized luciferase that allows measurement of rLuc activity in culture supernatant.

The rLuc8 had a single T5NT and was removed by PCR mutagenesis (ATT to ATC), to make rLuc8PV (PV = poxvirus). BstBI and Xbal sites were introduced by PCR and rLuc8PV was subcloned into the synthetic tPA-Pb9 cassettes as described above. *Nco*I digestion and religation was used to make a version lacking tPA. The addition of tPA allowed detection of rLuc activity in the culture supernatant as predicted in the paper by Leoning (156). *GalK* was subcloned downstream using *Xba*I to make a new recombineering cassette which was used for generating recombinant MVAs investigated in (chapters 4, 5 and 6). The cassette comprising tPA-Pb9-rLuc8PV and *GalK* was amplified with Phusion HF with the primers in Table 2.1.

Primer	LHA	RHA
B8R no tPA	taaaatatattatcacttcagtgacagtagtcaaataacaaac aacaccATGGatgatAGCTACATCCCTAGC	gcatgctaagaaaagtggtaacatcatttgatgtgctaccg agaaatttaTCAGCACTGTCCTGCTCCTT
B8R	ttaaaatatattatcacttcagtgacagtagtcaaataacaaa caacaccATGGATGCAATGAAGAGAGG	tgtttggcaccttcataaatttttatttctctattacaatttg catgttgTCAGCACTGTCCTGCTCCTT
F11L	aattataaaaagtgaaaaacaatattatttttatcgttggttgt tTcactATGGATGCAATGAAGAGAGG	tgaaatctagttctgcattgttggtacgcacctcaaatgt agtgttggatTCAGCACTGTCCTGCTCCTT
K6L	tagttatataacgtgaatcatgagtgcaaactgtatgttcaatc tggacaATGGATGCAATGAAGAGAGG	tgaagatttttaccaataatatgtagcggattctcaaagt atttatttacTCAGCACTGTCCTGCTCCTT
A44L	ccattatttagtaaaatagaataagtagtctgatattatgagtg gcagcaATGGATGCAATGAAGAGAGG	ctgatattatgagtggcagcaatggccgtgtacgcggttact ggtggtgcTCAGCACTGTCCTGCTCCTT
E5R	tcatttatatttttttcattattatgttgatattaacaaaagtga atatatATGGATGCAATGAAGAGAGG	tgaagatttttaccaataatatgtagcggattctcaaagtat ttatttacTCAGCACTGTCCTGCTCCTT
C11R	ttgtcataaacacacactgagaaacagcataaacacaaaatcc atcaaaaATGGATGCAATGAAGAGAGG	actttatacatttttgtaaaaatactcataaaaaaaatttat aaaaattaTCAGCACTGTCCTGCTCCTT
B2R	catggcgatgttttacgcacacgctctcggtgggtacgacga gaatcttcATGGATGCAATGAAGAGAGG	tggttacctcgatggtgccgccgatatagtagaagaaag attttctgtatTCAGCACTGTCCTGCTCCTT

Table 2.1: *GalK* Recombineering primers for amplifying tPA-Pb9-rLuc8PV.

The primers are 70bp in length and were designed to introduce the model antigen – tPA-Pb9-rLuc8PV for replacement with the ORF selected for deletion. The primers designated *B8R* no tPA was used for amplification of the model antigen that did not contain tPA used for the proof of principle study – chapter 3. The other primers *B8R, F11L, K6L, A44L, E5R, C11R* and *B2R* were used for amplification of the model antigen with tPA used for generation the viruses used in chapters 4, 5 and 6.

Primer	Forward	Reverse	PCR (bp)
B8R	ACGCGAACTTTACGGTCATC	TCCCCATGTTTCCTCTTCAG	506
F11L	ACGCGAACTTTACGGTCATC	ATCATTGGCAACACCCATTT	548
K6L	ACGCGAACTTTACGGTCATC	TTCATCCGTTTCCTTATGAAGAT	331
A44L	CCTCTATGCGCGATGATTTC	GTTCTGCATTGTTGGTACGC	295
E5R	ACGCGAACTTTACGGTCATC	TGGAGAAAGATGAAATGCCTA	352
C11R	ACGCGAACTTTACGGTCATC	AAATAACCATCTAGGTATAGGTATCG	742
B2R	ACGCGAACTTTACGGTCATC	TTCACACTGTCTTCCAGAGAGG	328

Table 2.2: Identity primers for confirmation of insert.

These primers were used for confirmation of the insert. These primers were used in the bacterial and virus stages for the generation of BAC derived MVAs.

Primer	Forward	Reverse	PCR (bp)
B8R	CCGCATTTCCAAAGAATGAT	GTGCTGTCACGCACACTTTT	318
F11L	ATAGAATGCGAGCAGCGATT	GCAGGATTTACGGCAACAAT	252
K6L	CGGGGTGTTAATCTTGGAAA	CCATGGGAGCAACAATTTCT	320
A44L	GCACGCGTACATTTTTAGCA	CCCAATAAACACGGTGATCC	484
E5R	GTGTCCGATGATCAATGACG	TCGGATTCTGTGATGCTTTG	310
C11R	TGATGTTGTTGTTCGCTGCT	AGCATAATACCGGGAGATGG	276
B2R	TGCTGCCTATGTTTGAGTGC	CCGATTCCGCTTAGAGCATA	110

Table 2.3: Purity primers to confirm the MVA ORF has been deleted and replaced by the model antigen.

These primers were used for confirmation that the MVA ORF selected for deletion has been replaced with the model antigen after recombineering. These primers were used in the bacterial and virus stages for the generation of BAC derived MVAs.

Primer	Forward	Reverse
B8R	CGTCGGATTTGATTCCATAG	TGAATAATCCGCCAGTTACG
F11L	ATCATTGGCAACACCCATTT	GCCGAGCATTCATTTACTCA
K6L	TTCATCCGTTTCCTTATGAAGAT	CAGTAGCCGTCTCCTTCCTC
A44L	TGGAAAAGATAACTATTGATGTCACAC	CCAATAACTCCACACGCTAATC
E5R	CGCCATTGATGTTTGTTCAT	TGGAGAAAGATGAAATGCCTA
C11R	TTTATGTTTGTACCCCGTATTCA	GATATATGGACTCCCGTCACA
B2R	TTACGACAAGTTTCGGCACA	TTCACACTGTCTTCCAGAGAGG

Table 2.4: Sequencing primers.

These primers were used for amplification of the insert to check for any mutations. Primers were used in the bacterial and virus stages for the generation of BAC derived MVAs.

Primer	Oligonucleotide
TKL-PhuUp-F (LHA)	CCGTGATAGGTATCGATGAAG
TKL-PhuUp-R (RHA)	TCGAGTGCGGCTACTATAACTAT

Table 2.5: Kanamycin (AphaI) recombineering primers for making p7.5 and SSP recombinants.

The primers were designed to amplify the pEP7.5TK cassette (Figure 4.4), which is the targeting cassette for insertion into the TK locus in MVA-BAC. The LHA and RHA are homologous to the thymidine kinase locus in genome of MVA and were used for the generation of the comparator p7.5 and SSP viruses.

Primer	Oligonucleotide
EPTK-ScrWtc-F	ttttgaagcattggaagcaa
EPTK-ScrAph-R	acactggcGTCGACATTACC
EPTK-Scrp75-R	TACCGGCATATGGCTATTCG
EPTK-Wtk-R	ctcggtttcctcacccaat

Table 2.6: Screening primers for pEPTK cassette.

These primers were used to screen the pEPTK cassette after recombineering. The EPTK-ScrWtc-F and PTK-Wtk-R confirms the entire AphA1 kanamycin resistant gene and insert were intact (2768bp). The EPTK-ScrWtc-F and EPTK-ScrAph-R confirms only the AphA1 kanamycin resistant gene (218bp). The EPTK-ScrWtc-F and EPTK-Scrp75-R confirms the p7.5 promoter was intact. Primers were used in the bacterial and virus stages for the generation of BAC deriven p7.5 and SSP recombinant MVAs

Primer	Oligonucleotide
SeqM13-EPTK-F	CCGTGATAGGTATCGATGAAG
SeqM13-EPTK-R	TCGAGTGCGGCTACTATAACTAT

Table 2.7: Sequencing primers for pEPTK cassette.

These primers were used for amplification of pEP7.5TK cassette to check for any mutations in AphAI and the insert. Primers were used in the bacterial and virus stages for the generation of BAC derived MVA.

2.1.4.3 pLuc - Photinus Luciferase

The photinus luciferase model antigen contains luciferase of Firefly (*Photinus pyralis)* and an AphA*I* (kanamycin resistant gene). The photinus luciferase was used because the $CD8^+$ T cell epitopes has been characterized in both C57BL/6 and Balb/c mice (157) while the AphAI was used because it has been used previously for rapid generation of recombinant viruses (158-160).

A plasmid carrying the AphAI was amplified by PCR using Phusion (Phusion PCR mix) to introduce *Xba*I and *MIu*I restriction sites. The 5' primer (primer here) encoded an *Xba*I restriction site while the 3' primer (primer here) encoded a *MIu*I restriction site. The resulting construct, *Xba*I-AphAI-*MIu*I, was digested with *Xba*I and *MIu*I and ligated with a Topo vector (give name), the resulting Topo vector now contains the AphAI.

To generate the pLuc-AphAI construct, a pLuc destination plasmid, pSc6.pLuc was digested with *Xba*I and *MIu*I and ligated with the AphAI – digested with *Xba*I and MIul from the Topo vector above. The resulting construct was screened with *ECONI* and *Age*I enzymes and the two expected bands 5804bp and 1057bp were confirmed on 1% agarose gel. The pLuc-AphAI was used as the second model antigen to generate the multivalent MVA after amplification with the primers below:

Primer	LHA	RHA
B8R	ttaaaatatattatcacttcagtgacagtagtcaaataacaa	gcatgctaagaaaagtggtaacatcatttgatgtgctaccga
pLuc	acaacaccATGGAAGATGCCAAAAACAT	gaaatttaTTAGAAAAACTCATCGAGCA

Table 2.5: Recombineering primers for amplifying pLuc.

The primers are 70bp in length and were designed to introduce the model antigen – pLuc for replacement with the B8R ORF selected for deletion.

Primer	Forward	Reverse
pLuc	TTACTGCAGAGGGAAAATGC	GCTCGTTGTAGATGTCGTTA

Table 2.6: Identity primers for confirmation of insert.

These primers were used for confirmation that the insert was intact and were used in the bacterial and virus stages for the generation of BAC derived MVAs.

Primer	Forward	Reverse
pLuc	CCGCATTTCCAAAGAATGAT	GTGCTGTCACGCACACTTTT

Table 2.7: Purity primers for confirmation that the B8R ORF has been deleted and replaced by the model antigen.

These primers were used for confirmation that B8R ORF selected for deletion has been replaced with the model antigen after recombineering.

2.1.5 rLuc Protein Purification

The rLuc expression plasmid (pBad-PelB-rLuc8-6xHis) was a kind gift from Andreas Leoning and the generation of the plasmid has been described in the paper by Leoning (156). Briefly, the pelB leader sequence encodes the first 22 codons of the pectate lyase B gene from *Erwinia carotovora* and directs protein expression into the bacterial periplasm. Using *Nco*I and *Hind*III restriction sites, a cassette comprising PelB and rLuc was inserted into the pBad-His A plasmid (Invitrogen, Carlsbad, CA, USA), which add a 6xHis tag, and a stop codon to the C-terminus of the gene.

Day 1: rLuc protein was generated by inoculating 10mL terrific broth (Sigma, USA) with pBad-PelB-rLuc8-6xHis plasmid in the presence of 2μl Ampicillin (name and dilution 1:200). The culture was incubated at 32^{o}C overnight in a shaking waterbath.

Day 2: 600mL terrific broth was inoculated with all overnight culture and 300μl Ampicillin followed by incubation at 32^{o}C in a shaking waterbath until OD_{600} of 0.6 was reached. 100mL aliquot was taken and kept as control. To induce expression, arabinose (Sigma Aldwich, UK) was added to a final concentration of 0.5%. The culture was then incubated for additional 5 hours in a shaking waterbath. The 100mL aliquot was also incubated at 32^{o}C for the same period in a parallel shaking waterbath. Cells were harvested by centrifugation at 4000 x g for 20 min. Cell pellets were kept on ice and re-suspended in 30 mM Tris.Cl, 20% sucrose, pH 8, at 80mL/gram. 1mL 500 mM EDTA was added drop-wise and incubated on ice for 10 min with swirling. The cell suspension was pelleted by centrifugation at 8000 x g for 20 min at 4^{o}C and the pellets re-suspended in same volume of ice-cold 5mM $MgSO_4$ (1.2mL of 1mM $MgSO_4$ + 240mL dH_2O). The cell suspension was swirled gently on ice for 10 min followed by centrifugation at 8000 x g for 20 min at 4^{o}C. To purify protein, 5mL Ni-

NTA slurry (Ni-NTA Superflow, Qiagen, UK) was added onto a 10mL PD-10 column (GE Healthcare Bio-Sciences AB), and then solution clarified by filtration with 0.2μm filter. The column was washed with 10mL wash buffer (10mM Imidazole, Sigma Life Science, UK) and eluted in 5mL elution buffer (250mM Imidazole). Protein was quantified with Pierce BCA Protein Assay Kit (Thermo Scientific, UK) and luciferase activity confirmed with the Luciferase Assay System (Promega, UK).

2.1.6 Methods: Recombineering work plan

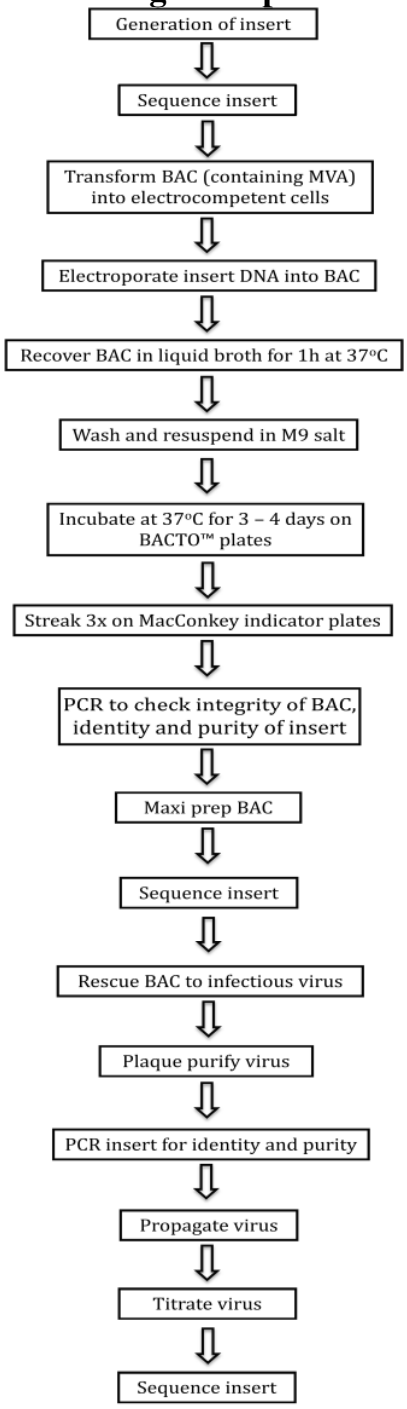


Figure 2.1: Flow chart recombineering.

Generation of rMVA by recombineering takes approximately 12 weeks, from the design of the MVA in seq builder, generation of the insert through to titration of the virus

2.1.6.1 Transformation of MVA-BAC into electrocompetent SW102 cells

Generation of the parent BAC carrying MVA (clone #26) has been described in details in the paper by Cottingham *et al* (153). The modification of the BAC was done with a modified bacterial strain, SW102. To transform BAC to electrocompetent SW102 cells the protocol below was used:

Few SW1O2 cells + 2mL Luria-Bertani (LB) medium + 2μl Chloramphenicol (12.5 μg/mL)

Box 2.4: Start-up culture for making electrocompetent SW102 cells

Day 1: Mixture was inoculated in 12mL culture tube (BD Biosciences, UK), incubated at 32^{o}C overnight in a shaking water bath (name of company). **Day 2:** Using a 250mL baffled cornical flask (Wheaton®, USA) 2mL overnight culture was diluted in 50mL (LB), 50μl chloramphenicol (12.5 μg/mL) followed by incubation at 32^{o}C in a shaking water bath until OD_{600} of 0.6 was reached. Following this, the 50mL culture was divided into two equal halves – 25mL transferred into another 250mL baffled cornical flask and heat-shocked (induced) at 42^{o}C for 15 min in a shaking water bath. The remaining 25mL culture was left at 32^{o}C as the uninduced control. After 15 min, both samples were cooled (induced and uninduced) for 5 min in ice/water bath slurry before transfer to two 50mL Falcon tubes (BD Biosciences, UK).

Samples were pelleted at 5000 RPM (Beckman) for 5 min at 0^{o}C. Following this, the

supernatant was poured off and pellet resuspended in 5mL ice-cold distilled water (dH_20) by gently swirling the tubes in ice/water bath slurry. 25mL dH_20 was added to the resuspended pellets to make up to 30mL before centrifuge at 5000 RPM for 5 min. The washing and centrifugation step was repeated once more followed by removal of the supernatant and pellets resuspended in ~50μl dH_20. The samples were kept on ice ready for electroporation.

2.1.6.2 Electroporation of DNA (model antigen) into BAC

To electroporate the DNA of the model antigens (Pb9-rLuc, tPA-Pb9-rLuc8PV or pLuc) into the BAC, 4μl DNA of PCR product of model antigen was electroporated with 50μl of induced or uninduced competent cells in a 0.1cm cuvette (Biorad, UK) at 25 μF, 1.75 kV and 200 Ω.

2.1.6.3 Recovery of BAC

To recover the bacteria, electroporated competent cell-DNA mixture was diluted in 1mL LB followed by incubation in a shaking water bath at 32^{o}C for 1 h.

2.1.6.4 Washing and plating of bacteria after recovery

The next step was to wash the bacteria with M9 minimal salt to get rid of any rich media prior to selection on minimal media. The 1mL culture was pelleted in 1.5mL eppendorf tube (Treff Laboratories, Switzerland) at 13, 200 RPM at 4^{o}C for 15 sec. Next, the supernatant was carefully removed with a pipette and pellet resuspended in 1mL M9 minimal salt. The washing and centrifugation step was repeated once more followed by resuspension of the pellet in 1mL M9 minimal salt.

The next step was to plate on Bacto™ agar minimum media plates. Plating was done serially by plating 10μl, 100μl and the remaining sample pelleted before plating. Plates were then incubated for 3 to 5 days at 32^{o}C in a cabinet type incubator (Heraeus, Thermo Scientific).

After colonies were observed, a colony PCR was carried out to check the integrity of the BAC using multiplex primers (Table 2.8) and to also confirm that the model antigen (insert) was intact using insert and MVA specific primers (Table 2.2). The PCR for identity of the insert and purity of the MVA-BAC was carried either by colony PCR or extracting the BAC DNA. To perform colony PCR, a singly colony was picked with a pipette tip and washed in 20μl H_2O in 0.2mL PCR tubes before heating at 98^{o}C for 5 min in the PCR machine.

2.1.6.5 BAC plasmid mini prep

Day 1: To extract the BAC DNA, individual colonies were inoculated in 2mL LB, 2μl chloramphenicol (12.5 μg/mL) in 12mL culture tubes followed by incubation overnight at 32^{o}C in a shaking waterbath. **Day 2:** 1.5mL overnight culture transferred to 1.5mL eppendorf tube and centrifuged at 5,000 RPM for 10 min in a tabletop centrifuge. Supernatant was aspirated and pellet resuspended in 70μl STET buffer or buffer P1 (Miniprep kit, Qiagen, UK). Bacterial pellets were resuspended by vortexing and at same time adding 200μl buffer P2 (Miniprep kit, Qiagen,UK) to lyse the bacterial cells followed by addition of 150μl of 7.5 M ammonium acetate while still vortexing. The neutralised lysate was placed on ice for 5 min before centrifugation at 13,000 RPM for 20 min at 4^{o}C. After the centrifugation step, supernatant was transferred to a fresh 1.5mL eppendorf tube followed by addition of 250μl room temperature isopropanol, and then mixing by repeated inversion of the

tube. Next, DNA was pelleted by centrifugation at 10, 000 RPM for 8 min. The next step was to wash the pellet. First, supernatant was aspirated followed by addition of 200μl 70% ethanol to wash the pellet before centrifugation at 10, 000 RPM for 8 min. After the centrifugation step, the 70% ethanol was aspirated. The tube containing the BAC DNA was inverted for approximately 20 min to air-dry the pellet. Finally, the DNA was resuspended in 50μl TE (miniprep kit, Qiagen).

2.1.6.5.1 PCR to check integrity of BAC, identity of insert and purity of MVA-BAC

The following PCR mixtures were used to determine the integrity of the BAC cassette and to also confirm the model antigen (insert) was still intact after recombineering.

Colony PCR mix for identity and purity of insert		BAC DNA PCR mix for identity And purity of insert	
Titanium Taq:	0.4μl	Titanium Taq:	0.4μl
dNTPs:	0.48μl	dNTPs:	0.48μl
TitTaq Buffer:	2.0μl	TitTaq Buffer:	2.0μl
Primer F:	0.4μl	Primer F:	0.4μl
Primer R:	0.4μl	Primer R:	0.4μl
Colony sample:	10μl	BAC DNA (1:100):	2μl
H_20:	6.32μl	H_20:	6.32μl
Total:	**20μl**	**Total:**	**20μl**

Box 2.5: Colony and BAC DNA PCR for identity and purity of insert

Identity and purity primers were dissolved at 100μM and diluted 1:10 of which 0.4μl each of forward and reverse primer was used.

Colony PCR to check integrity of BAC		BAC DNA PCR to check integrity of BAC	
Titanium Taq:	0.4μl	Titanium Taq:	0.4μl
dNTPs:	0.48μl	dNTPs:	0.48 μl
TitTaq Buffer:	2.0μl	TitTaq Buffer:	2.0μl
Multiplex primer mix:	4μl	Multiplex primer mix:	4μl
Colony sample:	10μl	BAC DNA (1:100):	2μl
H_20:	3.12μl	H_20:	11.12μl
Total:	**20μl**	**Total:**	**20μl**

Box 2.6: Colony and BAC DNA PCR for integrity of BAC.

BAC multiplex primers were dissolved at 100μM and the primer mix diluted 1:10 of which 4μl was subsequently used for the PCR. The 20μl mixture was ran on a *GalK* screen program: *Denaturation 98^{o}C for 15 sec, Annealing 50^{o}C for 10 sec, Extension 72^{o}C for 1 min, for 35 cycles*.

2.1.6.5.2 Multiplex PCR to check the integrity of the BAC

The multiplex PCR confirms that the BAC contains the full-length genome of MVA because insertion of the transgene under the control of poxviral endogenous promoters by recombineering can lead to deletion part of the genome. Insertion of a transgene under the control of a p7.5 promoter can also lead to of the right or left arm of the genome due to the presence p7.5 homologous region in the ITR. The multiplex PCR amplifies 4 regions in the genome and one region within the BAC backbone.

Primer Name	Oligonucleotide	Expected PCR product (bp)
Rikke/ BACid	Forward: ATAGAACTTACGCAAATATTAGCAAAAAT	444bp
	Reverse: TGGAAAGCGGGCAGTGA	
Kinase	Forward: TGCCGGATAAAAGTGGGATA	525bp
	Reverse: CAAAATTGGGCTCCATCAGT	
ITR	Forward: ACGGGATCGCAGTCTTTATG	232bp
	Reverse: CCGGAGACGTCATCTGTTCT	
R Poly	Forward: TACGGTTTTGGGGTGACATT	592bp
	Reverse: CGACCACCATATCCTCCATC	
ATPase	Forward: TTCACCTTCACAAAATACGGAGT	359bp
	Reverse: TTGCTGTCGCACAAAATCAT	

Table 2.8: Multiplex primers BAC

These primers were used for the amplification of four MVA specific regions and one region with the BAC backbone.

2.1.6.6 Plating on MacConkey indicator plates

After confirmation by PCR that the BAC cassette and insert were intact, the next step was to obtain single, bright red colonies. The colonies that appeared after 3 to 5 days of incubation on the Bacto™ agar plates should be galactose positive (Gal^+) but in order to get rid of galactose negative (Gal^-) contaminants (hitch hikers), it is important to obtain single, bright red colonies. Colonies from Bacto™ agar plate were streaked in a zigzag pattern on MacConkey indicator plates and incubated for 3 days at 32^{o}C in a cabinet type incubator. After 3 days incubation, a bright red colony was picked and streaked on a fresh MacConkey indicator plate. This step was repeated once more.

After streaking on the third plate a colony PCR was carried out to confirm identity of the insert using the same identity primers as before (Table 2.2) and the purity primers as shown in (Table 2.3). Upon confirmation of the insert and purity of the MVA-BAC the next step was to propagate the MVA-BAC for rescue to infectious MVA virus.

2.1.6.7 Propagation of MVA-BAC using QIAGEN Maxi-prep Plasmid Purification Kit – TIP 500

Day 1: A bright red colony was picked with a 1mL pipette tip and inoculated in a starter culture of 2mL LB medium and 2μl Chloramphenicol (12.5 μg/mL) before incubating overnight at 32^{o}C in a shaking waterbath. **Day 2:** Whole starter culture was inoculated in a 2000mL baffled cornical flask (Nalgene®, USA) containing 500mL LB medium and 500μl Chloramphenicol (12.5 μg/mL), then incubated overnight at 32^{o}C with vigorous shaking (~250 RPM). **Day 3:** 500mL culture was separated in two halves - 250mL each in (250mL Beckman tubes), before harvesting bacterial cells by centrifugation at 6000 x g for 15 min at 4^{o}C. The bacterial pellets were transferred in two separate 50mL Falcon tubes and resuspended in 10mL buffer P1.

The next step was to lyse bacterial cells by adding 10mL buffer P2 to each of the tubes followed by mixing with vigorous inversion (4 – 6 times). The tubes were further incubated at room temperature (15-25^{o}C) for 5 min. The next step was to precipitate the bacterial cells by adding buffer P3 followed by mixing with vigorous inversion (4 – 6 times) before incubation on ice for 20 min. The bacterial cells were then pelleted by centrifugation at 20, 000 x g for 30 min at 4^{o}C. After centrifugation, the supernatant were transferred to two clean 50mL Falcon tubes and centrifuged

again for 20, 000 x g for 15 min at 4°C. This second centrifugation step was done to avoid applying suspended or particulate material to the QIAGEN-tip because particulate materials in the supernatant can clog the QIAGEN-tip and reduce or eliminate gravity flow.

The next step was to equilibrate the QIAGEN-tip 500 by applying 10mL buffer QBT and the columns allowed to empty by gravity flow. After the column has been emptied, the supernatant from the two 50mL Falcon tubes were applied to the column and allowed to enter the resin by gravity flow. Following this, the next step was to wash the QIAGEN-tip by applying 2 x 30mL buffer QC. To elute DNA, buffer QF was first pre-heated to 65°C before adding 15mL to the QIAGEN-tip. The next step was to precipitate the DNA by adding 10.5mL room-temperature isopropanol to the eluted DNA followed by a gently mix before centrifugation at 15,000 x g for 30 min at 4°C. After centrifugation, the supernatant was carefully decanted and the DNA pellet washed with 5mL room-temperature (70%) ethanol followed by centrifugation at 15,000 x g for 10 min. At this stage the pellet is very unstable and is easily detached from the tube so handled with extra caution. After the centrifugation step, the supernatant was carefully decanted without disturbing the pellet. The next step was to air-dry the pellet for 20 - 30 min. After the pellet was completely dry, the DNA was redissolved in 100μl TE. The concentration of the DNA was then obtained using a Spectrophotometer (Nanodrop ND-1000 Spectrophotometer).

2.1.7 Methods – Molecular Virology

2.1.7.1 Rescue of BAC to infectious virus

A Fowlpox virus of the strain FP9 was used to provide transcriptional machinery for the BAC clones for rescue to infectious MVA. Day 1: A nearly confluent 6-well plate ((5 x 10^5/well) plate of BHK cells was infected (Figure 2.2 A) with FP9-LacZ (7.5 x 10^8 pfu/mL) at MOI = 1.0 in a volume of 1mL OptiMem (1.3μl of virus in 1mL OptiMem). The plate was then placed in an incubator at 37^{o}C (5% CO_2) for 2 hours. During the incubation period, Lipofectamine2k was mixed gently and 10μl added to 250μl OptiMem per transfection followed by incubation at room temperature for 5 min. The next step was to dilute 4μg of BAC DNA in 250μl OptiMem in a clean 1.5mL screw cap tube. 4μg of MVA-GFP2 was also diluted in 250μl OptiMem as control. After 5 min incubation the next step was to combine the Lipofectamine-OptiMem mix with the BAC DNA-OptiMEm mix. The sample was mixed gently by flicking the tubes gently followed incubation at room temperature for 20 min.

The next step was to transfect the BAC DNA with the FP9. After infecting cells with FP9 (2 hour incubation), the medium containing FP9 was aspirated and replaced with the transfection mixture (BAC DNA-OptiMem-Lipofectamine2k – 500μl mix) using a cut-off pipette tip (1mL pipette), added gently (drop wise) to avoid shredding of the BAC DNA. Plate was incubated at 37^{o}C for 2 to 4 hours, 500μl/well fresh pre-warmed OptiMem was added followed by overnight incubation at 37^{o}C (5% CO_2).
Day 2: Cells were examined by epifluorescence microscope (Leica). On examination there were lots of dim green cells (Figure 2.2 B). Upon confirmation, cell were further incubated for 4 days or until all cells were dead. Cells were freeze-thawed three times and re-plated on fresh BHK with neat, 1:10 and 1:100 dilutions followed by overlay

with 2mL Carboxymethylcellulose (CMC). The neat was 500μl of the freeze-thawed cells and dilution were made in complete Dubelco modified Eagle medium (DMEM). Cells were then monitored for 2-3 days for the presence of green virus infected cells or plaques because MVA carries a GFP marker gene. 2mL CMC was made in 1:3 dilution (1 part CMC and 2 parts DMEM).

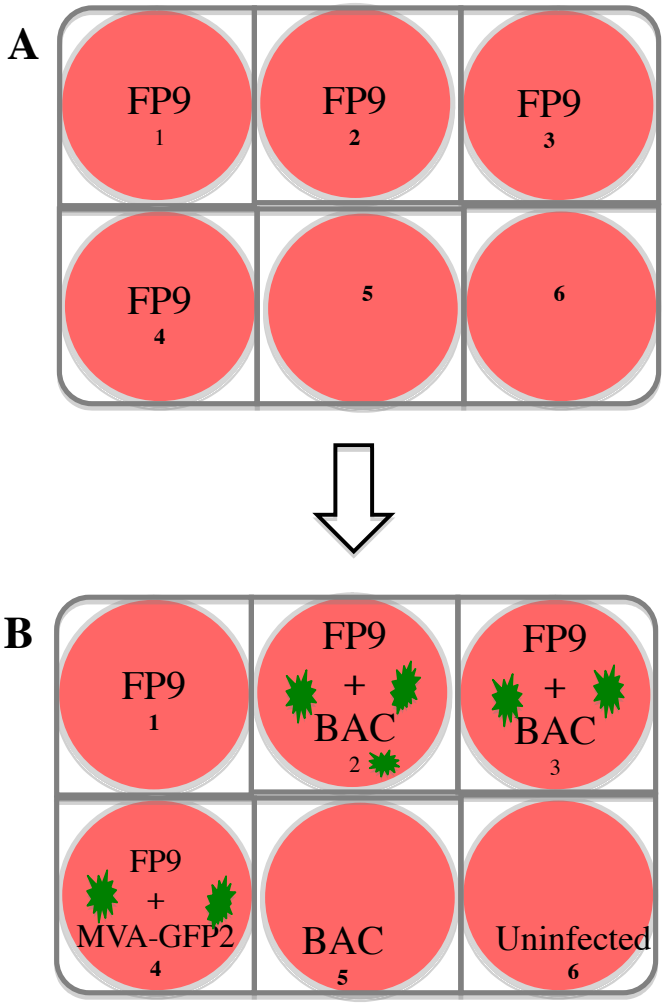


Figure 2.2: Rescue of BAC to infectious virus.

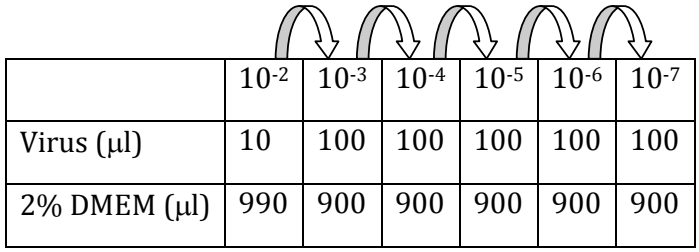
(A) Infection of cells with folwpox virus, FP9, (B) after transfection with BAC DNA

2.1.7.2 Plaque purification of recombinant virus by

The next step was to purify the rescued virus by plaque purification on CEFs. This

step was done to confirm that the rescued virus was not contaminated with FP9 or the parental MVA-GFP virus. Day 1: 100μl 10 mM Tris (pH9) was aliquoted into a 2mL screw capped tube. Using a fluorescent microscope and an object marker attachment a plaque identified for purification. In the safety cabinet, a P10 pipette was set to 10μl, then a 10μl pipette tip was taken and the pipette depressed such that it was ready to aspirate a sample. Using the microscope, the lid of the 6-well plate was carefully removed and the pipette tip used to circle the plaque in order to disrupt the cell monolayer around the plaque. The pipette tip was then used to carefully scrape the cells to release the cells from the plastic followed by aspiration of the cells into the pipette tip. The cells were then taken to the safety cabinet and added to the 2mL screw-capped tubes containing Tris.

The next step was to place the tube on dry ice and cells freeze-thawed three times. After this step, the virus was either stored at -80^{o}C or subjected to first round of plaque purification. For the first round of plaque purification, CEFs (~90% confluent) in 6-well plate/virus was prepared. **Day 1:** The viruses were diluted in 1.5mL sterile microcentrifuge tubes as described in the protocol below:



	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Virus (µl)	10	100	100	100	100	100
2% DMEM (µl)	990	900	900	900	900	900

Box 2.7: Dilution protocol for plaque purification.

Dilutions were first done in eppendorf tubes prior to infecting CEFs.

The next step was to aspirate the media from the 6-well plate followed by transfer of 900μl of virus suspension from the microcentrifuge tube as above to the relevant

wells of the 6-well plate (Figure 2.3). The plate was then incubated for 1 hour at 37°C (5% CO_2). After the incubation, the media containing virus was aspirated followed by overlay with 2mL/well of CMC (1:3 dilution). The plate was then incubated for 2-4 days and cells monitored for green virus infected cells. Once plaques were visible, a plaque was picked from the most diluted well (Figure 2.3 well 6) for the second round of plaque purification. This process was repeated once more or until a pure virus was obtained. Identity and purity PCR was carried out on purified viruses using primers as were used in the bacterial stage (Table 2.2 and 2.3 respectively).

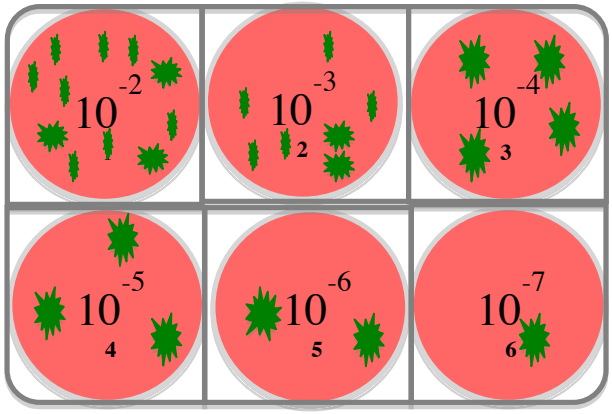


Figure 2.3: Plaque purification in CEFs

2.1.7.3 Propagation of recombinant MVA using BHK cell lines

BHK cell line was found suitable for the propagation of BAC derived MVAs instead of CEFs because it is the most suitable mammalian cell line for MVA replication. MVA-BACs are naked DNA molecules that contain genomic deletions and are co-transfected with a fowlpox virus, FP9. The FP9 provides the transcriptional machinery such as polymerases for rescue to infectious MVA virus. In addition, the use of BHK during propagation prevents fowlpox replication, which requires avian cell lines such as CEFs, though MVA still replicates in CEFs.

BHK proliferates at a very high rate and often results in cells overgrowing, dying and lifting before the virus completes a cycle of replication. So, in order to reduce the rate of cell proliferation, serum free media was used throughout the propagation stage.

2.1.7.3.1 Infection of T75 flask

Upon confirmation that a recombinant virus was pure, the same plaque was taken forward for propagation to achieve high viral titre. The first step was to seed T75 flask (Corning Inc., USA) with BHK (6 x 10^6) followed by incubation at 37°C (5% CO_2) and monitored for 2 days or until ~ 90% confluent. Cells were infected with 20μl of virus from the plaque that was confirmed pure. This was followed by incubation at 37°C (5% CO_2). Cells were monitored for cytopathic effect (CPE) and ~100% of cell infection was evident between 2 to 3 days. A cell scrapper was used to detach virus-infected cells from the flask before transfer to 50mL Falcon tube. The Falcon tube containing BHK infected cell was then placed on dry ice and cells freeze-thawed three times.

2.1.7.3.2 Infection of 10 x T150 flasks

The next step was to prepare 10 T150 flasks seeded with BHK cells for infection with virus lysates from T75 flask. Virus lysates from T75 flask were re-suspended in 15mL serum free media of which 1mL was aliquoted into a 2mL screw capped tube for storage at -80°C as the premaster stock. The remaining 14mL cell lysates was subsequently diluted to 150mL with serum free media. Media was aspirated from the 10 BHK flasks (~90% confluent) before infection with 15mL cell lysate/flask followed by incubation at 37°C (5% CO_2). Cells were then monitored for CPE and until all cells were dead, ~ 4 to 5 days.

2.1.7.3.3 Harvesting of virus-infected cells

The next step was to harvest virus-infected cells with a cell scrapper. **Step 1**: Cells were detached from the flasks with a cell scrapper, and transferred into a 250mL conical bottom tube (Beckman) followed by centrifugation at 670 x g for 10 min to pellet cell debris. After the centrifugation step, while retaining the pellet (kept on dry ice), the supernatant was transferred into an autoclaved 250mL flat-bottom Ultracentrifuge tube. **Step 2:** The 250mL tube was centrifuged at 33,000 x g for 60 min at 4^{o}C to pellet the virus particle (Beckam Coulter Optima L-80 XP Ultracentrifuge using Type 18 fixed-angle rotor). **Step 3:** During the 60 min centrifugation step, pellets from the 250mL conical bottom tube (as in **Step 1**) were processed by adding 3mL of 10 mM Tris (pH9) and resuspended thoroughly before transfer to 15mL Falcon tube. The tube was placed on dry ice and cells freeze-thawed three times followed by centrifugation at 670 x g for 5 min. **Step 4:** Supernatant from the 15mL Falcon tube (as in step 3) was transferred into a clean 15mL Falcon tube. The pellet left in the 15mL Falcon tube (as in step 4) was resuspended in 5mL 10 mM Tris (pH9) and sonicated (Sonics & Materials, USA) for 1 min at 57 Hz, followed by centrifugation at 1700 RPM for 5 min. The supernatant was then combined with that in the clean 15mL Falcon tube and made up to 10mL with 10 mM Tris (pH9). **Step 5:** Following 60 min centrifugation of the supernatant (as in step 2), the supernatant was aspirated from the 250mL bottle, leaving a virus-rich pellet. The virus-rich pellet was then resuspended with the 10mL virus-rich supernatant (as in step 4). This material was either stored at -20^{o}C or purified immediately in sucrose cushion

2.1.7.3.4 Purification of virus through 36% sucrose cushion

The next step was to prepare the ultracentrifuge bucket for the sucrose cushion purification step. **Step 1**: The bucket was placed in the safety cabinet and wiped thoroughly with 10% Microsol solution and the Microsol removed completely by spraying the bucket with 70% industrial methylated spirit (IMS). This was done because traces of Microsol in the bucket will inactivate the virus. The internal surface of the SW40 rotor (name of machine) was sprayed with 70% IMS and allowed to drain and dry properly.

Step 2: Material was resuspended thoroughly to ensure no clumps were present in the virus suspension, followed by centrifugation at 950 x g for 3 min at 4^{o}C. For 10 x T150 flask/virus, 2 x 7.5mL 36% sucrose (in 10 mM Tris pH9) was added to 2 x 13mL thin walled ultracentrifuge tube.

Step 3: The virus suspension was then layered equally onto the top of the sucrose, slowly and carefully with a sterile pipette without disturbing the sucrose layer. The tubes were then placed inside the buckets and balanced using a balanced scale. Balancing was achieved by adding droplet of 10 mM Tris (pH9) on the walls of the 13mL tubes until both tubes were balanced. This was followed by centrifuge at 30, 000 x g at 4^{o}C for 2 hours using a Optima L-80 XP Ultracentrifuge.

Step 4: After centrifugation, the supernatants were carefully removed and discarded using an aspirator. The supernatants were removed first by removing the top layer containing the sucrose so that it does not disturb the purified virus pellets at the base of the tubes. The virus-rich pellets were each resuspended in 300μl 10 mM Tirs (pH9) in total volume of 600μl per 10 x T150 flasks. The virus was then transferred to a 2mL screw cap tube. The tubes were washed with 200μl 10 mM Tris (pH9) and transferred to the screw cap tube to make up a total of 1000μl/virus/10 x T150 flasks.

Step 5: The virus was stored at -80°C for titration and until ready for use. After the purification step, viral DNA were extracted and screened for the presence of the insert and purity of the virus using the same identity and purity primers used at the bacterial stage.

2.1.7.3.5 Titration using CEFs

The microscopy method was applied to quantify the fluorescent maker, GFP, which was expressed in all recombinant viruses generated for this work. All viruses were titrated in CEFs and number of GFP infected cells (plaques) used as titre value for each virus.

Step1: 6-well plate per virus (1 x 10^6 CEF per/well) was prepared for use the next day or 0.5 x 10^6 CEF per well in 2mL of 10% FCS DMEM for use 2 days later. Once cells were confirmed ~90% confluent, the next step was to perform dilutions with the virus in 1.5mL sterile eppendorf tubes (Box 2.8). A reference virus with known titre value was also titred at the same time with the new recombinant virus. The dilution of the sample virus was done as described below

	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}
Virus (µl)	10	10	10	100	100
2% DMEM (µl)	990	990	990	900	990

Box 2.8: Dilution protocol for virus titration.

Dilutions were first done in eppendorf tubes prior to infecting CEFs.

Step 2: Before infecting cells, the 10% FCS DMEM in the wells were first aspirated and replaced with 1mL 10% FCS DMEM. If viruses were already diluted, cells were infected immediately otherwise cells were incubated at 37°C (5% CO_2) until required.

100μl of sample virus in 10^{-5}, 10^{-6} and 10^{-7} dilutions above were used to infect cells in duplicates as shown in Figure A. The dilution and plating of the reference sample was done as shown in Figure 2.3 B. The virus was mixed evenly in the wells by repeated movement of the plate left/right and forward/backward. Plates were then incubated for 1 hour at 37°C (5% CO_2). Step 3: After the incubation step, the media was aspirated and replaced with 2mL CMC (diluted 1:3 in 2% FCS DMEM). Plates were then incubated for 2 – 4 days before counting plaques under fluorescent microscope (Figure 2.3 A).

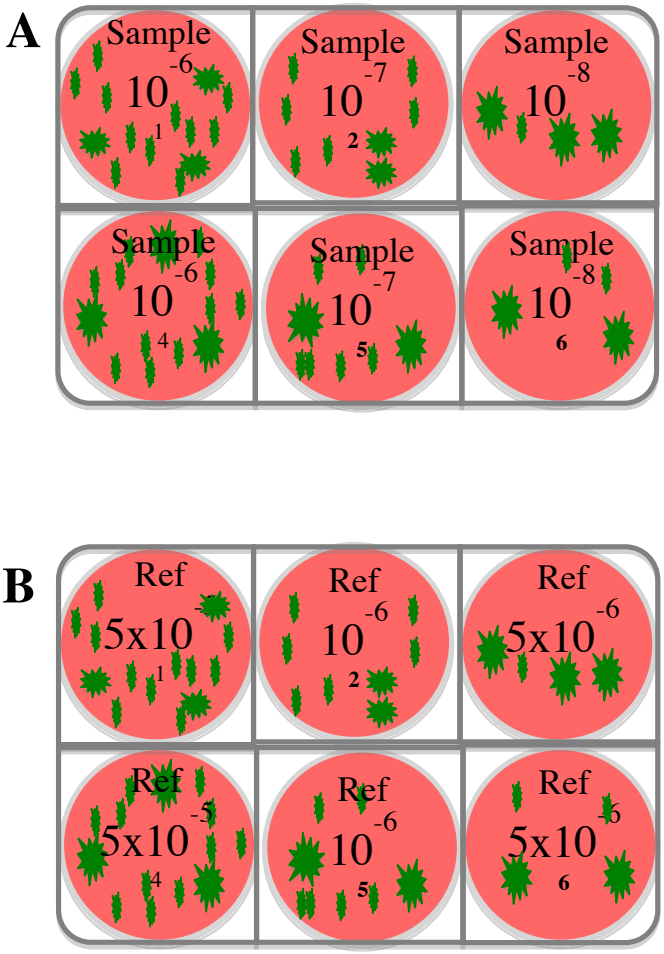


Figure 2.4: Virus titration in CEFs.

Test sample (A) and reference sample with known titre (B).

2.1.7.3.6 Virus titration

The number of plaques in duplicate wells containing 20 – 50 spots per well was counted to determine the titre of each virus generated. After counting, the formula presented below was used to determine the titre in pox forming unit (pfu), that is pfu/mL:

"*Average number of spots"* x "*serial dilution"* x "*dilution factor"* = "*x"* pfu/mL

e.g. $\frac{(24+20)}{2}$ = 22 x ($1x10^7$) x 10 = 2.2 x 10^9 pfu/mL

2.1.7.3.7 Sequencing of insert from BAC-DNA and viral DNA

The insert (tPA-Pb9-rLu8PV) was amplified from either BAC-DNA or viral DNA using the PCR mix below and the sequencing primers in Table 2.4

DNA (1:100)	2μl
Forward Primer	2.5μl
Reverse Primer	2.5μl
Phusion 2 x mastermix	25μl
H_2O	18μl
Total volume	**50μl**

Box 2.9: PCR mix for sequencing.

The 50μl PCR mix was performed on a Phusion program: *Initial denaturation 98^oC for 30 sec, Denaturation 98^oC for 10 sec, Annealing 60^oC for 30 sec, Extension 72^oC for 1 min, for 35 cycles, Final Extension 72^oC for 5 min and hold at 10^oC.*

At the end of the PCR program, 10μl ethidium bromide was added to the 50μl PCR mix and the whole sample (60μl – 20μl on each lane) separated on 1% agarose gel. Bands were excised, and DNA purified with Qiagen MinElute Gel Extraction Kit, followed by sequencing using universal M13 primers (M13 uni (-21) and M13 rev (-29)) by MWG Biotech (Ebersberg, Germany).

M13 uni (-21): TGTAAAACGACGGCCAGT

M13 rev (-29): CAGGAAACAGCTATGACC

2.1.7.3.8 Sterility testing for purified viruses

A sterility test was carried out for all purified viruses to determine if there were any contaminations with aerobic (not anaerobic) bacteria and fungi.

A Tryptic Soy Broth (TSB), (Soybean-Casein Digest Medium) was used for sterility testing because it is ideal for enrichment and cultivation of aerobic organisms that are not excessively fastidious, for example *Escherichia coli*, *Corynebacterium* and *Listeria*. To test the purified virus for any contaminations 10μl of the purified virus was added to the TSB medium and incubated at 30-35°C for 3 days to test for bacterial contamination. To demonstrate that the TSB was not contaminated, other vessels of TSB medium were also tested in the as shown in the protocol below:

- TSB only
- TSB + swab of skin surface
- TSB + test sample + swab of skin surface*
- TSB + test sample

Box 2.10: Protocol for sterility testing.

The swab of skin surface + one of the test samples is to control for any factors within the samples that may inhibit microbial growth (eg antibiotics carried through during purification).

2.1.7.3.9 Extraction of viral DNA from avian, mammalian cells or purified viruses

In order to perform identity and purity PCR, viral DNA needs to be extracted from purified viruses or avian and mammalian cells that were infected with recombinant MVA during the production stage. All kits and reagents used for viral DNA extraction were from (Sigma Genelute kit).

Step 1: Harvest virus infected cells and transfer into the appropriate tube, ie 1.5mL microcentrifuge tube followed by centrifugation at 13,000 RPM for 1 min. After centrifugation, the supernatant was completely removed then 10μl aliquoted for DNA extraction. If the sample was purified virus, 10μl was also taken for DNA extraction. **Step 2:** Resuspend the 10μl virus aliquots in 200μl resuspension solution; add 20μl Proteinase K and 200μl Lysis solution C. The sample was then mixed thoroughly by vortexing for ~15 sec followed by incubation at 70^{o}C for 10 min. Both steps above were performed in the safety cabinet. After the incubation step, the sample was transferred to a dedicated bench for virus work in the Jenner immunology laboratory.

Step 3: Add 500μl column preparation solution to Genelute Miniprep binding column followed by centrifugation at 12, 000 x g for 1 min. After the centrifugation, the flow-through was discarded. **Step 4:** Add 200μl of 100% ethanol to the lysate (sample) followed by vortex for 10 sec. **Step 5:** Load the lysate onto the column using a wide bore pipette tip to reduce DNA shearing followed by centrifugation at 6,500 x g for 1 min. Following centrifugation, the collection tube was discarded and the binding column placed in a new collection tube. **Step 6:** 500μl of wash solution was added to the column followed by centrifugation at 12,000 x g for 3 min. Following

centrifugation, the collection tube was emptied and the binding column was placed backing in the collection tube followed by centrifugation at 12,000 x g for 1 min. Following the second centrifugation, the collection tube was discarded and the binding column placed in a new collection tube. **Step 7:** 200μl of elution solution was added to the centre of the binding column followed by incubation at room-temperature for 5 min. The final step was to centrifuge the column at 6,500 x g for 1 min and the viral DNA collected in the collection tube.

2.1.7.4 Construction of P7.5 and SSP recombinants

A plasmid containing the insert tPA-Pb9-rLuc8PV (section 2.1.4.2 and Figure 4.4A) was first digested with Acc65I and NotI giving a product of 1081bp. A plasmid containing the pEP7.5TK cassette (Figure 4.4B) was also digested with Acc65I and NotI to give a product of 4.3kb. The two resulting products (insert - 1081bp) and destination vector (pEP7.5TK – 4.3kb) were ligated overnight (M+M section 2.3.3). Five colonies were screened with Xhol and EcoRV and only one was correct. The resulting product (was amplified with recombineering primers (as shown in Table 2.5) designed to introduce the pEP7.5TK carrying the model antigen (insert) into the TK locus of MVA-BAC. After recombineering, 10 colonies were screen for the presence of the insert using primers in (M+M Table 2.6). Of the ten colonies screened, three were positive but only one was taken forward for sequencing with primers in (M+M Table 2.5).

To generate the SSP recombinants, a pETK cassette lacking the p7.5 promoter as shown in (Figure 4.4B) was digested with BamHI and NotI giving a product of 4kb. The insert (section 2.1.4.2 and Figure 4.4A) carries the SSP promoter upstream of tPA, so it was digested with BamHI and NotI giving a product of 1129bp. The two

resulting plasmids, pETK without SSP and insert with SSP were ligated overnight and the ligation reaction plated on kanamycin plates next day. Five colonies were screened with NotI and EcoRV and only one was correct. The pESSPTK plasmid now carrying the model antigen was amplified with the same primers used for the amplification of pEP7.5TK cassette (M+M Table 2.5). After recombineering, 10 colonies were screened with primers (M+M Table 2.6) and 7 were correct but only one was taken forward for sequencing.

2.1.7.5 Generation of Multivalent MVA

To generate the multivalent MVA expressing the two model antigens (tPA-Pb9-rLuc8PV and pLuc), the BAC DNA already carrying the tPA-Pb9-rLuc8PV construct under the control of the pC11R promoter was used. The multivalent MVA was generated so that the pLuc is driven under the control of the pB8R promoter while the tPA-Pb9-rLuc8PV construct is driven under the control of the pC11R promoter.

To insert the pLuc model antigen into the BAC carrying the tPA-Pb9-rLuc8PV construct, the pLuc was PCR amplified to introduce 70bp homology arms (LHA and RHA), for replacement with the B8R ORF. The LHA and RHA primers are shown in Table 2.5.

A comparator virus expressing the pLuc from the pB8R was also constructed. Recombineering was done exactly as described previously. The resulting BAC containing the two model antigens (tPA-Pb9-rLuc8PV and pLuc) was sequenced to confirm the inserts were intact.

2.1.8 Methods – *In vitro* Assays

2.1.8.1 Luciferase Assay

Step 1: BHK cells (5 x 10^4 cells/well) in flat-bottom 96-well microtitre plates were inoculated in duplicate with rMVAs at 1 pfu/cell followed by incubation for 1h at 37°C (5% CO_2). A 20μl aliquot of supernatant was taken immediately after infection then at 1h before washing. **Step 2:** Cells were washed twice with 100μl PBS followed by addition of 150μl of DMEM containing 2% Fetal Calf Serum (FCS). Plates were incubated for 24h at 37°C (5% CO_2). Following washing, 20μl aliquot of supernatant was taken at 1h, 2h, 4h, 8h and 24h post-infection. Aliquots were also collected from duplicate infected cells containing 40μM cytosine arabinoside (AraC). **Step 3:** After 24h post-infection, the remaining media was aspirated, followed by washing twice with 100μl PBS. **Step 4:** Cells were lysed with luciferase lysis buffer and 10μl of cell lysate aliquoted. The rLuc activities in 10μl aliquots of these samples were quantified using the *Renilla* Luciferase Assay System (Promega) and a Varioskan Flash luminometer (Thermo Scientific).

2.1.8.1.1 Spinoculation and washing protocol

A duplicate luciferase assay was performed as above but adjusted to include a spinoculation and washing protocol prior to infection. The spinoculation step was performed in order to synchronize virus infection and washing performed to remove unbound rLuc from the inoculum.

Step 1: Infection of BHK cells was done on ice followed by centrifugation at 650 x g for 1h at 0°C before washing (3 x) with ice-cold DMEM containing 2% FCS. **Step 2:**

150μl pre-warm DMEM containing 2% FCS was added per well before incubation at 37°C for 24h. Collection of aliquots, cell lysates and quantification of rLuc activity was done as described before.

2.1.8.2 Growth rate of viruses

BHK cells ($5x10^4$cells/well) in black-walled, clear-bottom 96-well plate (Grand Plates™, Germany) were inoculated in duplicate with rMVAs (suspended in OptiMem) at 1, 0.5 and 0.25 multiplicities of infection (MOI). The growth rates were determined by quantifying GFP fluorescence every 6 min for 36 h using a BMG FluoSTAR fluorimeter equipped with 37 °C + 5% CO_2 incubation.

2.1.8.3 Viral genetic stability

2.1.8.3.1 Passage of viruses

Passage 1: Serial passage of rMVAs. How many CEFs seeded. **Step 1:** CEF cells in $25cm^2$ flasks were inoculated with 10μl of purified virus then incubated at 37^{o}C (5% CO_2) for 2-3 days or until all cells were infected as determined by epifluorescence microscopy. **Step 2:** Cells were harvested, pelleted at 1650 x g for 5 min then supernatant discarded. **Step 3:** Cell lysates were resuspended in 1mL DMEM containing 2% FCS and freeze-thawed three times. **Passage 2-10:** This process was repeated 9 times but this time cells were inoculated with 100μl crude lysate from the previous passage. After passage 10, viruses were titred on CEFs and titres fell within the range of 2.4 to 3.8×10^5 pfu/mL. The next step was to determine what proportion of viruses retained expression of the model antigen, tPA-Pb9-rLuc8PV.

2.1.8.3.2 Genetic Stability Assay

Day 1: CEF cells in 150cm^2 flasks were infected at 0.001 pfu/cell

Day 2: Cells were harvested and single GFP+ trypsinized cells were sorted into individual empty wells of 96-well plates using the CyCLONE robotic module of a MoFlo (Dako Cytomation) flow cytometer. Following this, the plate containing the GFP+ trypsinized cells were seeded with 5 x 10^4 BHK cells per well then incubated at 37^{o}C (5% CO_2) for 2-3 days. Next, wells were scored positive or negative for GFP[GFP] by epifluorescence microscopy before quantification of rLuc activity in the cell lysates as described in the luciferase assay above.

[GFP] We observed that not all BHK wells were GFP$^+$, owing to unavoidable errors in the MoFlo droplet identification combined with the imperfect correlation between presence of infectious virions and GFP positivity in the sorted CEF cells. Wells were scored positive or negative for rLuc based on a cut-off of three standard deviations above the geometric mean of the light units detected in GFP$^-$ (i.e., uninfected) wells. Any genetic instability at the tPA-Pb9-rLuc8PV insertion locus would result in absence of luciferase activity in a GFP$^+$ well.

2.1.8.4 Methods – Immunology

2.1.8.4.1 Animals

All procedures were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act Project Licence (PPL 30/2889) and were approved by the University of Oxford Animal Care and Ethical Review Committee. All mice were housed in the Wellcome Trust Centre for Human Genetics Animal Facility (Functional

Genomics Facility (FGF)), under Specific Pathogen Free (SPF) conditions. All experiments were carried out with female BALB/c ($H2K^d$) mice. Mice were 6 – 10 weeks old at the start of experiments and were purchased from Harlan Laboratories, Oxfordshire, UK or Charles River Laboratories, UK.

2.1.8.4.2 Anaesthesia

Mice were anaesthetized before each immunisation using inhalation anaesthetic comprised of a mixture of isofluorane (2 – 3.5%) and oxygen (1 – 21/min).

2.1.8.4.3 Route and dose

Intramuscular (i.m.) vaccinations were administered in a volume of 50μl using 0.5mL U-100 Insulin (0.33mm (29G) x 12.7mm) (BD, USA). Vaccines were divided equally into each *musculus tibialis* unless otherwise specified.

2.1.8.4.4 Preparation of viruses

Viral vectored vaccines were prepared in sterile, endotoxin free PBS. Recombinant adenoviruses were either dosed in viral particles (vp) or infectious units (ifu). Recombinant MVA were dosed in pfu/ml. Doses were calculated in a total volume of 50μl per mouse. When mixing two vaccines, each vaccine was first prepared before combining in a single vial and vortexed prior to immunisation. Vaccines were kept in ice buckets after preparation and until used for immunisation.

2.1.8.4.5 Peripheral Blood Mononuclear cell (PBMC) preparation

8-10 drops of blood were collected from the tail vein of mice into 200μl 10mM EDTA (in PBS) in 1.5mL eppendorf tube. 1mL of ACK lysis buffer was added and cells were centrifuged at 4000 RPM for 4 min. Supernatants were discarded and pellets were resuspended in 1mL ACK lysis buffer and centrifuged as before. Supernatants were removed and the cell pellet resuspended in approximately 1mL DMEM containing 10% FCS, and antibiotics (Penicillin and Streptomycin) (exact volume of DMEM was dependent on subsequent protocol used).

2.1.8.4.6 Splenocytes preparation

Mice were sacrificed by cervica1 dislocation and spleens removed by dissection. Spleens were crushed in PBS, passed through a sterile 70μm cell strainer in a total volume of 15 mL PBS and centrifuged at 1250 x g for 5min. Supernatants were discarded and pellets resuspended in ACK lysis buffer for 5 min before addition of 25mL PBS. The mixture was immediate1y centrifuged again as before and resuspended in 5mL complete medium (DMEM containing 10% FCS). Splenocytes

were counted using a CASY counter (Schärfe Systems, Germany) and resuspended at appropriate concentrations in complete medium.

2.1.8.4.7 ELISpot

Day 1: MAIP ELISpot plates were coated over night at 4°C or for a minimum of 1h at 37°C with anti-mouse-IFN-γ mAb at 5µg/mL in carbonate-bicarbonate buffer. Plates were blocked with complete medium for at least 1hr or until use. **Day 2: PBMC ELISpot.** PBMCs were isolated from peripheral blood and spleens as described previously. 50μl of PBMCs harvested from the blood were plated into duplicate wells and diluted two folds down the plate. 50μl peptide (1mg/mL) diluted in medium plus 0.25 x 10^6 naïve splenocytes were added to test wells. Medium and naïve splenocytes only were plated into the negative control wells. **Spleen ELISpot:** Spleens cells were re-suspended at 1x10^7cells/mL and 50μl of cells were plated in duplicate wells and diluted two folds down the plate. 50μl peptide diluted in complete medium was added to test wells and complete medium alone was added to control wells. Peptides were added at a final concentration of 5μg/mL. Plates were incubated at 37°C (5% CO2) in a humidified incubator for ˜18h. **Day 3:** Plates were washed 6 times with PBS and incubated for 2 hours with biotinylated anti-mouse-IFN-γ mAb (Mabtech, UK) diluted to 1µg/mL in PBS. After incubation, plates were washed 6 times as before followed by 1hr incubation with a streptavidin alkaline phosphatase polymer diluted to 1μg/mL in PBS. Spots were developed by addition of colour development buffer (AP Conjugate Substratre Kit, Bio-Rad Laboratories, Inc.) and counted using ELISpot software (Autoimmun Diagnostika, Germany). Results are expressed as spot forming cells (SFC) per million cells.

Background responses in media-only wells were subtracted from those measured in peptide-stimulated wells prior to analysis.

2.1.8.4.8 Intracellular Cytokine Staining (ICS)

To analyse the percentage of cytokine producing peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes, cells were collected from the spleen and blood as previously described. **Day 1:** Cells were restimulated for 5 hours at 37°C in the presence of Brefeldin A and peptides at a final concentration of 1μg/mL per peptide. Following restimulation, plates were stored at 4°C overnight. **Day 2:** Cells were transferred to V-bottom plates and centrifuged at 1200 x g for 5 min. Following a wash in FACS buffer (PBS + 1% FCS + 0.5% Sodium Azide), cells were surface stained with anti-CD8a PerCP-Cy5.5, anti-CD4 PB and CD16/CD32 (Fc block) for 30 minutes in the dark. After permeabilisation using Cytofix/Cytoperm, cells were washed and stained intracellularly with anti-IFN-γ APC, anti-TNF-α FITC and anti-IL-2 PE for 30 min in the dark. Samples were resuspended in 1% PBS Formalin and acquired on a LSRII and analysed using FloJo (TreeStar Inc.). Background responses in unstimulated cells were subtracted from the stimulated responses prior to analysis.

2.1.8.4.9 Whole IgG ELISA

Blood was collected from tail veins into Microvette tubes, or by cardiac puncture into eppendorf tubes following cervical dislocation and allowed to clot at 4°C for > 12 hours followed by centrifuged at 13,000 RPM for 4 min. Serum was removed, transferred into clean eppendorf tubes and stored at -20°C until use. **Day 1:** Nunc-Immuno Maxisorp Plates (Thermo Scientific, UK) were coated

at a concentration of 2μg/mL with protein overnight at room temperature. **Day 2:** Plates were washed with PBS/T and blocked with 10% skimmed milk powder in PBS/T. Sera were diluted 1:100 and 50μl plated in duplicate wells followed by diluting 3-fold down the plate. Plates were incubated for 2 hours at RT and then washed as before. Goat anti-mouse total IgG conjugated to alkaline phosphatase (Sigma Aldrich, UK) was added for 1 hour at RT.

Following a final wash with PBS/T, p-nitrophenylphosphate (PNPP) at 1mg/mL diluted in diethanolamine buffer was used as a developing substrate (Pierce, UK). Optical density (OD) was read at 405nm using a Model 550 Microplate Reader (Bio-Rad, UK). Serum antibody endpoint titres were taken as the x-axis intercept of the dilution curve at an absorbance value three standard deviations greater than the OD405 for serum from a naïve mouse. A standard positive serum sample and naïve serum sample were added as controls for each assay. Background responses in naïve mouse serum were subtracted from responses from test serum prior to analysis

2.1.8.4.10 Peptides

Peptides used in the cellular assays were commercially synthesized and obtained from Protein Peptide Research, Invitrogen, UK or Sigma Aldrich, UK and dissolved in DMSO as a concentration of 20mg/mL and stored at -20^{o}C. Working stocks were further diluted to 1mg/mL and stored at -20^{o}C until use.

2.1.8.4.11 Luciferase Immunoprecipitation System (LIPS) Assay

The LIPS assay is employed for the detection of antigen-specific antibody when antigen suitable for an ELISA (for example, purified protein) is not available. LIPS

assay harnesses light-emitting recombinant proteins for quantitatively measuring antibody titers to target antigen. The LIPS system employs renilla luciferase tagged to the antigen of interest to which an antibody-specific response is generated. The first step was to transfect the antigen of interest with a luciferase (rLuc) of the genus *Renilla reniformis*. The next step was to harvest the rLuc-antigen extract. The crude rLuc-antigen extract was then used to determine antibody-specific responses to the target antigen by quantifying rLuc activity. LIPS assay can also be used to determine antibodies to a mixture of antigens. For example, if the antibody titres to two or more antigens are to be determined, a mixture of crude rLuc-antigen extracts could be incubated with serum samples and rLuc activities quantified as a measure of antibody responses. In addition, LIPS assay have been used for profiling patient sera antibody responses to autoantigens and pathogen antigens associated with infection (161-165). The advantage of LIPS assay is that it eliminates time and effort needed to produce highly purified antigens as well as the labor-intensive assay optimization steps needed for standard ELISAs.

In the context of this work, the model antigen (Figure 4.1) consist of an epitope tagged luciferase (tPA-Pb9-rLuc8PV), so antibody responses were measured directly against crude rLuc extracts.

Generation of crude rLuc extracts and quantification of rLuc activity was done exactly as described in the Jenner LIPS Assay protocol, J211, which can be found in the appendix of this thesis.

2.1.8.4.12 Statistics

Statistical analysis was performed using Prism version 5 (Graphpad, USA). $CD8^+$ T cell antibody data were transformed by log 10. One-way ANoVA was used for multiple comparisons of parametric data with Bonferroni's or Newman-Keuls multiple comparison test for difference between more than two groups. Non-parametric data were analysed by Mann-Whitney U test for differences between two groups; or Wilcoxon Signed Rank test for differences in paired samples. $p<0.05$ was considered in all cases to be significant (*$p < 0.05$, **$p<0.01$, ***$p<0.001$).

CHAPTER 3

PROOF OF PRINCIPLE TO TEST THE "B8R"GENE IN MODIFIED VACCINIA VIRUS ANKARA AS AN INSERTION DRIVEN LOCUS

3 Proof of principle to test the "B8R"gene in Modified Vaccinia Ankara virus as insertion driven locus

3.1 Introduction

In vaccinia virus (VACV), classification of its genes (early, intermediate and late) and promoter functions are tightly conserved and the promoters have been classified on the basis of the class of genes they transcribe. A promoter can also exert its transcription activities in more than one gene class and an example is the vaccinia virus p7.5 that has both early and late promoter functions (135). In addition to the three classes of VACV genes, an immediate-early (IE) class of genes was recently identified (136). The distinction of this class of gene from traditional early genes is not well understood, but it was defined based on its kinetic description of preferential expression before the early genes. So because of its preferential expression before traditional early genes it was hypothesized that harnessing the endogenous promoters of some of these newly identified immediate early genes might have an advantage over the traditional early/late p7.5 and SSP promoters for the expression of recombinant antigens. In addition, these IE genes were recently categorised into E1.1 and E1.2 clusters by deep sequencing of vaccinia virus mRNA (166). Unlike other vaccinia virus promoters, no known promoter consensus sequence has been reported for this class of gene but has been shown to have A+T nucleotide sequences similar to vaccinia virus early promoters.

The conventional method for generating recombinant MVA is by insertion of the foreign gene of interest linked to a small number of early/late promoters (p7.5, mH5 and SSP) prior to insertion into the viral genome at TK locus (151), Del I to VI or HA (152, 167) by homologous recombination in infected cells. However, to our

knowledge, no previous work has shown that the robust and efficient recombination mediated technology could be employed to introduce the ORF of a transgene to replace the ORF of a non-essential or non-functional poxviral gene leaving the endogenous promoter intact for regulating the transgene. We hypothesized that with the application of this new technology – potent strong early or immediate early promoters that drive the expression of non-essential or non-functional genes in MVA could be harnessed as antigenic driven insertion loci to elicit antigen-specific $CD8^+$ T cell equal to or exceeding those of traditional p7.5 promoter.

3.2 Rationale for selecting the B8R gene as insertion locus

The sequencing of the entire genome of vaccinia virus Western Reserve (VACV WR) (168) has improved our understanding of how the virus modulates the host immune system and how best to improve it as a vaccine delivery system. It is well documented that the attenuated form of vaccinia virus, the modified vaccinia virus Ankara (MVA) has a highly conserved central region and variable terminal regions where there are mutations and deletions, rendering some genes inactive (167, 169). The central region encodes genes that are essential for virus replication - enzymes, transcription factor and structural proteins necessary for virus replication, while the terminal regions are made up of non-essential genes not required for viral replication, but affect features such as host range, virulence and immunomodulations. It has been reported in gene functionality studies that vaccinia viruses have evolved strategies for evasion of the host immune response which include the secretion of receptors for tumor necrosis factor (TNF), IL-1β, IFN-γ, IFN-α/β and CC chemokines (170). However, in MVA, most of the genes that encode these immunomodulatory molecules became fragmented or truncated during attenuation of MVA, rendering them non-functional

for the survival of the virus. The *B8R* is a classic example of a truncated and non-functional gene in MVA and studies have shown that MVA lacks IFN-γR (receptor) (171) by comparing the sequence of *B8R* in VACV WR with that of MVA and reported that both sequences were identical except for three silent nucleotide substitutions and three short deletions in MVA. They study concluded that, of the three short deletions, one deletion amounted to elimination of 24 nucleotides and caused a translational frame shift that truncated the region that encodes the C terminus of the *B8R* protein rendering it non-fucntional. Previous studies in murine models (172) have also shown that immunisation with VACV-*B8R* deleted mutants resulted in smaller skin lesions than animals immunised with wild type vaccinia virus with the functional *B8R*.

In addition, immunological studies have reported that of the 258 predicted ORFs in vaccinia virus, five peptide determinants (*B8R, A19L, A47L, A42R, K3L*) were identified in C57BL/6 mice. *B8R* was shown as the immunodominant determinant (IDD) accounting for >25% of the total VACV response in C57BL/6 mice and up to 40% when combined with the other four sub-dominant determinants (SDD – A19L, A47L, A42R and K3L)(144)

Putting these together we reasoned that (i) since the *B8R* is non-functional in MVA its ORF could be harnessed as antigenic driven insertion loci (ii) the *B8R* promoter sequence is conserved in MVA (iii) has immunodominant $CD8^+$ T cell epitope that could be turned to our advantage by replacing it with the ORF of a transgene (iv) has been deleted previously without having adverse effect on generation of rMVA (Cottingham *et al*., unpublished data) (v) BAC recombineering technology could be employed to introduce a model antigen into the ORF of the *B8R*.

3.3 Properties of B8R gene

In VACV, the B8R gene is located between positions 170571 and 171389 (Figure 3.1) and it encodes a 43 kDa glycoprotein that is secreted from infected cells early in infection as a homodimer. The protein has amino acid similarity with the extracellular domain of cellular IFN-y receptor (IFN-γR) and binds and inhibits IFN-y from a wide range of species (170, 172).

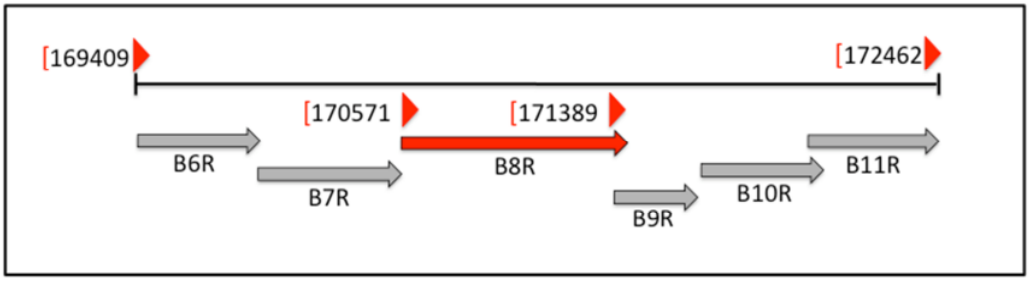


Figure 3.1: Open reading frame (ORF) of B8R.

In VV, the B8R ORF is located between position 170571 and 171389. Gray bars are neighbouring ORFs (GenBank: AY243312).

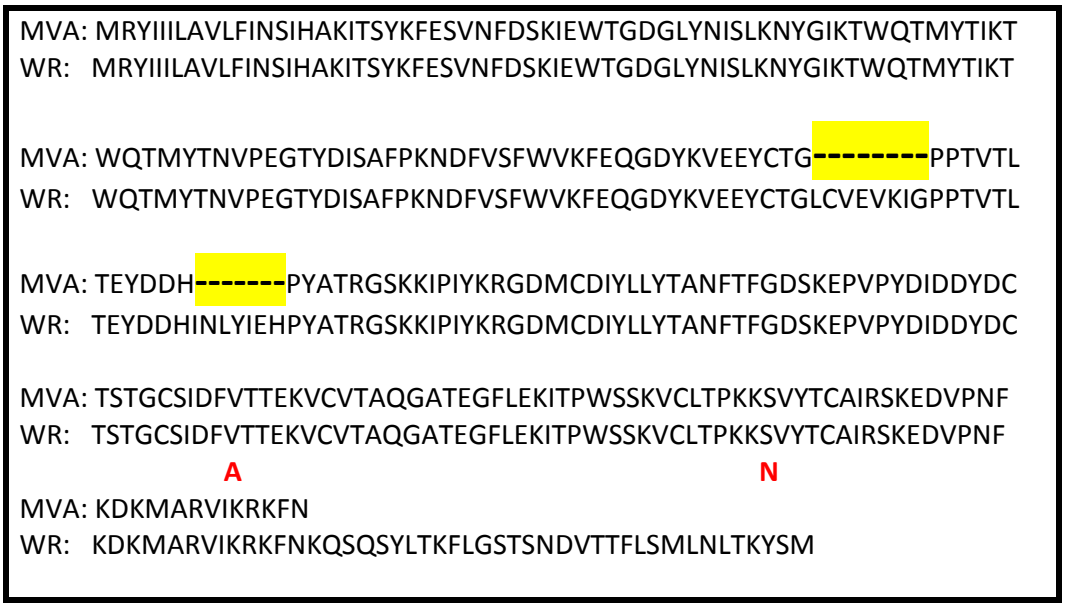


Figure 3.2: Sequence of the B*8R* gene in vaccinia virus Western Reserve (WR).

Yellow indicates two short deletions in MVA. Red indicates single nucleotide substitutions (GenBank: AY243312).

3.4 Results

3.4.1 Construction of Model Antigen

It was first hypothesized that a model antigen (epitope tagged luciferase) would allow *in vivo* ($CD8^+$ T cells) and *in vitro* (luciferase) assessment of the *B8R* endogenous promoter (pB8R). The model antigen comprises the H2-Kd restricted murine $CD8^+$ T cell epitope SYIPSAEKI (Pb9) from *Plasmodium berghei* circumsporozoite protein fused to a cDNA encoding a variant of *Renilla reniformis* (sea pansy) luciferase, rLuc, which has early gene transcription termination signal 5'-TTTTTNT-3' (T5NT). The T5NT sequence causes early transcription termination that result in sub-optimal gene expression. It was overlooked at the time of the proof of principle study but was subsequently removed from the rLuc used in chapter 4 in order to enhance protein expression (discussed in M+M sections 2.4.1, 2.4.2 and chapter 4)

The SYIPSAEKI (Pb9) epitope of *Plasmodium berghei* circumsporozoite protein is recognised by $CD8^+$ T cells in Balb/c mice and was included to detect antigen expression by the pB8R. The luciferase antigen, rLuc was positioned upstream of Pb9 and linked by a GS sequence. Inclusion of the luciferase allows quantification of luciferase in infected cells.



Figure 3.3: Pb9-rLuc-GalK model antigen.

The white open boxes are the left (LHA) and right (RHA) homology arms. They are each 50bp in length and were added by PCR to the ends of the cassette comprising the model antigen, Pb9-rLuc8PV (gray arrow) and the bacterial selectable marker *GalK* (red arrow), with its bacterial promoter (white "p" inside black triangle). The LHA and RHA were designed to replace the ORF of the *B8R* gene with the two ORFs shown in the figure above.

3.4.2 Construction of B8R-mutant BAC by Recombineering

The Pb9rLuc-GalK model antigen was amplified with Roche High Fidelity thermostable polymerase mixture (Roche) using long oligonucleotide primers (Eurofins MWG Operon) to introduce 50bp homology arms to the 5' and 3' ends, resulting in a product size of 2.3kb (Figure 3.5). The primers were designed so that the initiator ATG codon at the 5' end of the Pb9-rLuc-GalK cassette replaces that of the deleted viral gene. The 3' end primer was designed to delete the *B8R* ORF wholly. Introduction of the Pb9rLuc-GalK cassette replaced the ORF of the *B8R* gene, leaving its endogenous promoter (pB8R) intact to regulate the Pb9-rLuc-*GalK* (Figure 3.3).

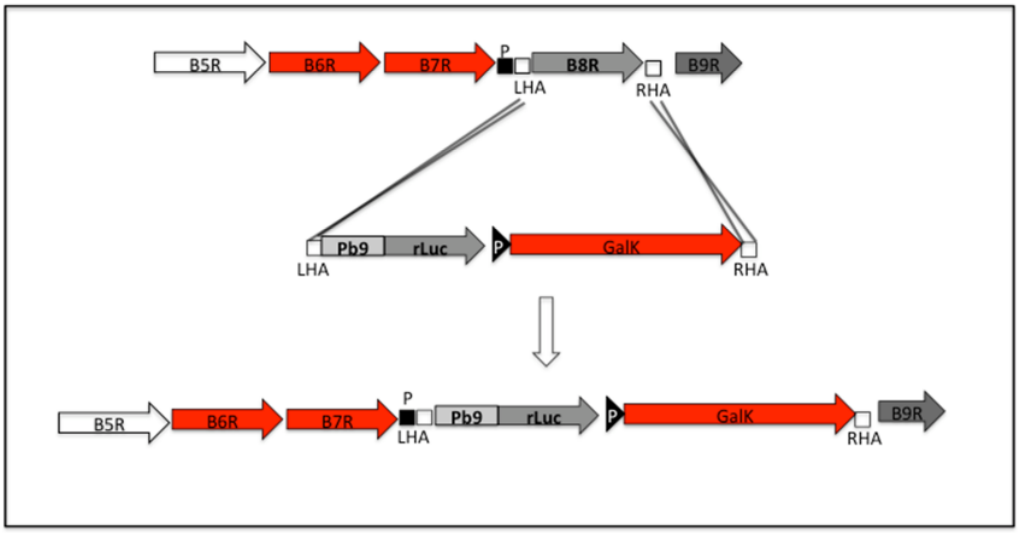


Figure 3.4: Schematic of transgene insertion at endogenous promoter driven locus of MVA using *B8R* as an example.

Surrounding MVA open reading frames (ORFs) are indicated by white arrows, with *B8R* highlighted in light grey. Black "p" above black bar indicates predicted *B8R* promoter core region. The LHA was designed to place the initiation codon of Pb9-rLuc in the same position as that of the deleted *B8R* ORF. After insertion by recombineering of the 2.3kb targeting DNA (Figure 3.5) into MVA-BAC, Pb9-rLuc8 was placed under control of the *B8R* promoter. Crossed lines indicate homology arm recombination events between targeting amplicon and MVA-BAC.

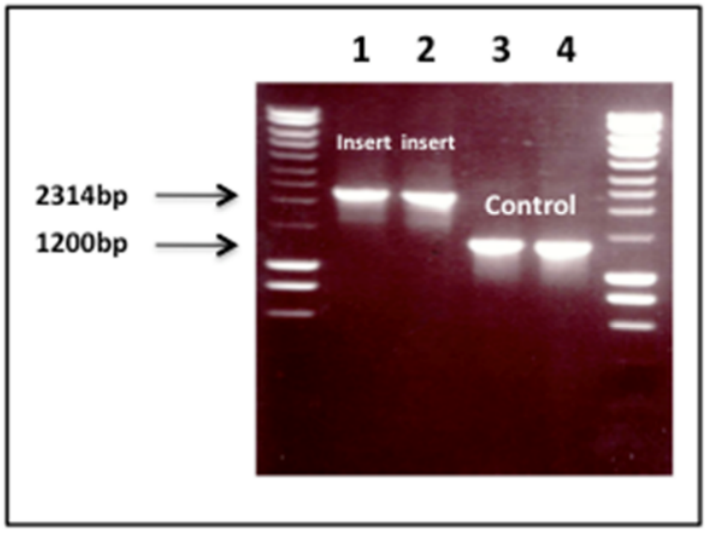


Figure 3.5: PCR to generate targeting DNA (insert) – Pb9-rLuc-GalK.

The insert was generated by PCR using B8R-F and B8R-R specific primers. Lane 1 and 2 represent the insert – 2314bp while lanes 3 and 4 are positive controls.

3.4.3 Screening of B8R-mutant BAC Clones

Four colonies were screened by PCR for the integrity of the BAC cassette (Figure 3.6) and the identity and purity of the model antigen (Figure 3.7). The purity of each colony was also screened by PCR using antigen and insertion-site specific primers using the protocol in 2.6.5.1 (M+M section 2.6.5.1 and primers in Table 2.2 and 2.3). Positive clones were further sequenced and thoroughly analyzed to ensure no mutations were found as this may result in sub-optimal translation of rLuc. No mutations were found in all clones sequenced.

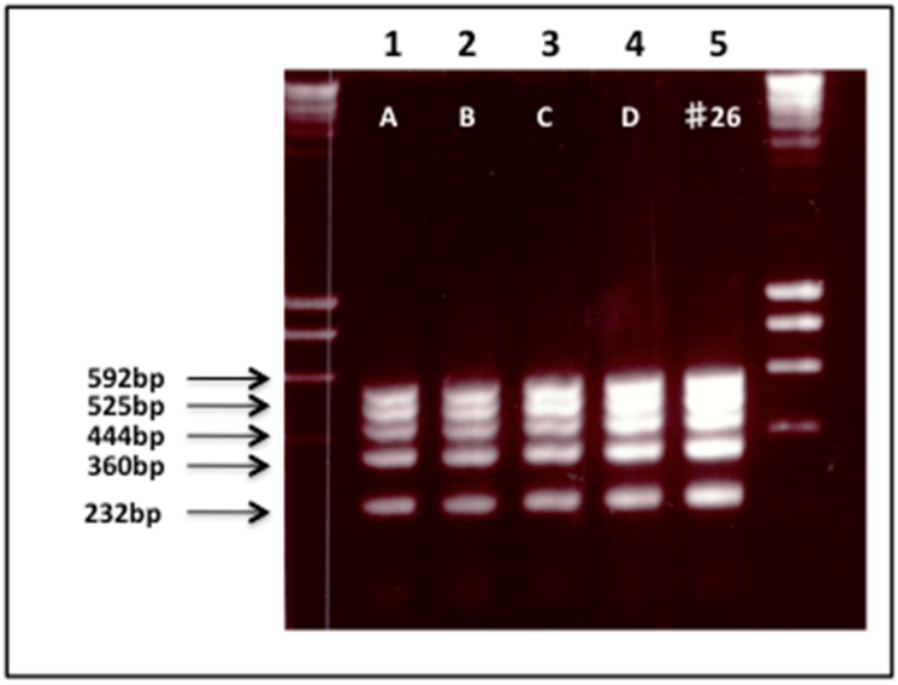


Figure 3.6: PCR to determine the integrity of the BAC cassette.

Five sets of forward and reverse primers specific for the BAC cassette were used to run a PCR. Lane 1, 2, 4 and 4 are four different clones of BAC after insertion of the model antigen, while lane 5 is positive control. The expected band sizes are 232bp, 360bp, 444bp, 525bp and 592bp. Lane 5 is the parent BAC that was used to generate the *B8R* BAC. Lane 5 is another clone of the BAC.

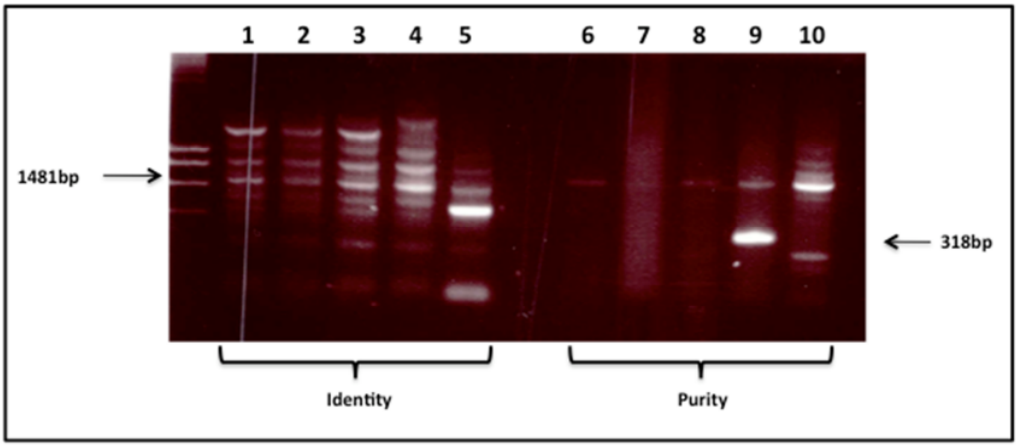


Figure 3.7: PCR to confirm identity and purity of the insert in MVA-BAC.

Insert specific primers, Screen-GalK-F and B8R-R were used to run a PCR to confirm the presence of the insert. Lane 1, 2, 3, 4 are BAC clones after insertion of the model antigen, while lane 5 is control. The expected band was 1481bp and was present in lanes 1, 2, 3 and 4 and was incorrect in lane 5. Clone 3 was chosen because it is brighter suggesting high DNA concentration. Lanes 6, 7, 8, 9 and 10 were replicates of BAC clones in lanes 1, 2, 3, 4 and 5 to test for purity. PCR reaction was run with *B8R* ORF specific primers, B8R-Ind-F and B8R-Ind-R to confirm the deletion of the *B8R* ORF. The expected band 318bp is seen in lane 5, indicating the *B8R* ORF was still intact in that clone.

3.4.4 Rescue of B8R-mutant MVA-BAC to infectious virus

Following recombineering, the next step was to confirm the integrity of the BAC cassette. Four colonies were screened for the presence of specific regions in the BAC using Multiplex primers (discussed in M+M section 2.7.1). Upon confirmation that the BAC was intact, one clone (clone 3 Figure 3.7) was taken forward for rescue to infectious MVA viruses. BHK cell line was found suitable because it supports MVA replication (120) and has been used previously for this purpose (153). BHK cells were

first infected with a Fowlpox virus helper (FP9), which provided transcriptionary machinery, followed by transfection with DNA from the BAC clone. The BAC clone was successfully converted to infectious MVA by plaques formation. Plaques were purified in BHK cells, screened by PCR for purity and identity using the same PCR assays as were used at the bacterial stage (M+M section 2.6.5.1 and primers in Table 2.2 and 2.3).

3.4.5 Recombinant Viral Identity and Purity PCR

Recombinant viral identity and purity was confirmed by PCR using antigen and insertion-site specific primers – Screen-GalK-F and B8R-R.

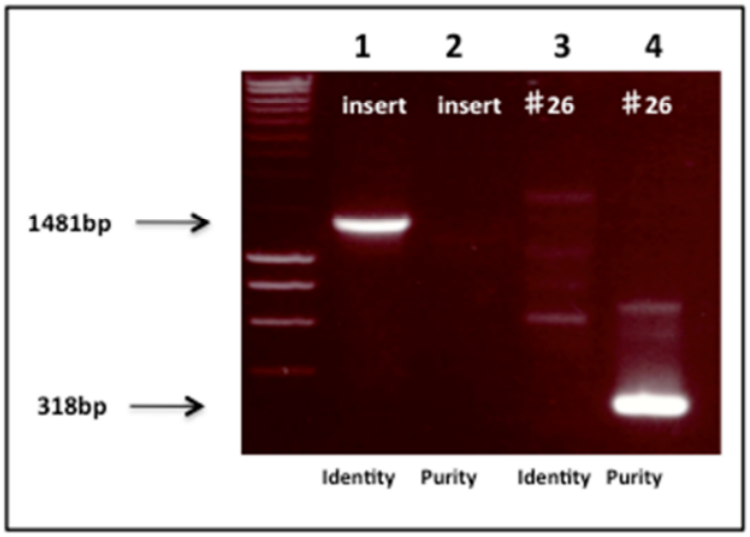


Figure 3.8: PCR to confirm identity and purity of recombinant virus.

Insert specific primers, Screen-GalK-F and B8R-R were used to run a PCR assay to confirm the presence of the insert in the rMVA. Lane 1 confirms the presence of the insert – 1481bp. Lane 2 confirms the purity of the rMVA as no band was seen. Lanes 3 and 4, same identity and purity primers were used for the parent BAC. Lane 3, no insert was found, while lane 4 shows presence of the *B8R* ORF – 318b.

3.4.6 Generation of pB8R-rMVA

The generation of the parent BAC containing the MVA genome (clone #26) has been described previously (153). To generate the rMVA carrying the model antigen (Pb9-rLuc), recombineering primers were designed to replace the *B8R* ORF with that of the model antigen in place. Following recombineering, four positive BAC clones were screened by PCR for the integrity of the BAC cassette (Figure 3.6) and for identity and purity of model antigen. The insert was confirmed in four of the BAC clones (Figure 3.7), and all four clones were subjected to three further rounds of screening on MacConkey agar plates to get rid of GalK-negative "hitch-hikers" which is sometime problematic in BAC selection system (155). After the third round of streaking, the insert was PCR amplified from the three BAC clones and sequenced for any possible mutations because we initially found poor protein translation from an rLuc with a point-base mutation. Upon confirmation of correct sequence of the insert, only one clone was taken further for rescue to infectious virus.

rMVA employing the traditional p7.5 promoter driving the same Pb9-rLuc construct at the TK locus was generated by conventional homologous recombination (Sumi Biswas, Thesis submitted). The next step was to rescue the BACs to infectious MVA viruses. BHK cell line was found suitable because it supports MVA replication (173) and has been used previously for this purpose (153). The BACs employing the pB8R was successfully rescued back to infectious viruse, amplified and purified. Titration was done on CEFs and resulted in yield of 1.9×10^9 pfu/mL (final volume ~0.5mL from $1500cm^2$ flask area), which lies within the expected range for conventional BAC-derived MVA at the Jenner Laboratories. The identity and purity of the recombinant viruse was verified using the same PCR assays as were used at the

bacterial stage (M+M section 2.6.5.1 and primers in Table 2.2 and 2.3). The recombinant virus was further sequenced for any possible mutations.

3.4.7 *In vitro* expression of rLuc by B8R endogenous promoter

The luciferase antigen, rLuc, serves well as a model reporter gene for expression studies because it can be quantified in cell lysates (174). The pB8R promoter was capable of driving expression of rLuc after 24 hpi (Figure 3.6). In the presence of AraC (black bars), the pB8R promoter drove expression of rLuc equivalent to that of p7.5, but when post replicative DNA expression was allowed in the absence of AraC (open bars), the p.75 promoter produced rLuc higher than the pB8R promoter but the difference was not statistically significant ($p = 0.06$ by one-way ANoVA). The data from the AraC treated cells (green bars) confirms that the pB8R promoter is capable of driving early protein expression that was equivalent to the protein expressed by the traditional early/late p7.5 promoter.

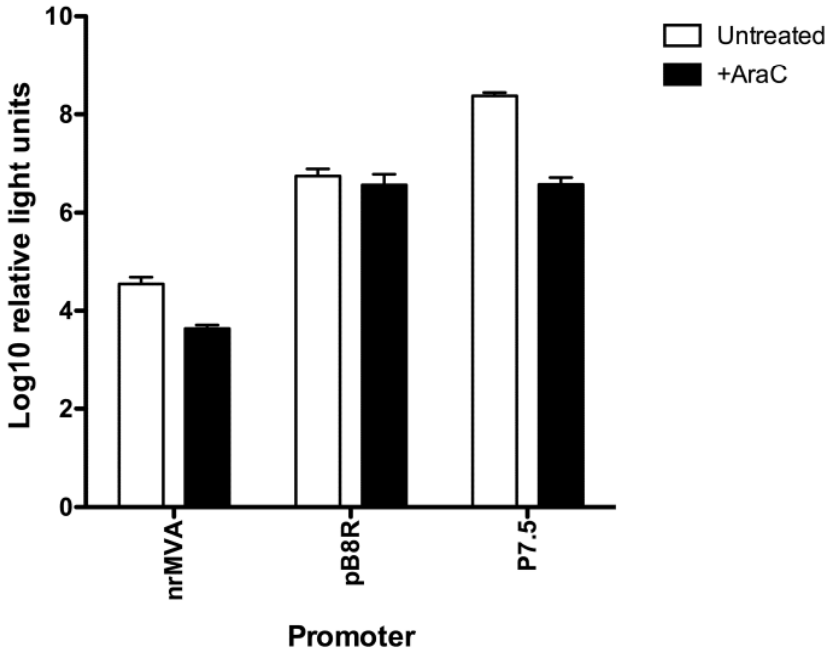


Figure 3.9: Promoter activity in BHK cells infected with rMVA.

BHK cells (~ 80% confluent) were infected with rMVA at 1 pfu/cell. Cells were infected with rMVAs expressing model antigen - Pb9-rLuc under the control of B8R promoter in comparison to p7.5 and control vector (nrMVA). At 24 hpi, cell lysates were analysed for luciferase expression. Cells were either untreated (open bars) or treated with AraC (black bars). Data shown are the mean and standard deviation of duplicates after subtraction of signal from uninfected wells. Data is a representative of three independent experiments.

3.4.8 Virus-cell infectivity to determine equality of infection

All viruses (rMVAs and nrMVA) carried the GFP maker gene, which is driven by the p4B late promoter of fowlpox virus. To investigate whether there was equality of infection, the GFP marker gene was used to measure viral-cell infectivity using real-time fluorimetry. A revised version of this assay that allows continuous assessment

of GFP was previously used to show that derivation of MVA by BAC technology does not affect viral growth (158). GFP +ve cells for all viruses including MVA lacking the rLuc-Pb9 construct (nrMVA) were approximately equivalent (data not shown). This data shows that expression of rLuc by the pB8R promoter is not due to excess virus in the pB8R group but the ability of the promoter to drive early protein expression equivalent to the traditional p7.5 promoter.

3.4.9 Immunogenicity of recombinant antigen driven by B8R endogenous promoter

To test the ability of the *B8R* endogenous promoter to generate $CD8^+$ T cells we used the Pb9 epitope fused to the N-terminus of the model antigen – Pb9-rLuc. Seven days post vaccination with 10^6 pfu/mouse of rMVAs and nrMVA, splenocytes were assayed for the frequencies of Pb9-specific $CD8^+$ T cells by intracellular cytokine staining (ICS). Interestingly, the pB8R endogenous promoter drove high expression of the recombinant protein *in vivo* and elicited $CD8^+$ T cells that were indistinguishable from the traditional p7.5 promoter (Figure 3.7). However, there were undetectable $CD8^+$ T cells to the nrMVA virus lacking the Pb9-rLuc construct and the difference between the groups was statistically significantly ($p<0.05$ by Newman-Keuls post-test following one-way ANoVA; overall $p = 0.0005$). This data confirms that the $CD8^+$ T cells elicited by rMVA employing the pB8R promoter was Pb9 specific and shows that the pB8R perform as well as the traditional p7.5 promoter in terms of $CD8^+$ T cells.

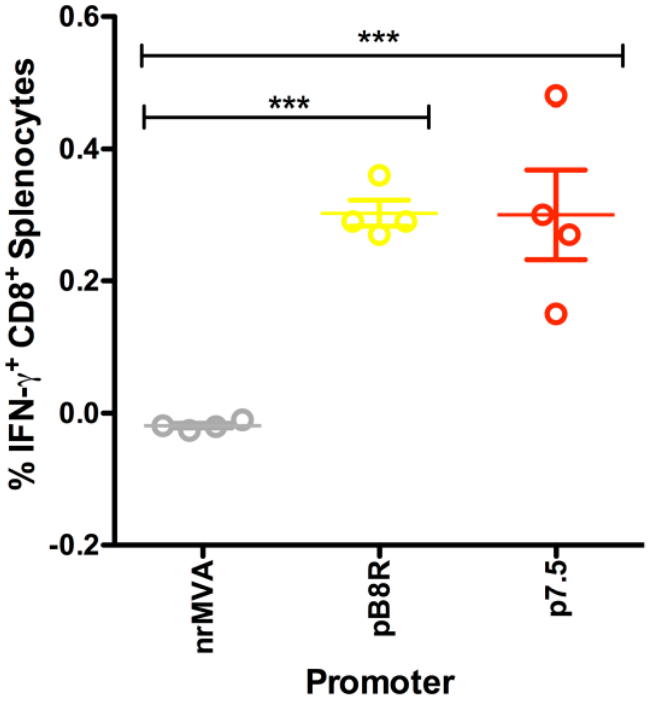


Figure 3.10: Cellular immune responses in mice immunised with rMVAs.

Each group of mice (n = 4) was immunised (i.m) with 10^6 pfu/mouse of rMVAs employing the pB8R and p7.5 promoters or an empty vector (nrMVA). Seven days post vaccination splenocytes were assayed for Pb9-specific $CD8^+$ $IFN\gamma^+$ by intracellular cytokine staining (ICS). Circles represent responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is a representative of two independent experiments.

3.5 Discussion

To develop a BAC derived MVA, we employed the BAC recombineering technology previous used by Domi and Moss (175, 176) to insert a model antigen (Pb9-rLuc) into the insertion driven loci of the *B8R* ORF. The insertion of the model antigen into the natural locus of the *B8R* allowed expression of protein and immunogenicity by its endogenous promoter.

To our knowledge, this is the first time this technology has been applied for inserting the ORF of a transgene to replace that of a poxviral ORF as against the traditional method where a transgene is linked to a poxviral promoter prior to insertion into the viral genome at the TK locus. In addition conventional method for generation of recombinant MVA vectors often rely on inefficient endogenous recombination events in poxvirus-infected cells but the BAC technology was shown to be straightforward as > 90% efficiency was achieved in generating the recombinant pB8R-MVA-BAC (pB8R-MVA). In addition, application of the BAC technology facilitated the discovery of a novel insertion site and a poxviral endogenous promoter – pB8R.

The ability of the pB8R to express exogenous protein from its authentic locus was investigated by infecting BHK cell lines with rMVA and luciferase measured 24 hpi. As expected the comparator p7.5 expressed higher protein in the absence AraC. The high protein expression observed was due to combination of early and late promoter element of p7.5. However, in the presence of the inhibitor, AraC, protein expression for the pB8R was indistinguishable from the traditional p7.5 promoters (Figure 3.6).

The ability of the pB8R promoter to elicit potent $CD8^+$ T cells to the recombinant antigen *in vivo* was based on the immunodominant properties of the *B8R* gene it

originally transcribes, as reported in the paper by Tscharke (144). So we reasoned that the endogenous promoter of *B8R* would elicit $CD8^+$ T cells equal to or exceeding the traditional early/late promoter p7.5 promoter. After priming with the rMVA, the pB8R was capable of eliciting comparable $CD8^+$ T cells to the traditional p7.5 promoter (Figure 3.10). This data is in agreement with the protein expression data and it is suggestive that late promoter element might not be required for generating $CD8^+$ T cells because when the late promoter core of the p7.5 was blocked; indistinguishable protein expression was found between pB8R and p7.5 promoter (Figure 3.6).

These findings are suggestive that (i) other non-essential or non-functional poxviral genes could be harnessed as antigenic driven insertion loci (ii) the promoters of these genes could be utilized for transgene expression in their authentic locus for generating $CD8^+$ T cells equal to or exceeding the traditional p7.5 and SSP promoters (iii) these promoter if carefully selected could be utilized for vaccines that require multiple antigens either from the same or different pathogens (iv) the methodology employed in modifying the mH5 promoter (177) and the recent pHyb promoter (early promoter core of p7.5 arranged in five tandem repeats (178) could be applied to these promoters to enhance their activities.

CHAPTER 4

IN VITRO ASSAYS TO TEST KINETICS, GROWTH RATE AND GENETIC STABILITY OF VIRUSES

4 In vitro assays to test kinetics, growth rate and genetic stability of viruses

4.1 Introduction

Having obtained proof of principle with the *B8R*, this chapter describes how the approach was extended to six other candidate promoters (*F11L, K6L, A44L, K5L, E5R and B2R*). Having found the endogenous promoter of *B8R* to elicit $CD8^+$ T cells equivalent to p7.5, in addition to the immediate early gene identified by Assarsson and colleagues (136) we hypothesised that the endogenous promoters of the new candidate genes could transcribes foreign antigens as well as the p7.5 and SSP promoters. We selected the new candidate genes on the basis of their functionality in MVA. For example the *E5R*, which encodes structural protein in VACV (179) is reported to have 30bp deletion in MVA and it is one of the genes identified to have immediate early gene expression by Assarson *et al*. (136). Another example is the *C11R* that encodes vaccinia viral growth factor (VGF). Though while this gene is functional in VACV (180, 181) it is not known to have any specific functions in MVA. Other examples of immediate early genes identified in VACV that have either been fragmented, mutated or with unknown functions in MVA are (*K6L, A44L, B2R, O1L* and *F8L*).

Reporter genes, such as luciferases have repeatedly been shown as good cost-effective and noninvasive probes for studying biological activities in cell cultures and small animal imaging which provide useful information for the design of subsequent preclinical studies. The most widely studied of these luciferases is the *Photinus pyrallis* (Firefly) and was recently reported to have dominant $CD8^+$ T cell epitopes in

C57BL/6 mice BALB/c mice (157). The luciferase from *Renilla reniformis* (Sea pansy) is also widely used in gene reporter studies for studying the regulatory element that control its expression such as a promoter. Studies have shown that the bioluminescence of these luciferases are unstable especially in serum applications but (156) recently used a consensus sequence driven strategy to generate eight specific mutations in *Renilla* luciferase (RLuc8) that enhanced its stability in serum and light output.

In biological studies, several strategies have been employed to improve expression of reporter genes such as luciferases or antigens from pathogens that cause infectious diseases. One of these methods is to include the leader sequence of the human tissue plasminogen activator (tPA), positioned upstream of the target antigen. Recent studies have shown that inclusion of tPA improved antigen expression in a DNA malaria vaccine (182) and in viral vectored malaria vaccines (60). In addition, inclusion of tPA has been shown to enhance antigen secretion and by directing them to the endoplasmic reticulum (183). In another DNA flu vaccine encoding the nuclecleoprotein (NP) (184), it was reported that inclusion of tPA enhanced expression and secretion of the recombinant protein *in vivo*.

Based on these studies, this chapter sought to build on the success of the proof of principle study (Chapter 3) and explore whether these newly identified strong early or IE promoters, inclusion of tPA, in addition to the modified luciferase (rLuc8) would enhance expression of the recombinant protein for assessment both *in vitro* and *in vivo*.

The genetic stability of an insertion locus is important for generating recombinant poxviral vaccines and examples of insertion loci in MVA are deletion I-VI (del I-VI)

(167), thymidine kinase (TK) (151), heamagglutini (HA) (152). Since the deletion of the ORF of *B8R, F11L, A44L, K6L, E5R*, *C11R* and *B2R* will be concomitant with insertion of the tPA-Pb9-rLuc8PV construct, this chapter will explore whether deleting these genes will have any effects on viral growth.

This chapter will also investigate whether the transgene is genetically stable, as previous studies have shown that the choice of promoter might affect genetic stability of recombinant virus and immunogenicity of transgene (140, 177, 185).

4.2 Results

4.2.1 Selection of non-essential, highly expressed genes for replacement with a transgenic ORF

Building on the results of the pilot study to test *B8R* as an insertion driven loci, one early gene (*F11L*) and five immediate-early (IE) genes (*K6L, A44L, E5R, C11R and B2R*) were identified from the vaccinia virus microarray data (136). Of the six genes selected in MVA (Table 4.1), three are fragmented and one truncated. The functions of two of these genes are unknown (*K6*L and *B2R*) owing to limited published literatures. *K6*L and *B2R* are each one bigger VACV ORF but became fragmented to two small genes (*K5L + K6L*) and (*B2R + B3R*) respectively, during the attenuation of MVA. In MVA, *B2R* is further fragmented into (168 and 169). The criteria used for selecting the *B8R* (refer to chapter 3) as an insertion driven locus was used in addition to the results from the proof of principle study (chapter 3).

Firstly, in addition to *B8R* (refer to chapter 3 for details) which has lost its function in MVA (169, 170, 172), Assarsson *et al*. (136) identified some immediate early genes in VACV which are non-functional in MVA so it was hypothesized that since the BAC recombineering technology proved effective for deleting the *B8R* ORF concomitant with insertion of the model antigen, the technology could be employed for deleting these newly selected strong early or immediate early genes (Table 3.1). It was also hypothesized that since deletion did not affect generation of rMVA-pB8R, other non-functional genes could be deleted without impairment of viral growth.

Secondly, it was hypothesized that since the *B8R* endogenous promoter expressed protein and elicited $CD8^+$ T cells similar to that of the traditional p7.5 promoter

(Figure 3.6 and 3.7), that it is possible to achieve better expression with the endogenous promoters of these immediate early genes. Both *A44L* (186) and *C11R* (181, 187) encodes proteins important for vaccinia virus virulence factors and previous studies have reported that deletion of *A44L* (153) or *C11R* (Cottingham et al., unpublished data) did not affect MVA immunogenicity.

Thirdly, we reasoned that a locus in the conserved central region might have advantage in terms of genetic stability, so the *E5R* was chosen based on this context. Other immediate early genes in the conserved region such as *A35R*, *E3L* and *E4L* were excluded because they are essential for the growth of the virus *in vitro* (179, 188, 189). In addition the *E3L,* which encodes anti-apoptotic protein, expressed the most abundant mRNA in the micro array data (136) but was excluded because it is essential for the growth of the virus in CEFs. The BAC DNAs were rescued back to infectious virus on CEFs (M+M section 2.7.1), so if we had selected *E3L* the virus would not have rescued.

Lastly, there are ORFs in MVA that carry mutations in their upstream regions and these were avoided since the mutations might affect promoter function. The F11L falls within this category but was not excluded; instead an MVA-specific substitution just upstream of the initiation codon was reverted back to identity with vaccinia virus (Table 4.2).

Vaccinia Ortholog	MVA ORF	Function in vaccinia virus	MVA specific function	Predicted promoter activity*	Position in Gene Bank U94848 (152)
B8R	*176R*	IFN-γ soluble receptor (170, 172)	3' inactivating truncation	Early (E | E1.1)	157621 (top strand)
F11L	*041L*	Cell motility (RHoA inhibitor) (190, 191)	Fragmented *(040L+041L)*	Early (E | E1.1)	33771 (bottom strand)
K6L	*027L*	Unknown function	Fragmented *(026L+027L)*	Early (IE | E1.1)	24694 (bottom strand)
A44L	*157L*	3β hydroxysteroid dehydrogenase (186)	1 amino acid substitution	Early (IE | E1.2)	15377 (bottom strand)
E5R	*052R*	Structural protein in virosome (179)	30bp deletion	Early (IE | E1.1)	44810 (top strand)
C11R	*005R*	Vaccinia viral growth factor (VGF) (180, 181, 187)	None	Early (IE | E1.2)	10203 (top strand)
B2R	*168R*	Unknown; fragmented *(B2R+B3R)*	Fragmented *(168R+170R)*	Early (IE | E1.1)	152144 (top strand)

Table 4.1: Loci selected for insertion of endogenous promoter driven transgene by replacement of MVA open reading frame (ORF).

*Designations in brackets refer to categorisation into immediate early (IE) or early (E) clusters by microarray analysis (136), and into the corresponding E1.1 and E1.2 clusters by deep sequencing (166), of vaccinia virus mRNA.

4.2.2 Optimised model antigen with secreted luciferase (tPA-Pb9-rLuc8PV)

It was hypothesized that modification of the model antigen Pb9-rLuc (Figure 3.3), used for the proof of principle study would allow detection of renilla luciferase in the culture supernatant for in-depth analysis of the kinetic activities of these promoters. To improve expression of the model antigen, the leader sequence of the human tissue plasminogen activator (tPA) was added upstream of the Pb9-rLuc8PV to allow detection of protein activity in the supernatant. This is because a previous study showed that addition of a tPA to the AMA improves it expression (60). The luciferase was modified at eight stability-enhancing point mutations (156), such that the protein is readily detected in the culture supernatant. The resulting construct, tPA-Pb9-

rLuc8PV, now encodes a Pb9-tagged and a more stable version of rLuc with improved stability and light output for assessing poxviral early expression.

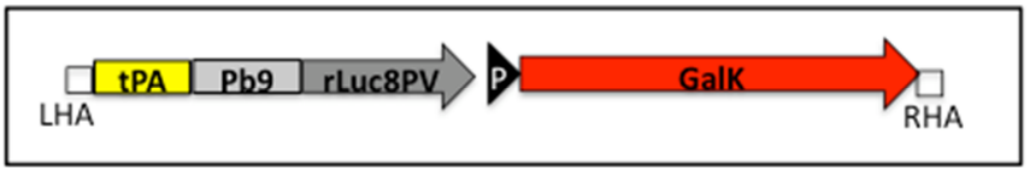


Figure 4.1: tPA-Pb9-rLuc8PV model antigen.

The white open boxes are the left (LHA) and right (RHA) homology arms. They are each 50bp in length and were added by PCR to the ends of the cassette comprising the model antigen, tPA-Pb9-rLuc8PV (yellow and gray arrows) and the bacterial selectable marker *GalK* (red arrow), with its bacterial promoter (white "p" inside black triangle). The LHA and RHA were designed to replace the ORF of the B8R gene with the ORF in the figure above. The MDD sequence linked the tPA and Pb9 (green open box), while the GS sequence linked the Pb9 the rLuc8PV (red open box).

4.2.3 Generation of recombinant viruses by BAC recombineering

To generate recombinant viruses, primers were designed to insert the new model antigen, tPA-Pb9-rLuc8PV (Figure 4.1) to replace the viral ORF (Figure 4.2) of the six newly selected genes including *B8R* (Table 4.1). This was done so that the ATG of the model antigen will replace that of the deleted viral ORF, followed by the bacterial selectable maker. PCR primers (50bp each for both LHA and RHA) were designed for each individual gene selected for deletion and were used to amplify the insert (Figure 4.1) giving products of approximately 2.4kb (Figure 4.2). Recombineering reactions were efficient in all cases and as evident in the proof of principle study. Following recombineering the BACs were rescued back to infectious viruses, amplified and purified and the six resulting viral preparations titred and had yields

ranging from 1.8 x 10^9 to 5.4 x 10^9 pfu/mL (final volume ~0.5mL from 1500cm^2 flask area). PCR analysis was carried out on viral stocks to verify purity and identify (M+M section 2.6.5.1 and primers in Table 2.2 and 2.3). Insertion of the model antigen into the six loci did not affect generation of the recombinant viruses (rMVA).

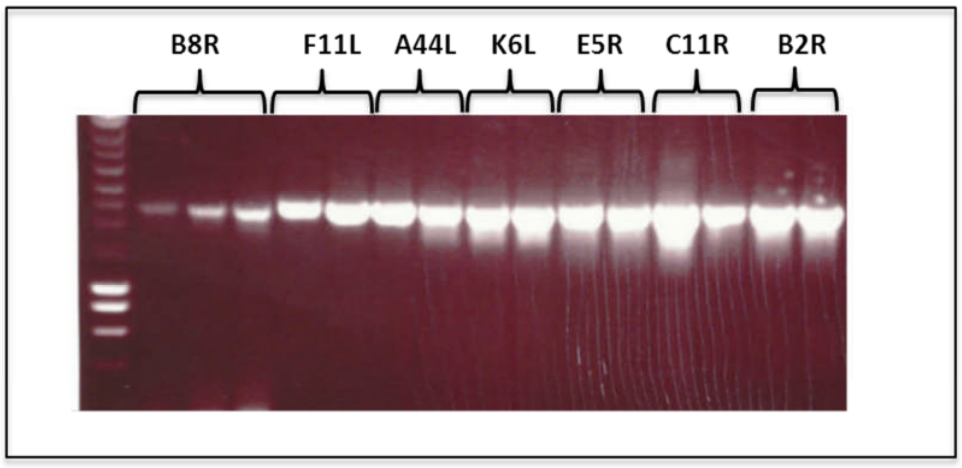


Figure 4.2: PCR to generate the targeting DNA – tPA-Pb9-rLuc8PV-GalK.

The DNA was generated by PCR using primers specific for the ORF of individual gene selected for deletion. The size of the PCR products ~2.4kb was the same for the individual genes because the LHA and RHA are each 50bp in addition to the size of the insert.

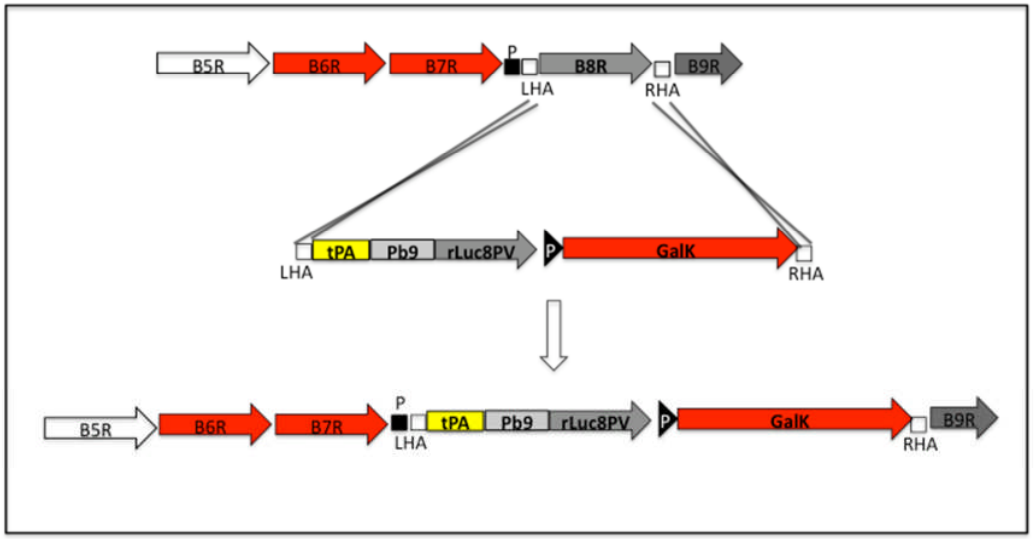


Figure 4.3: Schematic of transgene insertion at endogenous promoter driven locus of MVA, using *B8R* as an example.

To delete the B8R ORF (dark grey arrow) concomitant with insertion of the model antigen, 50bp primers (white open boxes) corresponding to left homology arm (LHA) and right homology arm (RHA) were designed and introduced by PCR to the end of the cassette comprising the model antigen, tPA-Pb9-rLuc8PV and the *GalK* (red arrow) which is driven by a late p4b fowlpox promoter (white "p" inside black triangle). The first step is to introduce the insert (DNA of the model antigen – Figure 4.2) by electroporation to replace B8R ORF. The 5' end of the primer (LHA) was designed to replace the initiator ATG codon of tPA-Pb9-rLuc8PV with that of the *B8R* ORF. After recombineering the tPA-Pb9-rLuc8PV was placed under control of the *B8R* promoter (black solid box). The next step is selection on minimal medium containing galactose and chloramphenicol. By introducing *GalK* and in the presence of galactose the bacteria become phenotypically Gal^{+}.

4.2.4 Generation of comparator viruses with p7.5 and SSP promoters

The short synthetic promoter (SSP) was included as a comparator promoter in addition to p7.5 in order to have a broad comparative analysis of traditional promoters. The rationale for this was to investigate whether these strong early or immediate early promoters (Table 4.1) would express the model antigen to levels comparable to SSP, which is considered as one of the strongest traditional early/late promoters (140). A plasmid containing the insert tPA-Pb9-rLuc8PV (Figure 4.4A) was by conventional cloning into the pEP7.5TK cassette (Figure 4.4B). The resulting product (Figure 4.4C) was amplified with recombineering primers (M+M Table 2.5), for electroporation into the TK locus of MVA-BAC (Figure 4.4D). After recombineering (Figure 4.4E), 10 colonies were screened for the presence of the insert using primers in (M+M Table 2.6). Of the ten colonies screened, three were positive but only one was taken forward for sequencing with primers in (M+M Table 2.7).

To generate the SSP recombinants, a pETK cassette lacking the p7.5 promoter as shown in (Figure 4.4B) ligated by conventional cloning with the insert (Figure 4.4A) carrying the SSP promoter upstream of tPA. The resulting plasmid, pESSPTK, was amplified with the same recombineering primers used for the amplification of pEP7.5TK cassette (M+M Table 2.5). After recombineering, 10 colonies were screened with primers (M+M Table 2.6) and 7 were correct but only one was taken forward for sequencing.

Upon confirmation the sequences were correct, the BAC were rescued back to infectious viruses, amplified, purified and the two resulting viral preparations titred

and had yields of 4.5 x 10^9 pfu/mL (p7.5) and 4.7 x 10^9 pfu/mL (SSP), (final volume ~0.5mL from 1500cm^2 flask area). PCR analysis was carried out on viral stocks to verify purity and identify (M+M section 2.6.5.1 and primers in Table 2.2 and 2.3).

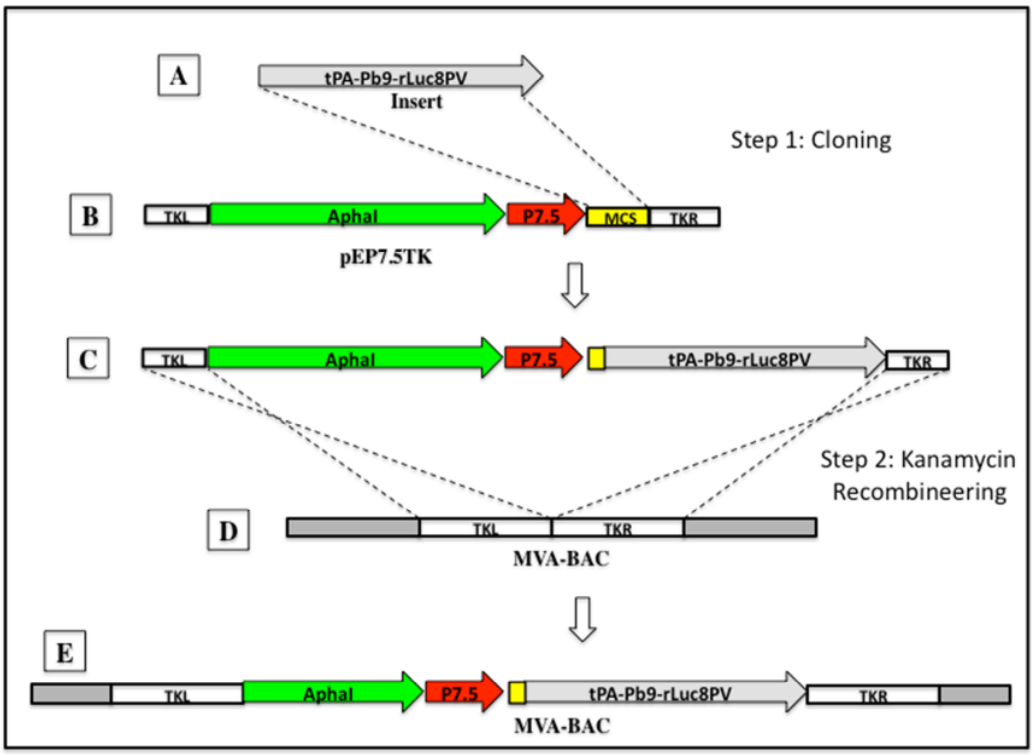


Figure 4.4: Schematic of transgene insertion at TK locus of MVA using p7.5 as an example.

The pEP7.5TK is the targeting cassette for insertion of the model antigen under the control of the p7.5 promoter (red bar). The left and right ends of the pEP7.5TK cassette are the 50bp homology arms (TKL and TKR) for recombination with TK. The yellow bar (multiple cloning sites) allows removal of p7.5 and insertion of another promoter. To insert the model antigen (tPA-Pb9-rLuc8PV) into the pEP7.5TK cassette (Figure B), the model antigen (Figure A) was digested and ligated into the mutiple cloning site of the pEP7.5TK plasmid (yellow bar in Figure B). After insertion of the model antigen into pEP7.5TK cassette, the plasmid was amplified by PCR with TK primers as shown in (M+M Table 2.5). After amplification, the PCR

product (Figure C) is electroporated into MVA-BAC for recombinant with TK (Figure D). After recombineering, the MVA-BAC (Figure E) is shown to carry the pEP7.5TK-tPA-Pb9-rLuc8PV inserted at the TK locus.

4.2.5 In vitro expression of improved luciferase with 8-point mutations

Viruses employing all seven promoters (Table 4.1) were able to drive expression of rLuc (Figure 4.5), which was measured 4 hours post infection (hpi) in BHK cells in the absence or presence of AraC. In presence of AraC, viruses employing *B8R, F11L, A44L* and *C11R* promoters (pB8R, pF11L, pA44L and pC11R), produced rLuc similar to that produced by p7.5 or SSP recombinants (Figure 4.5). As expected, in the absence of AraC, the late promoter element of p7.5 and SSP were un-inhibited and allowed higher protein expression. However, protein expression for pE5R, pK6L and pB2R were unexpectedly poor in the absence or presence of AraC.

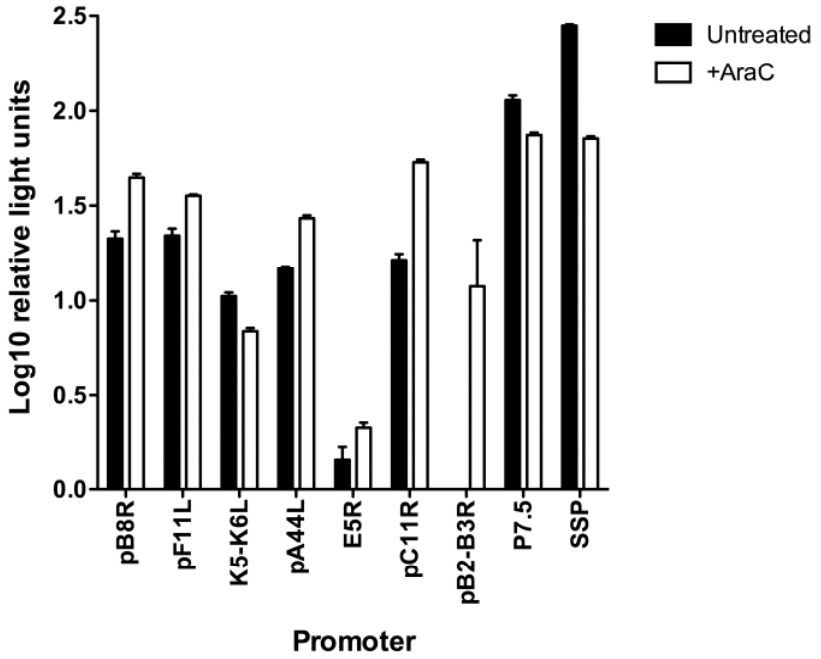


Figure 4.5: Activity of endogenous promoters compared to p7.5 and SSP *in vitro*.

BHK cells were infected with 1 pfu/cell of recombinant MVA carrying tPA-Pb9-rLuc8PV under the control of the indicated promoters. Renilla luciferase activity in the culture supernatant was quantified at 4h post-infection. Cells were either untreated (grey bars) or exposed to 40μM AraC during and after infection (open bars), to inhibit post-replicative gene expression. Data shown are the mean and standard deviation of duplicates after subtraction of signal at 1h post-infection. Data is a representative of three independent experiments.

4.2.6 Non-authentic initiation codons of K6 and B2R affected rLuc expression

To investigate whether the poor protein expression by rMVAs employing the pK6L and pB2R promoters was due to poor expression from their promoters, a careful analysis of the core regulatory regions of these two genes was carried out. Sequence

analysis revealed that the 5' ends in our constructs were fragmented and resulted in a frame shift mutation, which truncates the authentic ORF and produces an MVA ORF encoding a protein with an alternative out-of-frame N-terminal sequence. In addition, the functions of these two genes are unknown because of limited information in the literature about the proteins they encode. However, these two genes were selected because they were among the immediate early genes that drove very high expression of protein in the micro array data (136).

These observations reveled that we mistakenly used the downstream, non-authentic ATG for the regulation of tPA-Pb9-rLuc8PV (Table 4.2), which might have resulted in inefficient initiation translation, and poor expression of pK6L (Figure 4.6). However, when the two promoters were compared over 24 hpi, the kinetic pattern differed from other endogenous promoters including the traditional p7.5 and SSP promoters. The trend of protein expression resembled that of intermediate gene expression (compare rLuc expression for pK6L and pB2R between 1 to 8 hpi in Figures 4.6 A and B) and we could not establish whether the promoter core regulatory region have consensus sequences with intermediate promoters. Viruses employing the pK6L and pB2R promoters were not investigated further because of inconsistency in their transcriptional pattern in comparison to other endogenous poxviral promoters being investigated.

B8R	atattattcaaaatatgatttttaaaAatttaaaatatattatcacttcagtgacagtagtcaaataacaaacaacaccATG
F11L[a]	aaaaaagtgaaaaacaatattattttTatcgttggttgtt*c*actATG
A44L	gtaaaatagaataagtagtctgatattaTgagtggcagcaATG
K6L[b]	ataaaacataaaaataatatgatcatcaAacgaactgttaatattgatagttatataacgtgaatcATGagtgcaaactgtatgttcaatctggac aATG
E5R[c]	taacaaaagtgaatataT—ATG
C11R	atattactgaattaataatAtaaaattcccaatcttgtcataaacacacactgagaaacagcataaacacaaaatccatcaaaaATG
B2R[b]	cgataaaaattaaaaaaTaacttaatttattattgatctcgtgtgtacaaccgaaatcATGgcgatgttttacgcacacgctctcggtgggtacg acgagaatcttcATG
P7.5[d]	taaaagtagaaaatatattctaatttatTgcacggtaaggaagtagaatcataaagaacagt---MCS
SSP[d]	taaaaattgaaattttattttttttttttTgaatataaataa---MCS

Table 4.2: Sequences of promoters, with transcriptional and translational (ATG) start sites shown capitalised, and the predicted early promoter core sequences underlined (166).

[a]The italicised 't' in F11L was mutated during recombineering to identity with vaccinia virus Western Reserve strain (VACV-WR).

[b]The second (downstream) ATG was used in the recombinant viruses described in the text. This is the ATG of the annotated ORF in MVA. The additional (upstream) ATG, however, probably represents the authentic translational start site, as this is the one annotated in VACV-WR.

[c]Two base pairs present in VACV-WR but missing in MVA are indicated by dashes.

[d]Conventional insertion of promoter linked to ORF. Dashes indicate appendage of multiple cloning site (MCS).

Gene	Recombineering	Virus Made	Luciferase Assay	ELISpot	Genetic Stability	Growth Kinetics
B8R	✓	✓	✓	✓	✓	✓
F11L	✓	✓	✓	✓	✓	✓
K6L	✓	✓	✓	×	×	×
A44L	✓	✓	✓	✓	✓	✓
E5R	✓	✓	✓	×	×	×
C11R	✓	✓	✓	✓	✓	✓
B2R	✓	✓	✓	×	×	×

Table 4.3: Production chart of viruses employing the seven candidate promoters. Viruses employing the K6L, E5R and B2R were discontinued because of we used the wrong ATG in addition to inconsistency in transcriptional pattern.

✓: Done

×: Discontinued

4.2.7 Kinetic analysis of transgene expression in vitro

The next step was to analyze the kinetic expression pattern of these promoters by measuring protein production by tPA-Pb9-rLuc8PV producing viruses. Unexpectedly, rLuc was readily detected in sucrose-purified viral preparations and immediately after infection – 0 hpi. This was evident especially in the case of SSP, the strongest late promoter. As expected, the rLuc activity of pK6L was lowest in both untreated and AraC treated cells which is in agreement with data in (Figure 4.4). Interstingly, the rLuc activities of pE5R and pB2R were different from other promoters (see legend between 3 to 8 hpi) and more evident after 24 hpi in AraC treated cells (Figure 4.6 A

and B). The remaining four endogenous promoters (pB8R, pF11L, pA44L and pC11R) were able to drive rLuc expression similar to p7.5 and SSP in the presence of AraC (Figure 4.). However, when post-replicated expression was allowed, in the absence of the inhibitor, p7.5 and SSP drove high expression of rLuc, which was evident between 8 – 24 hpi (Figure 4.6 C and D – data adjusted to exclude pK6L, pE5R and pB2R). Approximately two-log difference in rLuc activity was observed at 24 hpi between p7.5 and SSP, and is suggestive of the weak late promoter element of p7.5 (see legend in Figure 4.6 A and C). The virus employing the pE5R promoter expressed protein exceeding that of the strong early/late SSP promoter, this was however surprising so we reasoned that deletion of *E5R* might have affected the growth pattern of the virus. The recombinant viruses employing the non-authentic initiation codons of *K6L* and *B2R* and the poorly understood *E5R* were not investigated further in this study.

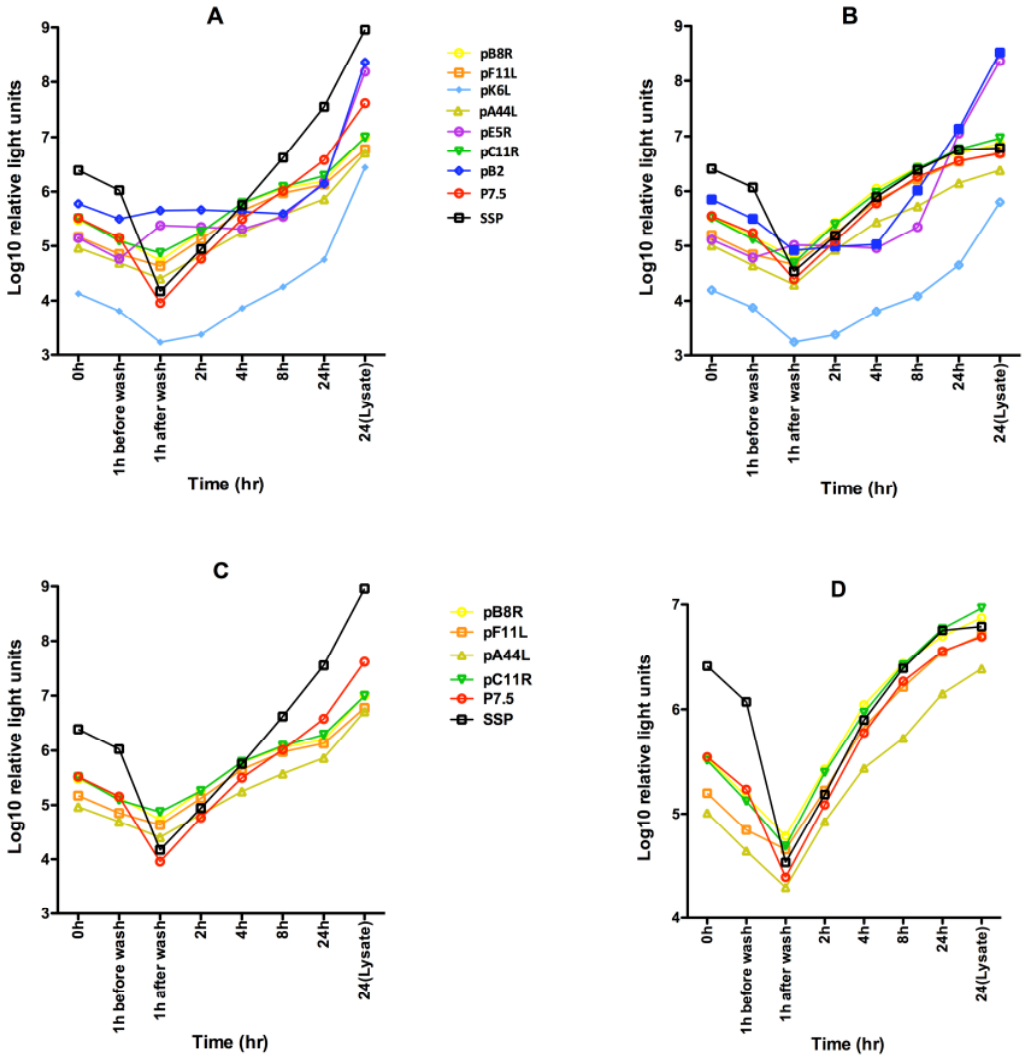


Figure 4.6: Timecourse of endogenous promoter activities after compared to p7.5 and SSP *in vitro*.

BHK cells were infected with rMVAs at 1 pfu/cell. All viruses expressed the model antigen tPA-Pb9-Galk-rLuc8PV under the control of the indicated promoters. Renilla luciferase activity was quantified at, 0'h, 1h, 2h, 4h, 8h, 24h and in the cell lysates at 24h post infection. Cells were either untreated (A) or treated with 40μM AraC (B), to inhibit post-replicative gene expression. Figures C and D are replicas of Figures 4. A and B respectively and have been adjusted to exclude *K6L, E5R* and *B2R* promoters.

Data shown are the mean and standard deviation of the duplicates. Data is representative of four independent experiments.

4.2.8 Spinoculation removed rLuc from sucrose-purifed viral preparations

Having detected rLuc in the sucrose-purified viral preparations and at 0 hpi (Figure 4.6), it was unclear whether they were unbound or incorporated proteins because studies have shown that certain recombinant proteins are incorporated within compartments of vaccinia virions (192, 193). A spinoculation and washing protocol at 0^{o}C (194, 195) was employed to remove soluble rLuc and subsequently enhance viral entry upon return to 37^{o}C. Infection of cells was done in an ice-cold compartment followed by centrifugation for 650 x g for 1hr at 0^{o}C which allowed viruses to synchronise into the cells. Cells were washed with ice-cold dH2O before return to 37^{o}C for the virus to grow. The application of this procedure effectively removed rLuc from the sucrose-purified viral preparations because rLuc were found in the inoculum confirming that its presence was as a result of carry-over of soluble proteins into the viral preparations (Figure 4.7 – see difference between inoculum and 0 hpi). After 1 hpi, the kinetic pattern of all endogenous promoters including p7.5 and SSP were similar to that observed before the application of the spinoculation and washing protocol and it continued until 24hpi (compare Figure 4.6 C and D, and 4.7 A and B).

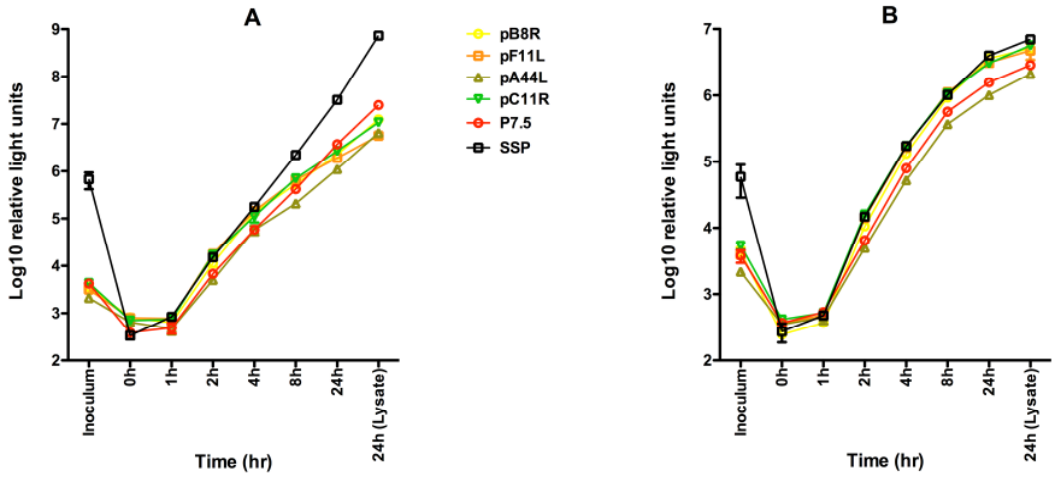


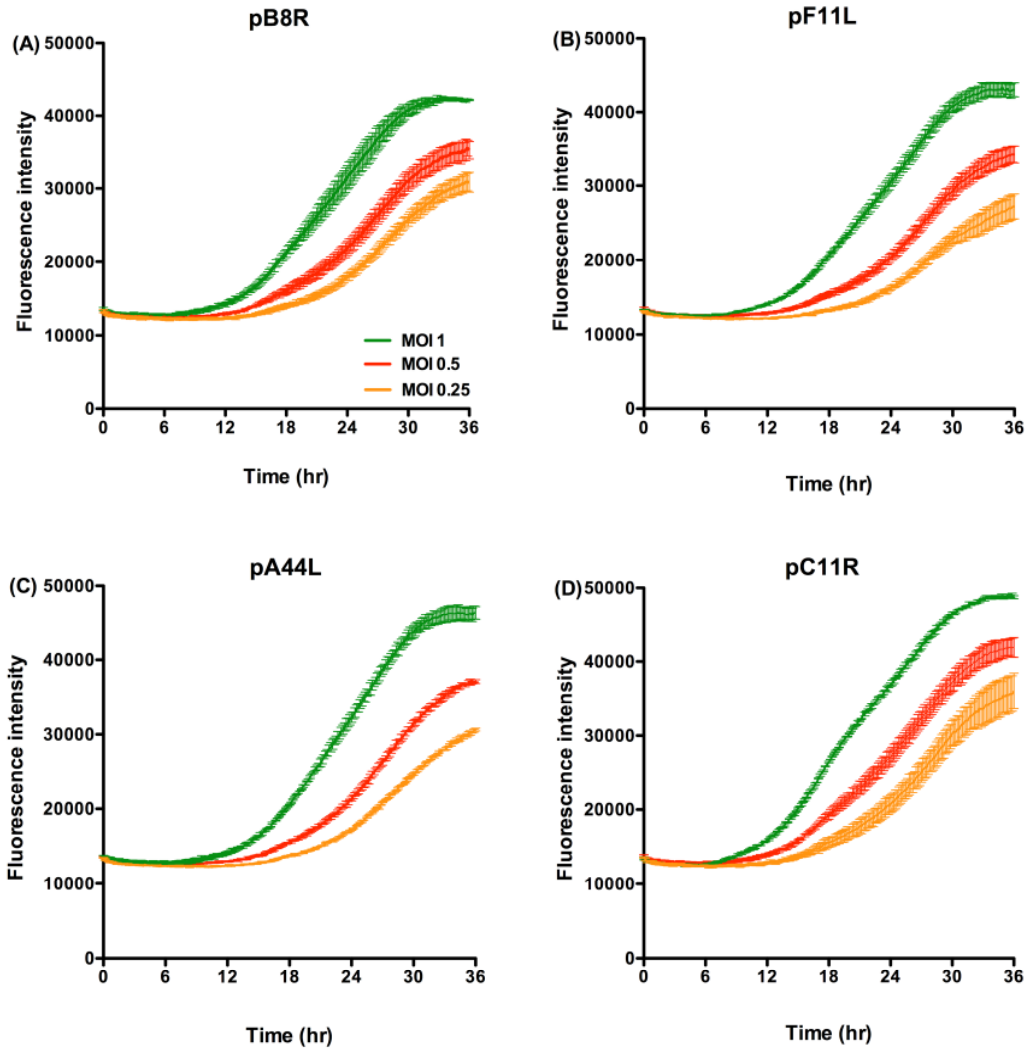
Figure 4.7: Timecourse of endogenous promoter activities after spinoculation.

BHK cells were spinoculated with rMVAs at 1 pfu/cell. All viruses expressed the model antigen tPA-Pb9-Galk-rLuc8PV under the control of the indicated promoters. Renilla luciferase activity was quantified at, 0'h, 1h, 2h, 4h, 8h, 24h and in the cell lysates at 24h post infection. Cells were either untreated (A) or treated with 40μM AraC (B), to inhibit post-replicative gene expression. Data shown are the mean and standard deviation of the duplicates. Data is representative of four independent experiments

4.2.9 Transgene insertion at endogenous promoter driven loci does not affect viral growth

To investigate whether the viral growth of BAC derived rMVAs expressing the tPA-Pb9-rLuc8PV inserted to replace viral ORF were impaired, a fluorimeter was used to measure viral growth rate because all BAC-derived rMVAs carried a GFP marker driven by the P4b late promoter of the fowl pox virus (M+M section 2.8.2). Cottingham et al. (158) has previously shown that derivation of MVA by the BAC technology does not impair viral growth. Equivalent growth pattern was seen for

rMVAs employing the pB8R, pF11L, pA44L and pC11R promoters upon infection of BHK cell lines at 1, 0.5 and 0.25 pfu/cell (Figure 4.8 A-D). To further investigate whether the growth pattern observed was equivalent to BAC derived MVA (MVA-BAC) lacking the tPA-Pb9-rLuc8PV (but containing the GFP marker) a head-to-head comparison was made and the growth curves of MVA-BAC overlaid exactly with pB8R, pF11L and pA44L, but the rMVA employing pC11R exhibited a faster growth and reached a slightly higher plateau at 36 hpi (Figure 4.8 E-G). The high plateau observed for rMVA-pC11R was unexpected as it lacks the C11R protein that encodes the vaccinia growth factor (187). However, insertion of the transgene at any of these insertion loci, concomitant with deletion of the targeted viral ORF, did not impair viral growth


pB8R
(A)
Fluorescence intensity
Time (hr)
MOI 1
MOI 0.5
MOI 0.25
pF11L
(B)
Fluorescence intensity
Time (hr)
pA44L
(C)
Fluorescence intensity
Time (hr)
pC11R
(D)
Fluorescence intensity
Time (hr)

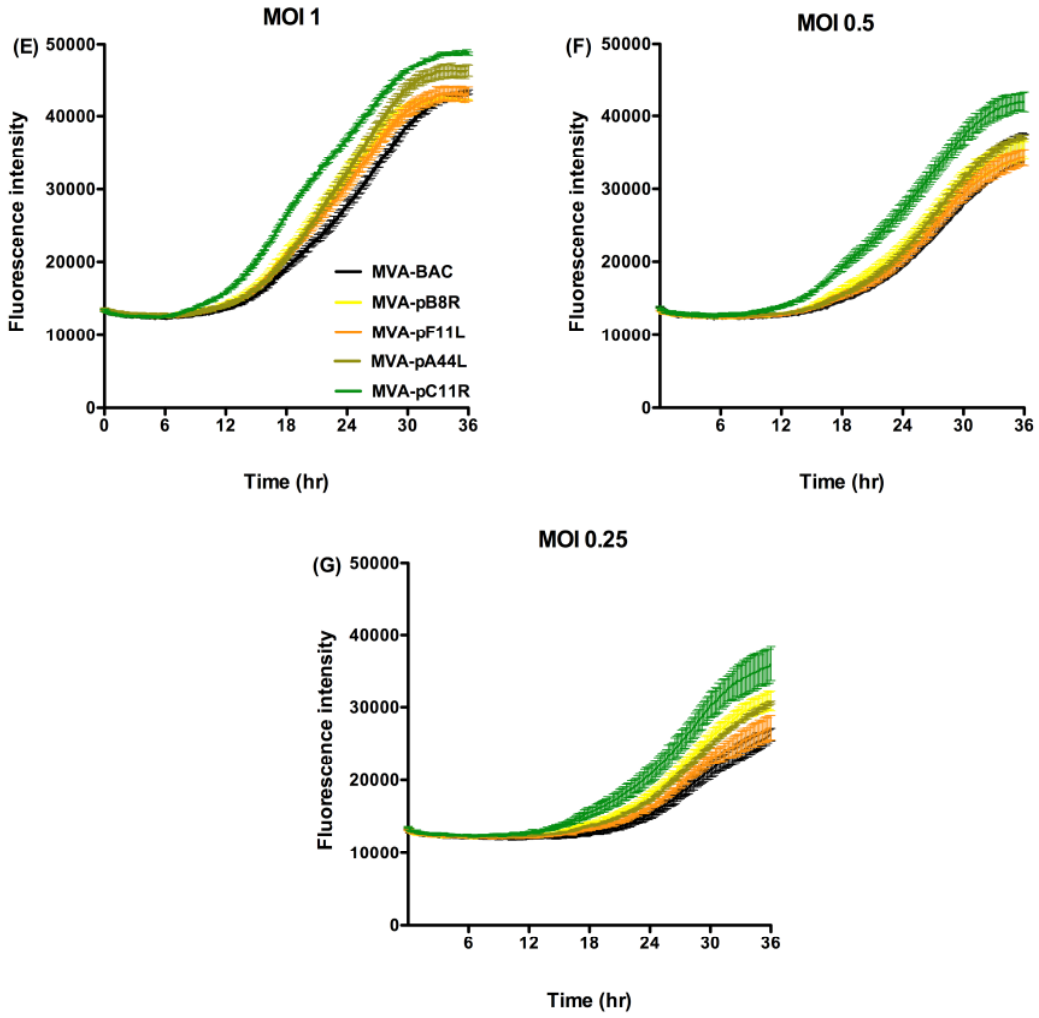


Figure 4.8: Growth rate of rMVAs expressing tPA-Pb9-rLuc8PV under the control of the indicated MVA endogenous promoters and in comparison to MVA lacking the tPA-Pb9-rLuc8PV but containing the GFP marker gene (nrMVA).

BHK cells were infected with the rMVAs (A – D) at 1 (green), 0.5 (yellow) or 0.25 (tangerine) pfu/cell and fluorescence of viral GFP marker gene was quantified every 6 minutes for 36 h using a BMG FluorSTAR equipped with 37 ^{o}C and 5% CO2 incubation. E, F and G are showing infection at the indicated multiplicities of infection with MVA endogenous promoters in comparison to MVA lacking the tPA-Pb9-rLuc8PV. The data shown are representative of 3 different experiments and the mean of two replicates with error bars showing standard error of the mean.

4.2.10 Genetic stability of rMVA with transgenes inserted at endogenous poxviral driven insertion loci

To investigate the stability of these novel insertion loci, the four rMVAs employing the pB8R, pF11L, pA44L and C11R were subjected to ten rounds of serial passages in CEFs. Crude viral stock from passage 1 and 10 were analyzed by PCR to confirm the insert was intact at the beginning and at end of the passage (Figure 4.9), using the same PCR assays as were used at the bacterial stage. To investigate whether each rMVA sustained expression of tPA-Pb9-rLuc8PV, crude viral stocks from passage 10 were titred and used to infect CEF cells at low multiplicity of infection before flow sorting into microtitre plate. Viruses were amplified by addition of BHK cells before quantification of rLuc in uninfected versus infected wells, which were differentiated via the viral GFP maker gene. rMVA employing the pB8R had almost equal numbers of uninfected (GFP+) and infected GFP – GFP negative) wells, while the remaining three rMVAs employing pF11L, pA44L and pC11R had < 25% GFP – wells. All rMVAs had > 50% GFP+ wells (Table 4.4), which contained detectable rLuc (Figure 4.10) indicating that the novel insertion sites are genetically stable.

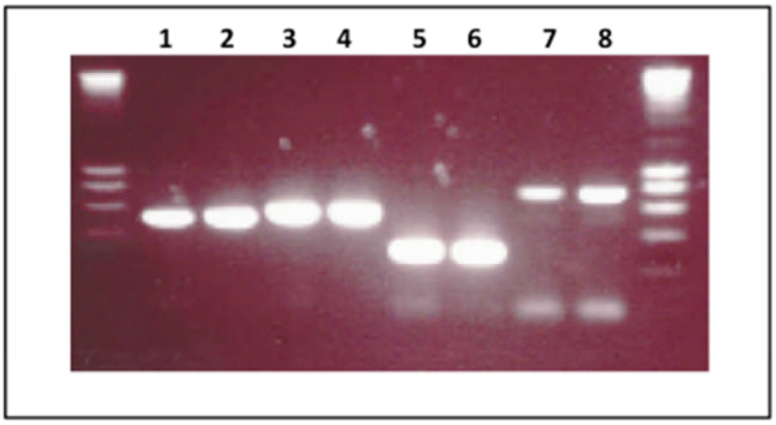


Figure 4.9: PCR to confirm insert was intact at passage 1 and 10.

PCR was carried out with crude virus stock at the beginning of the passage (passage 1) and at the end of the passage using same primers as were used in the bacterial stage (M+M section 2.8.3). Lanes 1 and 2 represent passage 1 and 10 for *B8R* (506bp). Lanes 3 and 4 represent passage 1 and 10 for *F11L* (548bp). Lanes 5 and 6 represent passage 1 and 10 for *A44L* (295bp), while Lanes 7 and 8 represent passage 1 and 10 for *C11R* (742bp).

Promoter	**GFP+ Luc+**	**GFP- Luc-**
pB8R	53/53	42/42
pF11L	60/60	23/23
pA44L	72/72	22/22
pC11R	**72/72**	**24/24**

Table 4.4: Well scores for all viruses.

Wells were scored for GFP+ and GFP-. Wells that were GFP+ expressed luciferase (Luc+), while wells that were GFP- did not express luciferase (Luc-)

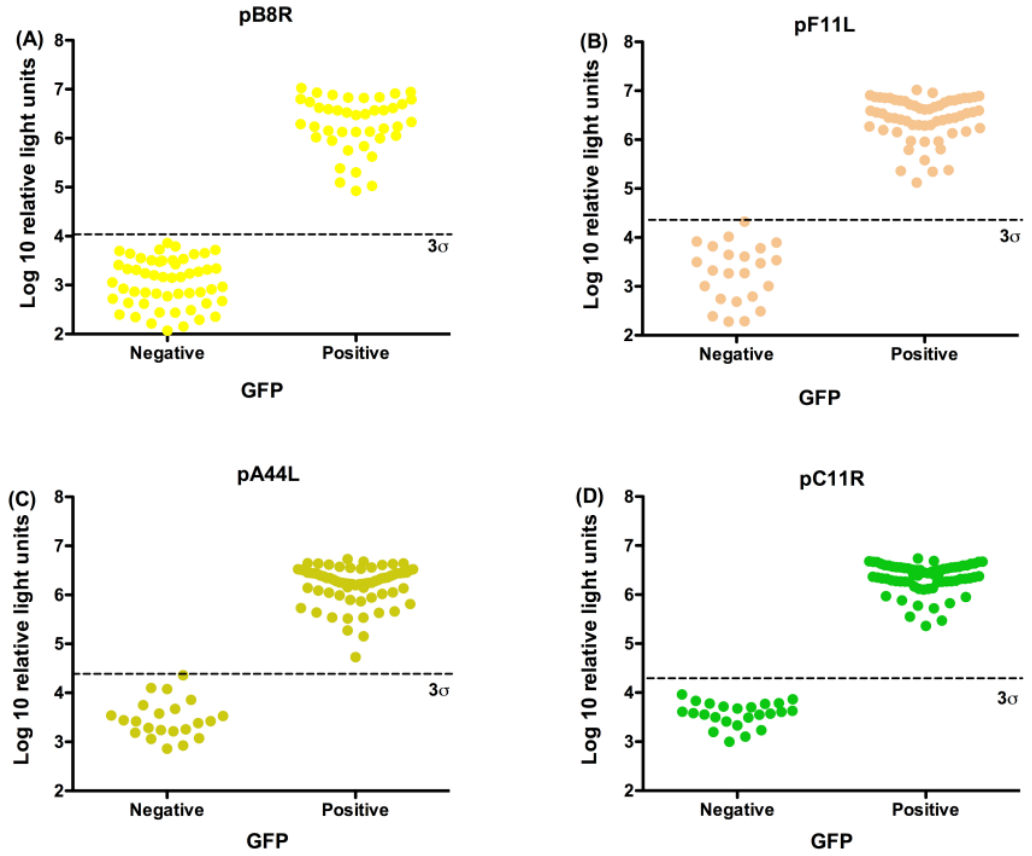


Figure 4.10: Genetic stability of rMVA expressing tPA-Pb9-rLuc8PV under control of endogenous promoters.

Viruses were subjected to ten serial passages in CEFs, titred, and inoculated onto BHK cells at 0.001 pfu/cell. After 2 days, the cells were harvested and individually sorted into the wells of a 96-well plate using the CyCLONE attachment of a MoFlo flow cytometer. Two days later, renilla luciferase activity in the cell lysates was determined after scoring of wells as positive (+) or negative (-) for the viral GFP marker gene, indicating infection in the well. A cut-off of three standard deviations above the geometric mean of the GFP (dashed line labelled 3σ) was used to score GFP+ and GFP- wells luciferase positive (Luc+) or negative (Luc-). Wells in which cell monolayers were lost during processing were excluded. Raw data for promoters (Figures A - D).

4.3 Discussion

Building on the results of the proof of principle study – Chapter 3 and studies from published literatures, six additional (*F11L, K6L, A44L, E5R, B2R, C11R*) non-essential poxviral genes as well as *B8R* were selected for replacement with the ORF of the optimized model antigen - tPA-Pb9-rLuc8PV. The robust BAC technology was employed to replace the ORF of these genes with that of the tPA-Pb9-rLuc8PV for expression by their endogenous promoters. The activities of each promoter were assessed by protein expression for 4 hours. The kinetic analysis of these promoters was also analyzed over 24 hpi. The rMVAs employing pB8R, pF11L, pA44L and pC11R were further assessed for genetic stability and growth. Early protein expression for pB8R, pF11L, pA44L, pC11R were similar to that achieved by traditional p7.5 and SSP promoters both expressing the optimized model antigen and inserted at the TK locus. Three of the seven endogenous promoters, pK6L, pE5R and pB2R did not express protein comparable to other endogenous promoters (Figure 4.6 A and B). Analysis of the promoter sequences of pK6L and pB2R revealed that there was mis-positioning of the model antigen owing to expression from an inferior ATG codon that was overlooked at the time of selection (Table 4.2). Sequence analysis of the MVA orthologs of *K6L* and *B2R* showed their ORFs are fragmented in MVA that arose from an upstream frame-shift, such that the authentic ATG initiates a severely truncated ORF.

The kinetic patterns of all seven endogenous promoters were investigated further for 24 hours and rLuc expression for pB8R, pF11L, pA44L, pC11R resembled that of traditional p7.5 and SSP promoters when post-replicative gene expression was inhibited. However, two of the seven promoters, pB2R and pE5R showed promoter

activity that resembled that of intermediate promoters (compare legend from 1 to 8 hpi in Figure 4.6 A and B). Assarsson *et al*. (136) classified *E5R* and *B2R* as immediate early gene and most recently into E1.1 and E1.2 clusters (166), so the endogenous promoters of both genes were expected to express protein in similar kinetic pattern to other endogenous promoters. Analysis of their promoter sequences showed they are both A+T rich, which is suggestive of intermediate promoter that have been reported to carry an A+T rich upstream element (137), but we did not take this further to establish any intermediate promoter activities.

During the purification stage (M+M section 2.7), all rescued viruses (conversion of MVA-BAC to infectious viruses) were subjected to two rounds of plaque purifications to obtain pure viral clones, but it was difficult to obtain pure viral clones from viruses employing the *E5R* and *B2R* promoters. To overcome this impurity, the viruses were subjected to six additional rounds of plaque purification making a total of eight plaque purifications. This problem was attributed to (i) contamination with unrecombined MVA (ii) functionality of the genes which are unknown (iii) their large sizes, *E5R* (45kDa) and B2R (57kDa) (168, 179) which could have affected deletion. In VACV, the function of *B2R* is unknown (Table 4.1) while the *E5R* is reported to have 30bp deletion in MVA.

Timecourse assessment of the kinetic pattern of the endogenous promoters showed that there were proteins readily detected in the viral preparations. It was first hypothesized that the proteins were either unbound rLuc or incorporated in the virions. A spinoculation and washing protocol was applied to understand this in great details because previous studies have shown that application of spinoculation enhances viral infection and allows assessment of virions that are associated with

cells (195). Application of the spinoculation step removed unbound rLuc by three logs and allowed "real time" kinetic assessment of the endogenous promoters (Figure 4.7 A and B). This observation has several implications for recombinant proteins expressed with viral vectors (i) immunisation with recombinant proteins in combination with viral vectors expressing the same recombinant protein could enhance immunogenicity (ii) that humoral immunity generated with recombinant viral vectors might not necessarily be exclusive to transgene expressed by the viral vector but soluble proteins in the viral preparations or both (iii) immunogenicity studies to assess soluble proteins in viral preparations should be considered. However, this is the first time recombinant proteins have been reported in viral vectored vaccines in the laboratory so these findings might be exclusive to the rLuc protein. Though the quantity of the rLuc found in the viral preparations were not verified, so it is possible that these were just traces rather than abundance of proteins.

In addition, previous studies have shown that treatment of viral vectored vaccines with psolaren and ultraviolet light (UV) inactivates viral replication without abolishing the ability of the virus to infect cells (196). This procedure could be harnessed to inactivate an rMVA expressing a transgene such as the tPA-Pb9-rLuc8PV in order to assess immunogenicity of soluble proteins in the viral preparations.

It was first considered that deletion with concomitant insertion of tPA-Pb9-rLuc8PV might affect viral growth. The growth rate of rMVAs employing the *B8R, F11L, A44L* and *C11R* promoters were assessed in comparison to BAC derived MVA lacking the tPA-Pb9-rLuc8PV (nrMVA) and all four rMVAs showed equivalent growth rate similar to that achieved by the nrMVA. However, rMVA employing the *C11R* endogenous promoter showed a slightly higher growth pattern (Figures 4.8 E, F

and G). Secondly, it was postulated that expression from these novel insertion loci might affect genetic stability. In addition to the strength of these endogenous promoters we also considered the stability of the insertion loci because studies have shown that genes that are located in the conserved regions of poxviruses are more stable than those located nearer to the terminal regions (168). The positions of the insertion loci of *B8R* and *A44L*, which are located in the right terminal region, the *C11R* located in the left terminal region and *F11L* in the conserved region did not affect the stability of the rMVAs after ten passages (Figures 4.9 and 4.10).

The data represented here demonstrates the generation of four endogenous promoters (pB8R, pF11L, pA44L and pC11R) in MVA and showed that rMVAs employing the four promoters were capable of driving expression of a model antigen equivalent to or exceeding the traditional p7.5 and SSP recombinants in terms of protein expression. We also showed that the endogenous driven loci of these four genes are genetically stable for transgene expression and that deletion with concomitant insertion did not impair viral growth.

CHAPTER 5

IMMUNOGENICITY OF RECOMBINANT ANTIGEN DRIVEN BY ENDOGENOUS PROMOTERS

5 Immunogenicity of recombinant antigen driven by endogenous promoters

5.1 Introduction

Induction of $CD8^+$ T cells against recombinant antigens from pathogens that cause infectious diseases is becoming more attractive in the field of vaccinology. Viral vectored vaccines such as recombinant modified vaccinia virus Ankara (rMVA) encoding antigen (Ag) to which a $CD8^+$ T cells is required have shown promise both in pre-clinical and clinical trials for infectious diseases such as malaria (89), HIV/AIDS (101) and influenza (9, 197, 198). In addition rMVA can also elicit humoral immunity to pathogens that require potent antibody responses (58).

Despite these promising results, rMVA vaccines still faces major obstacles in generating robust $CD8^+$ T cells because of competition for $CD8^+$ T cells (199) or a phenomenon known as "immunodominance" (144, 200). *In vitro* and *in vivo* studies have been carried out to demonstrate that it is possible to overcome immunodominance in order to improve $CD8^+$ T cells to a minigene by employing UV/psoralen treatment to reduce vector gene expression (201) or immunisation with different vectors encoding the same recombinant antigen such as "prime-boost" – prime with an adenovirus and boosting with MVA (59, 69, 89).

The mechanism underlying induction of $CD8^+$ T cells by MVA is well characterized and studies have shown that MVA preferentially infect professional antigen presenting cells (APCs) such as dendritic cells (DCs). *In vitro* studies have also shown that when MVA infects DCs only viral early genes are expressed and the viral life cycle is arrested before late gene expression occurs (146, 147).

As discussed previously, a study involving expression of the human Her-2/neu antigen (147) driven by the p7.5 promoter also showed that MVA infection of human DC is restricted to the production of viral early proteins. The study reported that analysis of early and late viral proteins on immature DC (iDC) and mature DC (mDC) showed that only early viral polypeptides were made and that late viral proteins were not detectable on either iDC or mDC.

Since only viral early genes are expressed in DC we reasoned that the endogenous promoters of these newly identified early or immediate-early genes (*B8R, F11L, A44L and C11R*) in their authentic location might be able to drive expression of recombinant protein *in vivo* to elicit $CD8^+$ T cells exceeding or equal to the traditional p7.5 and SSP promoters.

This chapter explores whether poxviral promoters in their authentic location would elicit potent $CD8^+$ T cell equal to the p7.5 and SSP promoters. It also seek to explore whether the immediate early promoter kinetics hypothesized for these endogenous promoters might have any preferential advantage in generating $CD8^+$ T cell because it is possible that a shift from early (early promoters) to very early times (immediate-early promoters) might favor rapid generation and expansion of $CD8^+$ T cells to foreign antigens because antigens might be presented to DC faster. Though the difference between early and immediate-early might be small, it is possible that if antigens are presented to $CD8^+$ T cells very early (immediate-early) after infection, it might facilitate rapid control and clearance of infection. This chapter will further investigate whether the rMVAs employing these endogenous poxviral promoters are good at boosting Pb9-specific $CD8^+$ T cells in three doses of rMVA immunisation. As previously mentioned, when MVA was used as the boosting vector, it was capable of

inducing protective ME-TRAP-specific $CD8^+$ T cells against malaria in a DNA-MVA regime (91) and in FP9-MVA regime (52, 71). MVA has been shown to boost protective Ag85-specific $CD8^+$ T cells against TB when used in BCG-MVA regime (8). In addition, MVA has also been shown to boost natural immunity by inducing protective NP+M1-specific $CD8^+$ T cells against influenza A virus challenge (11).

5.2 Results

5.2.1 Endogenous poxviral promoters elicit potent CD8$^+$ T cells after single-shot MVA

The Pb9 epitope fused to the N-terminus of the model antigen tPA-Pb9-rLuc8PV was used to determine the ability of viruses employing the pB8R, pF11L, pA44L and pC11R promoters to assess Pb9-specific T cell responses in comparison to the conventional p7.5 and SSP promoter driven recombinants. Seven days post immunisation with 10^6 pfu/mouse of rMVA, splenocytes were assayed for the frequency of IFN-γ from CD8$^+$ T cells by either intracellular cytokine staining (ICS) (Fig 5.1A) or ELISpot (Fig 5.1B). In the IFN-γ ELISpot readout viruses employing the four endogenous promoters elicited potent Pb9-specific CD8$^+$ T cells which were indistinguishable from the comparator p7.5 and SSP promoters and no significant difference was observed between the groups ($p = 0.08$ by one way ANoVA). However, in the ICS readout (Figure 5.1A), the frequencies of Pb9-specific CD8$^+$ T cells elicited by pF11L and pC11R was statistically significantly higher than pA44L but not p7.5 and SSP ($p = 0.05$ by one way ANoVA).

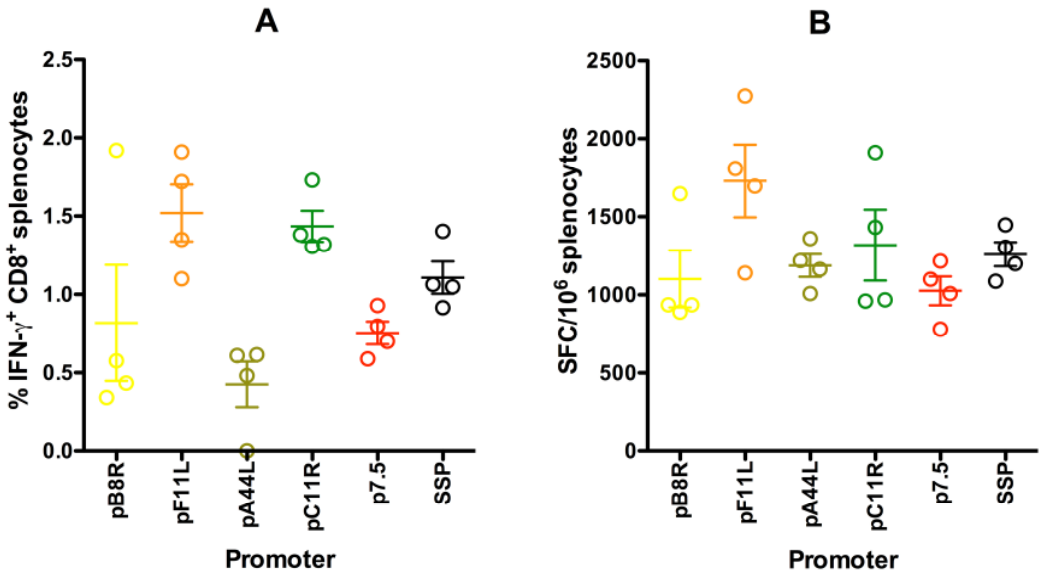


Figure 5.1: Cellular immunogenicity in mice immunised with recombinant MVA antigen (tPA-Pb9-rLuc8PV) driven by endogenous promoters compared to p7.5 and SSP.

Six groups of female Balb/c mice (n = 4) were immunised intramuscularly (i.m) with 10^6 pfu/mouse rMVA. Seven post immunisation, spleenic $CD8^+$ T cell responses to Pb9 were determined by (A) intracellular cytokine staining (ICS) and flow cytometry or (B) IFN-γ ELISpot. Circles represent Pb9-specific $CD8^+$ T cell responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is representative of four independent experiments.

5.2.2 Homologous prime-boost regimen to observe differences between endogenous promoter hierarchy

Next was to investigate $CD8^+$ T cell responses against recombinant tPA-Pb9-rLuc8PV construct expressed under the control of pB8R, pF11L, pA44L and pC11R promoters in Balb/c mice after one, two and three immunisations with 10^6 pfu/mouse of rMVAs. The rational for this was because previous studies have shown that vaccination strategy employing rMVA in a homologous prime-boost regimen can

shape promoter hierarchy (178). Mice were bled at week 4, 8 and 12 following vaccination at week 0, 4 and 8. The Pb9-specific $CD8^+$ T cells responses were determined by intracellular cytokine staining (ICS) for IFN-γ, IL-2 and TNF-α after stimulation with the H2-k^d restricted, Pb9 peptide – SYIPSAEKI. Figure 5.2 shows the gating strategy and representative ICS staining of Pb9-specifis $CD8^+$ T cell. Four weeks after the first immunisation we observed similar proportions Pb9-specific $CD8^+$ T cells regardless of the promoter used (Figure 5.3).

When the Pb9-specific $CD8^+$ T cells were analyzed for the three cytokines after one, two and three immunisation, we found that the proportion of $CD8^+$IFN-γ+ T cells induced by *F11L, A44L* and *C11R* promoters increased only after the second immunisation and did not increase after the third immunisation. The trend was the same for $CD8^+$IL-2+ T cells and even decreased for $CD8^+$TNF-α+ T after the third immunisation. Interestingly, the proportion of $CD8^+$IFN-γ+ T cells induced by virus employing the *B8R* promoter increased by four fold after the second immunisation and by another three fold after the third immunisation,making a total of seven fold increase between the first and third immunisation. In fact, the trend of $CD8^+$IL-2+ T cells and $CD8^+$TNF-α+ T cells observed between the first and third immunisation was similar to that observed in the $CD8^+$IFN-γ+ T cells populations. These data shows that after the third immunisation, virus employing the *B8R* induced a slightly higher proportion of Pb9-specific T cells than the other three promoters (*F11L, A44L* and *C11R*), though the difference was not statistically significant ($p = 0.05$ by Repeated measures of One way ANoVA).

The proportion of cells secreting more than one cytokine were further assessed and the biggest percentage of $CD8^+$ cells were single positive IFN-γ and double-positives

IFN-γ+TNF-α+ cells (Figure 5.4). While there was no triple positive cell population, the IL-2 population was slightly above background level. Virus employing the C11R promoter secreted the highest proportion double positive cells while the virus employing the B8R promoter secreted the highest proportion of single positive cell population.

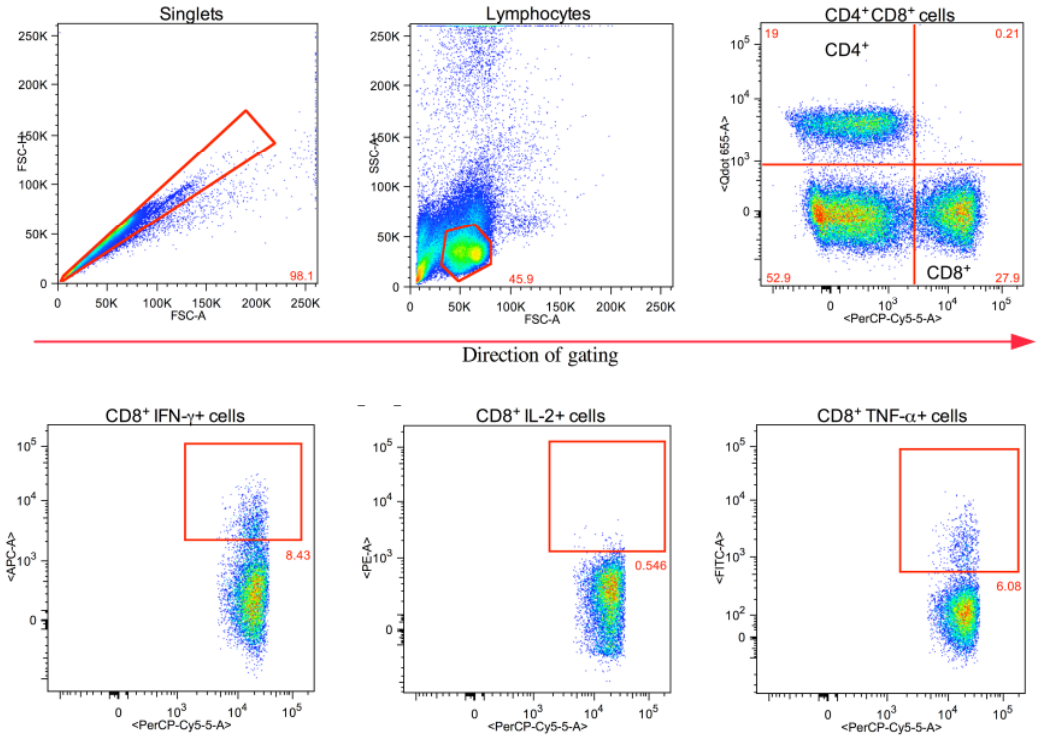


Figure 5.2: Gating strategy to identify CD8+ T cells

Balb/c mice (n = 4/group) were immunised i.m at week 0, 4 and 8, with 10^6 pfu/mouse of rMVAs expressing the model antigen (tPA-Pb9-rLuc8PV) under the control of pB8R, pF11L, pA44L and pC11R promoters. At week 4, 8 and 12 blood was collected from the tail vain of each mice and PBMC surfaced stained as described in (M+M section 2.8.4.8). The sequential gating strategy is shown above. Gates show the percentage of parent.

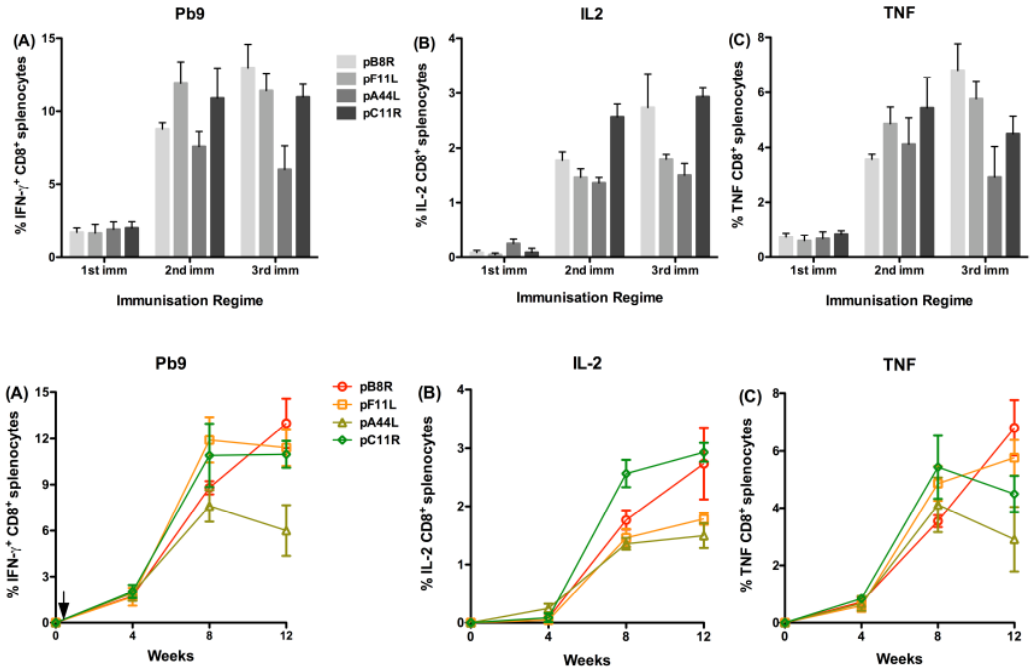


Figure 5.3: Cellular immunogenicity in homologous prime-boost immunisation regime.

$CD8^+$ T cells against the recombinant antigen – Pb9, expressed under the control of pB8R, pF11L, pA44L and pC11R promoters were determined in Balb/c mice after one, two and three immunisations with 10^6pfu/mouse of rMVAs. Pb9-specific $CD8^+$ T cells were determined by ICS for IFN-γ (**A**), IL-2 (**B**) and TNF-α (**C**). Top and bottom panels are the same experiment – represented in bar chart **top**, connecting line bottom panel. Each data point, bar or symbol represents responses of each group of mice, with error bars showing SEM. Data shown is a representative of one experiment.

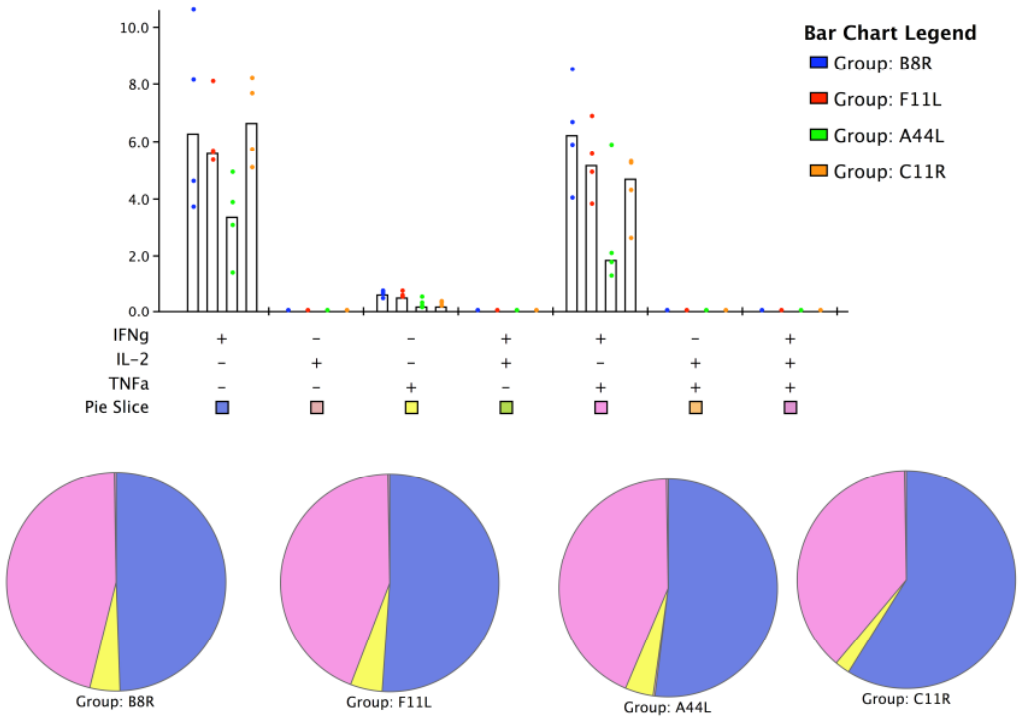


Figure 5.4: Polyfunctionality of T cell responses following homologous prime-boost immunisation with rMVAs.

$CD8^+$ T cells against the recombinant antigen – Pb9, expressed under the control of pB8R, pF11L, pA44L and pC11R promoters were determined in Balb/c mice after one, two and three immunisations with 10^6pfu/mouse of rMVAs. Pb9-specific $CD8^+$ T cells determined by ICS is shown for IFN-γ, IL-2 and TNF-α. Pie charts show the proportion of cells producing 1, 2 or all 3 cytokines

5.2.3 Heterologous prime-boost vaccination enhanced $CD8^+$ T cells

An heterologous prime-boost immunisation regimen was employed to measure the frequencies of Pb9-specific $CD8^+$ T cell responses because previous studies have shown that vaccination strategy employing an recombinant adenoviral vector prime followed by 8 week rMVA boost is capable of initiating prompt recall response to the recombinant antigen. In addition, the prime-boost immunisation have be shown to expand the frequencies of $CD8^+$ T cells in mice (93), monkeys (59) and humans ((12).

To investigate production of IFN-y from CD8+ T cells after prime-boost immunisation regime, a chimpanzee adenovirus serotype 63 (ChAd63) expressing recombinant TIP antigen [TIP contains Pb9 epitope as described in details in M+M section 2.4.1] was used to prime because recent ChAd63-MVA prime-boost trials have elicited the highest magnitudes of $CD8^+$ T cells ever reported in humans (94). Two weeks post ChAd63-TIP prime, mouse PBMCs were stimulated with Pb9 peptide and analyzed by ELISpot. Pb9-specific $CD8^+$ T cells were detectable in all animals (data not shown). Eight weeks after prime, rMVAs expressing model antigen (tPA-Pb9-rLuc8PV) under the control of the four endogenous poxviral promoters (pB8R, pF11L, pA44L and pC11R) and the comparator p7.5 and SSP were used to boost. Two weeks after the boost, splenocytes were assayed by IFN-γ ELISpot for the production of Pb9-specific $CD8^+$ T cells. Recombinant MVAs employing the pB8R, pF11L, pA44L and pC11R were capable of boosting Pb9-specific CD8+ T cells to levels equivalent to the comparator p7.5 promoter (Figure 5.4). Surprisingly, SSP elicited the lowest $CD8^+$ T cell and was statistically significant worse than pC11R ($P = 0.04$ by one-way ANoVA). These data shows that the four endogenous viral

promoters are capable of boosting recombinant antigens as well as traditional p7.5 and SSP promoters.

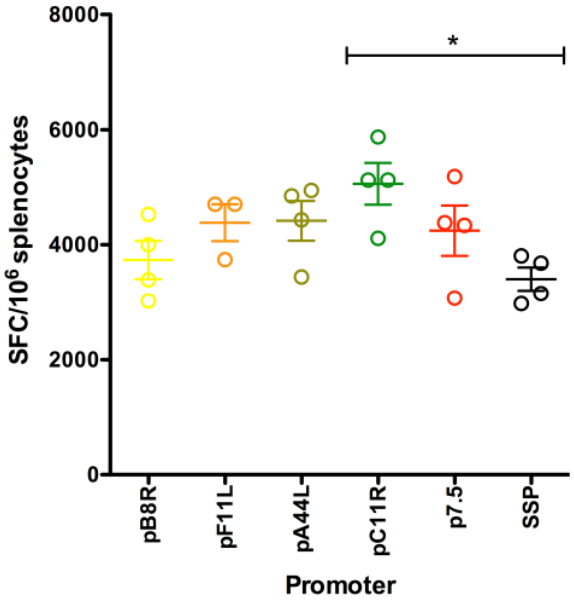


Figure 5.5: Cellular immunogenicity in heterologous prime-boost immunisation regime.

Six groups (n=4) of mice were immunised intramuscularly (i.m) with 10^8 infectious units of AdCh63-TIP and 56 days (8 weeks) later boosted with 10^6 pfu/mouse rMVA. At day 70 (2 weeks post-boost), splenic CD8+ T cell responses to Pb9 peptide were determined by IFN-γ ELISpot. Circles represent Pb9-specific $CD8^+$ T cell responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is representative of four independent experiment.

5.3 Discussion

Recombinant MVAs employing the four endogenous promoters (pB8R, pF11L, pA44L and pC11R) and the comparator p7.5 and SSP promoters were assessed for their ability to elicit cellular and humoral immune responses against the model antigen – tPA-Pb9-rLuc8PV. The frequencies of Pb9-specific $CD8^+$ T cell were determined by IFN-γ ELISpot and intracellular cytokine staining (ICS), and antibodies profiled by ELISA and Luciferase Immunoprecipation Systems Assay (LIPS) (162). To determine the frequencies of $CD8^+$ T cells mice were either vaccinated in a single-dose rMVA, homologous prime-boost rMVA regime or heterologous Ad-MVA prime-boost vaccination regime. Splenocytes were assessed for the production of IFN-γ from $CD8^+$ T cells and mouse serum assessed for antibodies.

Viruses employing the four endogenous promoters (pB8R, pF11L, pA44L and pC11R) and comparator p7.5 and SSP recombinants were capable of eliciting potent $CD8^+$ T cells to the recombinant antigen *in vivo* as demonstrated in single-dose MVA regime, homologous prime-boost rMVAs or as a boost after priming with an adenoviral vector.

Based on the results from the all three experiments, single dose MVA, homologous prime-boost and heterologous prime-boost vaccination regimen, it is difficult to rank the promoters based because there alteration in the rank order between these experiments. In the single MVA vaccination regimen pF11L and pC11R were the highest while pB8R and pA44L the lowest. In the homologous prime-boost vaccination regimen pB8R and pC11R were the highest while pF11L and pA44L were the least. In heterologous Ad-MVA prime-boost vaccination regimen the rank

pC11R and pF11L were the highest while pA44L and pB8R took the least positions. (The rank order has been used here based on the actual count of Pb9-specific $CD8^+$ T cell rather that statistical significance). Overall, pA44L was the least in two of these experiments while the pC11R promoter was consistently at the top position in all three experiments and assumed to be the strongest.

Antibodies to the rLuc protein were assessed in the heterologous Ad-MVA prime-boost immunisation regime and the four endogenous promoters (pB8R, pF11L, pA44L and pC11R) were capable of inducing antibodies in the LIPS assay readout. However, this result is inconclusive as it was assumed that the LIPS assay might be less sensitive compared to a more sensitive assay such as ELISA so we employed an ELISA assay to confirm this result. In addition, there was no proper positive control for the LIPS assay so the result remains inconclusive.

Assessment of anti-rLuc antibodies with an ELISA assay employing a laboratory made rLuc protein showed that viruses employing the four endogenous promoters induced antibodies equivalent to p7.5 but were significant lower than SSP ($p = 0.005$ by one-way ANoVA). Several attempts to reproduce the data obtained with ELISA assay were unsuccessful because of (i) aggregation of the laboratory made recombinant rLuc protein (ii) rLuc protein was not commercially available at the time of the setting up the experiment. However, the humoral immunogenicity therefore remains to be fully characterized because the main aim was to characterise $CD8^+$ T cells.

Data presented here shows that rMVAs employing (pB8R, pF11L, pA44L and pC11R) promoters in their authentic location drove expression of a model antigen (tPA-Pb9-rLuc8PV) and elicited Pb9-specific $CD8^+$ T cells similar to or exceeding

that generated by the traditional p7.5 and SSP promoters. rMVAs employing these promoters were good at boosting Pb9-specific $CD8^+$ T cells already primed with rMVA or ChAd-63 adenovirus vector expressing the same antigen – tPA-Pb9-rLuc8PV.

CHAPTER 6

GENERATION AND IMMUNOGENICITY OF BIVALENT MVA VACCINE EXPRESSING TWO ANTIGENS

6 Generation and immunogenicity of bivalent MVA vaccine expressing two luciferase antigens

6.1 Introduction

The ability of recombinant vaccinia virus to express multiple antigens from different pathogens was first demonstrated in the 1980s (202, 203). In one of these studies (202) the coding sequences for hepatitis B virus surface antigen (HBsAg), herpes simplex virus glycoprotein D (HSV), and the influenza virus hemagglutinin (InfHA), were expressed by VACV under three different promoters from three different insertion sites. The study reported that the vaccine induced antibodies against the three recombinants antigens in rabbits. Since then, many studies have employed this technology with different strains of vaccinia virus for vaccine against malaria (85, 90, 204), HIV/AIDS (101, 205) and influenza virus (206).

The rationale for designing a recombinant poxviral vaccine expressing multiple antigens either against a single pathogen or multiple pathogens is to generate broad effective immune responses to the recombinant antigens *in vivo*. The malaria parasite is an example of a pathogen that requires a multiantigen vaccine because of the different stages of the parasites lifecycle, which occurs in the liver, blood and in the mosquito (sexual stage). A previous multiantigen, multistage malaria vaccine used the NYVAC strain (85) to express seven *P. falciparum* malaria antigens (NYVAC-Pf7) targeting the three stages of the plasmodia lifecycle. The seven antigens were expressed under seven different poxviral promoters from four different insertion sites. The vaccine induced antibodies to only four (CSP, SSP2, MSP-1 and Pf25) of the seven antigens, though the total antibody responses achieved with the entire NYVAC-

Pf7 vaccine was significantly lower than that achieved with a single recombinant antigen.

In another study (204) employing a polyprotein (L3SEPTL). The vaccine was designed based on six malaria antigens (liver stage antigen 1 (LSA), liver stage antigen 3 (LSA3), sporozoite threonine and asparigine rich protein (STRARP), exported protein (Exp1), Pfs16 and the thrombospodin related adhesion protein (TRAP). A sequence coding the six antigens, L3SEPTL, was expressed by a plasmid DNA vector and by two attenuated poxviral vectors, FP9 and MVA, under the control of the early/late p7.5 promoter. The study concluded that only two of the antigens (TRAP and LSA) were boosted by the full-length rMVA polyprotein when primed with single antigen TRAP- or LSA1-expressing DNAs. This study also reported that heterologous prime-boost regimen involving the two viral vectors expressing the full-length polyprotein (FP9 prime followed by MVA boost) resulted only in the induction of anti-LSA $CD8^+$ T cells.

Recommendations from these studies highlighted the importance of testing the immunogenicity of each antigenic component for their individual contribution to vaccine efficacy and that combination of subunit vaccines based on single antigens might be more effective than a multiantigen vaccine approach. This hypothesis was recently explored in a *Plasmodium yoelli* (Py) mouse malaria study by co-administering rMVAs expressing the circumsporozoite protein (CSP) and the 42-kDa C terminal domain of the merozoite surface protein 1 ($MSP\text{-}1_{42}$) in an adenoviral vector prime and MVA boost (Ad-MVA) immunisation regimen (207). The study reported that there was competition for $CD8^+$ T cells after the MVA boost when the vectors were co-administered as a mixture but competition was eliminated when the

vectors were given at separate sites. The study concluded that antigenic competition for $CD8^+$ T cell in the mixture group was not due to competition for antigen presentation on MHC class I molecules but partly due to lack of physiologic space for high-magnitude responses against multiple antigens. However, this explanation might not fully explain the rationale for $CD8^+$ T cell competition because previous studies have shown that $CD8^+$ T cell directed against multiple viral antigens is determined by the timing of viral gene expression in infected APCs, which could be determined by the quality of the promoter used.

There are approximately 258 predicted open reading frames ($ORFs^{ORF}$) in VACV (144) with each encoding viral proteins that competes for $CD8^+$ T cells which shows how difficult it is for foreign genes to elicit $CD8^+$ T cells when expressed in recombinant poxviral vaccines. Heterologous prime-boost immunisation strategies employing an adenovirus vector prime and MVA boost (59, 69, 89) have been employed to overcome this competition to appreciable levels because the strategy reduces the immunodominant effects of the vectored vaccine (homologous prime-boost) and directs the responses from viral antigens to the target antigen. This is because there is a pre-existing population of cells induced by the foreign antigen, which preferentially expands after the boost.

Firstly, the mathematical illustration below explains one of several possible theories of $CD8^+$ T cell competition in recombinant poxviral vaccines using VAVC WR (V) as an example:

[ORF] I have used 258 $CD8^+$ T cell epitopes to VACV just for the purpose of illustration because not all poxviral genes have been characterised to have $CD8^+$ T cell epitopes. For more details of $CD8^+$ T cell epitopes to VACV please refer to paper by Tscharke *et al* (143, 144, 208).

If V = 258 antigens (258 CD8+ T cell epitope in vaccinia virus – VACV WR)

A = Antigen A or epitope A

B = Antigen B or epitope B

Single vaccination of A = 1 copy of 258 VACV antigens + 1 copy of A

Single vaccination of B = 1 copy of 258 VACV antigens + 1 copy of B

Single vaccination of multivalent = 1 copy of 258 antigens + 1 copy A + 1 copy of B

Mixed vaccination of A + B = 2 copies of 258 antigens + 1 copy of A + 1 copy of B

Separate vaccination of A + B = 2 copies of 258 antigens + 1 copy of A + 1 copy of B

This means there are 2 $CD8^+$ T cell epitopes to antigen A and B competing with 2 copies of 258 $CD8^+$ T cell epitopes from two rVACV illustrating the amount of poxviral genes competing for $CD8^+$ T cell when two single rVACVs are co-administered as a mixture or given separate sites.

But, if antigens A + B are expressed from one rVACV there will be 1 copy of 258 poxviral epitope competing with 2 $CD8^+$ T cell epitopes to antigen A and B which might reduce competition for $CD8^+$ T cells. These illustrations demonstrate the importance of a bivalent MVA vaccine expressing two antigens and the purpose of this chapter is to test the hypothesis that bivalent will be more immunogenic than two monovalent MVA vaccines, owing to reduced antigen competition.

Most studies employing multiple antigens as vaccine candidates have overlooked the importance of multiple insertion loci and the choice of promoter in driving expression of foreign genes partly because the focus has been on immunogenicity of the recombinant antigen rather than interference with parameters of the delivery vehicle in the host such as $CD8^+$ T cell competition in VACV. A plausible strategy to overcome competition for $CD8^+$ T cell is to develop a multiantigen vaccine that will

express multiple antigens from more than one insertion loci and under the control of different promoters. Though the NYVAC Pf7 (85) employed a similar approach but in our study we aim to use endogenous poxviral promoters in their authentic location, which might preferentially transcribe foreign genes better than when a promoter is inserted exogenously.

In addition to minimising competition for $CD8^+$ T cell, a multivalent MVA vaccine will be more cost effective against infectious diseases of global health importance such as malaria, HIV/AIDS, influenza and TB where subunit vaccines have not been able to achieve sterile immunity. This is because for some pathogens such as malaria where two or more antigens are desired as vaccine candidates, it will be more cost effective to develop a multivalent MVA vaccine targeting the pre-erythrocytic, blood and sexual stages of the parasite's life cycle than developing multiple rMVAs for routine immunisation.

Secondly, a multivalent MVA vaccine in comparison to combining two or three monovalent MVA vaccines would have the potential to reduce reactogenicity, such as local reactions (pain, redness, swelling, itching, warmth and scaling) and systemic symptoms (oral temperature, feverishness, myalgia, arthralgia, nausea or vomiting, lethargy, headache and malaise) which have been reported with monovalent MVA vaccines in clinical trials in malaria (209), HIV/AIDS (7) and tuberculosis (210).

Thirdly, due to antigenic polymorphism, a multivalent MVA would be suitable for expressing different strains of a particular antigen, for example the 3D and FVO alleles of the *P. falciparum* malaria AMA-1 antigen (60). The influenza virus, which frequently undergoes antigenic drift (211, 212) is another infectious pathogen for which a multivalent MVA would be beneficial.

Lastly, it would be more advantageous to introduce a multivalent MVA (multi-disease MVA) comprising pathogens from different infectious diseases into the expanded programme on immunisation (EPI) than introduction of multiple rMVAs expressing single antigens

Data from chapter 4 shows the immunogenicity and kinetics of four poxviral endogenous promoters (pB8R, pF11L, pA44L and pC11R) that drove high early expression of the model antigens – tPA-Pb9-rLuc8PV that were indistinguishable from the traditional p7.5 and SSP promoters. These data also showed that the ORF of *B8R, F11L, A44L* and *C11R* were genetically stable for the expression of the foreign gene.

With this in mind and in addition to the BAC technology, this chapter will explore whether these newly identified poxviral promoters and insertion loci could be harnessed for the generation of a bivalent MVA vaccine. It will further explore whether a bivalent MVA expressing two recombinant antigens (renilla luciferase, tPA-Pb9-rLuc8PV and photinus luciferase, pLuc) from the insertion driven loci of two the newly identified promoters (ORF of *B8R* and *C11R*) would elicit Pb9 and pLuc-G9V$^{pLuc\text{-}G9V}$-specific CD8$^+$ T cell equal to or exceeding that generated by (i) rMVA expressing a single antigen (monovalent) (ii) mixture of two monovalent MVAs (iii) or the two monovalent MVAs given at separate sites. Antibody production against the recombinant rLuc and photinus luciferase (pLuc) proteins will also be assessed *ex vivo*.

Immunogenicity of the bivalent (double-pLuc-Pb9) in comparison to monovalent MVA vaccines (single-pLuc or single-Pb9), coadministration of two monovalent

$^{pLuc\text{-}G9V}$ pLuc-GV9 CD8$^+$ T cell epitope in Balb/c mice (GFQSMYTFV) identified in the paper by Limberis *et al*. (157).

MVAs given as mixture or at separate sites will be explored first in single-shot MVA vaccination regimes and thereafter as a boost after priming with mixing two recombinant adenoviruses. These experiments will test the hypothesis that boosting with a bivalent MVA in comparison to boosting with a combination of two monovalent MVAs reduces competition for $CD8^+$ T cells because of lesser amounts of viral antigens in the bivalent MVA.

6.2 Results

6.2.1 Selection of promoters and antigens for generation of bivalent MVA

Two of the newly identified poxviral promoters, pC11R and pB8R were chosen for the expression of tPA-Pb9-rLuc8PV and pLuc respectively because (i) immunogenicity data from chapter 5 suggest that rMVA employing the pC11R promoter elicited the highest frequency of $CD8^+$ T cell in comparison to the traditional p7.5 and SSP promoters (Figure 5.1 and 5.4) (ii) deleting the ORF of *C11R* and *B8R* with concomitant insertion of the model antigen (tPA-Pb9-rLuc8PV) did not affect the growth of the virus (Figure 4.8) (iii) the insertion driven loci of *B8R* and *C11R* were found to be genetically stable for transgene expression (Figure 4.10).

In addition, the pB8R promoter was found suitable for generation of the bivalent MVA vaccine because (i) the promoter sequence is conserved in MVA (ii) the B8R has immunodominant $CD8^+$ T cell epitope in C57BL/6 and Balb/c mice that could be turned to our advantage by replacing its ORF with a recombinant antigen under the control of its natural promoter (iii) rMVA employing the pB8R promoter elicited $CD8^+$ T cell comparable to p7.5 and SSP in single dose and Ad-MVA immunisation regimes and elicited the highest frequency of Pb9-specific $CD8^+$ T cells in the homologous prime-boost immunisation regime (Figure 5.3)

In addition to the model antigen – tPA-Pb9-rLuc8PV used for the generation of rMVAs in chapter 4 (Figure 4.1), a recombinant firefly (*Photinus pyralis*) luciferase was selected for insertion under the control of the pB8R promoter. The photinus luciferase (pLuc) was selected because $CD8^+$ T cell epitopes to the recombinant

protein has been identified *in vivo* in C57BL/6 mice (LMYRFEEEL) and in Balb/c mice (GFQSMYTFV and VPFHHGFGM, VALPHRTAC) (157) and can be easily quantified independently from rLuc. The immunodominant GFQSMYTFV $CD8^+$ T cell epitope was used in quantifying $CD8^+$ T cell induced by pLuc and referred as pLuc-G9V

6.2.2 Generation of bivalent MVA expressing two antigens

To generate rMVA expressing the tPA-Pb9-rLuc8PV and pLuc, a BAC already carrying the tPA-Pb9-rLuc8PV under the control of the pC11R promoter was engineered using the *AphAI* recombineering technology (158) to insert pLuc. Briefly, the pLuc construct comprises a photinus luciferase and the *AphAI* cassette. *AphAI* is a kanamycin resistance gene that enables selection of recombinants in *E. coli*. The application of *AphAI* recombineering instead of recycling of *GalK* was done because (i) the *AphAI* recombineering technology is faster than *GalK* recombineering (ii) recycling of *GalK* could be difficult and time consuming (iii) to explore a combination of the two recombineering technology (iv) the aim was to test the pre-clinical development of a bivalent MVA vaccine.

Generation of the bivalent MVA (Figure 6.1) was achieved by designing primers (50bp each for LHA and RHA) to insert the pLuc-AphAI construct to replace the viral ORF of *B8R*. The 5' primer was designed so that the ATG of the pLuc will replace that of the deleted viral ORF, followed by the *AphAI* selectable maker. A comparator virus expressing only one antigen (single-pLuc) under the control of the pB8R promoter was also made. The rescue of the BACs to infectious viruses, amplification and purity of the viruses was carried out exactly as described for the proof of principle study – chapter 3 (section 2.6 and 2.7). The two inserts, tPA-Pb9-rLuc8PV and pLuc

were PCR amplified and sequenced for any mutations. In two cases, a base deletion was found on the pLuc insert at position 5 (which resulted in a frame-shift) and we avoided proceeding with the virus because the deletion was found in the core region of the protein, which might result in inefficient translation of the protein *in vivo*. In subsequent viral preparations, no mutation was found and the two resulting viral preparations were titred and had yields of 3.0 x 10^{10} pfu/mL each (final volume ~0.5mL from 1500cm^2 flask area). PCR was carried out to verify identity insert and purity of the bivalent and single-pLuc rMVAs (Figure 6.2) (M+M section 2.6.5.1 using primers in Table 2.2, 2.3).

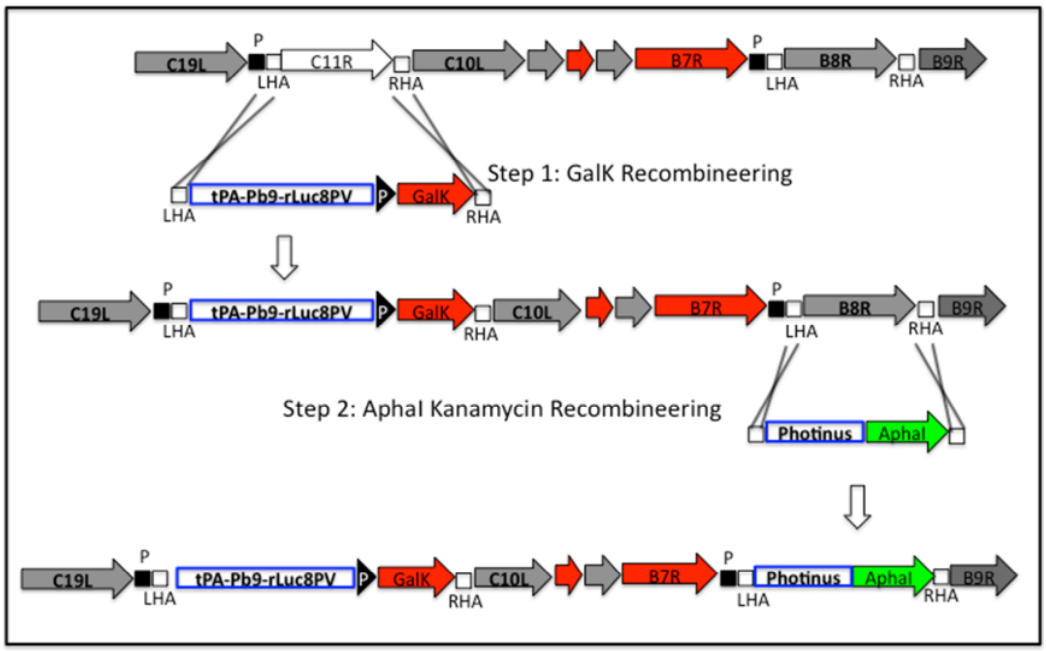


Figure 6.1: Schematic of bivalent MVA vaccine expressing two antigens under the control of the pC11R and pB8R promoters.

Step 1: Deletion of *C11R* with concomitant insertion of the model antigen, tPA-Pb9-rLuc8PV, was done exactly as described in chapter 4. **Step 2:** The BAC now carrying the tPA-Pb9-rLuc8PV was modified by kanamycin recombineering (158) to delete the *B8R* ORF with concomitant insertion of photinus luciferase (pLuc) antigen. The 5'

end of the primer (LHA) was designed to replace the initiator ATG codon of pLuc with that of the *B8R* ORF. After recombineering the pLuc was placed under control of the *B8R* promoter (black solid box "p"). The next step was to select positive clones on kanamycin agar plates followed by virus rescue, plaque purification and propagation.

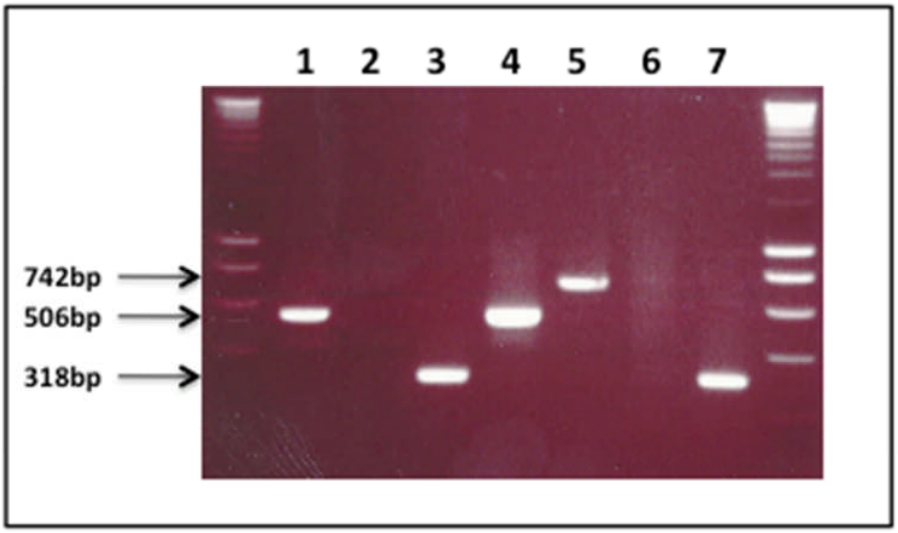


Figure 6.2: PCR to confirm identity and purity of bivalent MVA.

Insert specific primers for Screen-AphA-1 and Screen-B8R, purity primers for B8R and C11R primers (M+M Table 2.3 and 2.3) were used to run a PCR to confirm the presence of the insert and purity of virus. Lane 1 confirms the pLuc insert and 2 confirms the purity of the single-pLuc recombinant. Lane 3 is a positive control. Lane 4 confirms the pLuc insert in the bivalent MVA, while lane 5 confirms the tPA-Pb9-rLuc8PV insert in the bivalent MVA. Lane 6 confirms the purity of the bivalent MVA, while lane 7 is a positive control.

6.2.3 Immunogenicity of the bivalent MVA vaccine

The immunogenicity of the bivalent MVA vaccine (double-pLuc-Pb9 (Double x 1)) was assessed by Pb9 and pLuc-G9V-specific $CD8^+$ T cells elicited by the recombinant constructs (tPA-Pb9-rLuc8PV and pLuc) *in vivo*. The Pb9 $CD8^+$ T cell epitope is (SYIPSAEKI) and the pLuc-G9V epitope (GFQSMYTFV). Both these epitopes are H2-K^d restricted in Balb/c mice (157, 213).

To investigate whether pLuc-G9V or Pb9-specific $CD8^+$ T cell generated by the bivalent MVA vaccine equal or are inferior to those generated by the two comparator monovalent MVA vaccines (rMVA-pB8R-pLuc and rMVA-pC11R-tPA-Pb9-rLuc8PV referred to as single-pLuc and single-Pb9 respectively). Seven days after vaccination with 10^6 pfu/mouse of rMVAs (Table 6.1), splenocytes were assayed for frequencies of Pb9 and pLuc-G9V-specific $CD8^+$ T cells by IFN-γ ELISpot (Figure 6.3). In the double-pLuc-Pb9 group ((Double x 1) refer to Table 6.1), $CD8^+$ T cell responses were measured against both Pb9 and pLuc-G9V peptides, while in the single recombinant groups $CD8^+$ T cells was measured against either the Pb9 or pLuc-G9V peptide. The frequencies of pLuc-G9V and Pb9 specific responses elicited by the double-pLuc-Pb9 were indistinguishable from the single recombinants (Figure 6.3). Despite the robust $CD8^+$ T cell elicited by the double-pLuc-Pb9, it appears that the $CD8^+$ T cell responses are skewed towards the immunodominant peptide epitope, Pb9, while the pLuc-G9V appears to be sub-dominant.

S/N	Groups	Total Dose (MVA) (pfu/mouse)	Total Dose (Antigen)	Left Leg	Right Leg
1	Single-pLuc	1×10^6	1×10^6 A	5×10^5 A	5×10^5 A
2	Single-Pb9	1×10^6	1×10^6 B	5×10^5 B	5×10^5 B
3	Mixture A+B	2×10^6	1×10^6 A + 1×10^6 B	5×10^5 A + 5×10^5 B	5×10^5 A + 5×10^5 B
4	Separate A+B	2×10^6	1×10^6 A + 1×10^6 B	1×10^6 A	1×10^6 A
5	Double A+B (Double)	1×10^6	1×10^6 A + B	5×10^5 AB	5×10^5 AB
6	Single-pLuc + Vector	2×10^6	1×10^6 A	5×10^5 A + 5×10^5 V	5×10^5 A + 5×10^5 V
7	Single-Pb9 + Vector	2×10^6	1×10^6 B	5×10^5 B + 5×10^5 V	5×10^5 B + 5×10^5 V
8	Double + Vector (Mixture)	2×10^6	1×10^6 A + B	5×10^5 AB + 5×10^5 V	5×10^5 AB + 5×10^5 V
9	Double + Vector (Separate)	2×10^6	1×10^6 A + B	1×10^6 AB	1×10^6 V

Table 6.1: Single-shot MVA immunisation regime.

Mice were immunised intramuscularly (i.m) with recombinant MVAs as shown in each group. Mice were immunised in a total volume of 50µl divided into 25µl per lower limb. Each 25µl contained the indicated antigen dose as shown in columns: left leg and right leg.

pLuc = Antigen A, tPA-Pb9-rLuc8PV = Antigen B

Non recombinant MVA = V

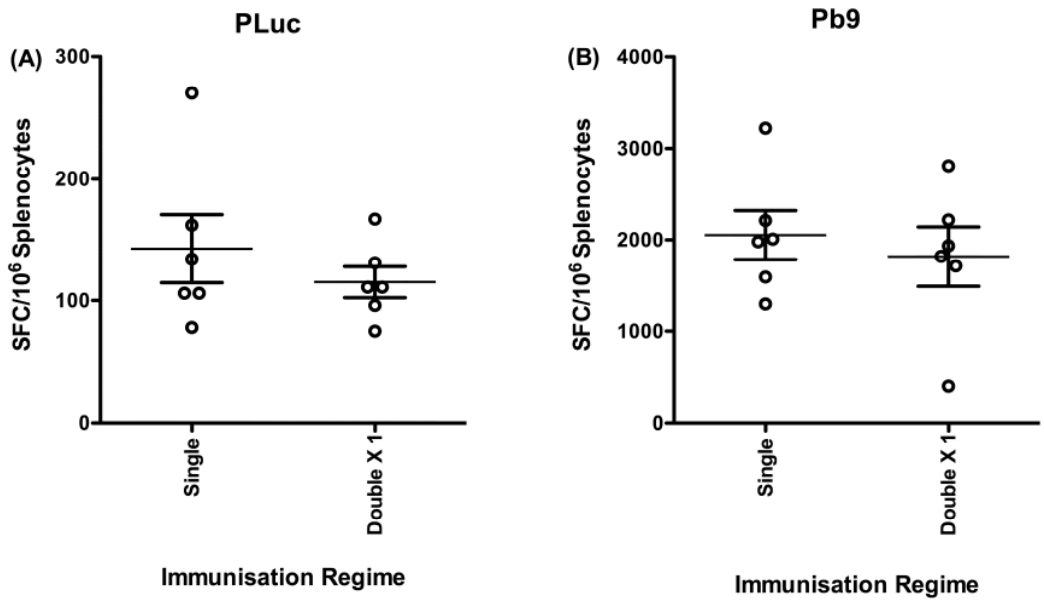


Figure 6.3: Cellular immunogenicity in mice immunised with bivalent MVA expressing two antigens (tPA-Pb9-rLuc8PV and pLuc).

3 groups of female Balb/c mice (n = 6) were immunised intramuscularly (i.m) with 10^6 pfu/mouse of rMVA (Table 6.1 – groups 1, 2 and 5). Seven days post immunisation, splenic $CD8^+$ T cell responses to pLuc-G9V (A) or Pb9 (B) were determined by IFN-γ ELISpot assay. Circles represent responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is a representative of two separate experiments.

6.2.4 $CD8^+$ T cells elicited by bivalent MVA equal or exceeded mixture, separate groups in single dose MVA immunisation.

Next was to determine whether Pb9 and pLuc-G9V-specific $CD8^+$ T cells generated by the bivalent MVA would equal or exceed that generated by mixing the two monovalent MVAs or given at separate sites because competition of $CD8^+$ T cells has been reported after boosting with two rMVA vaccines co-administered as a mixture but the competition eliminated when administered at separate sites (207). Seven days

after vaccination of mice with rMVAs as shown in (Table 6.1 – groups 3, 4 and 5) mouse splenocytes were assayed for frequencies of Pb9 and pLuc-G9V-specific $CD8^+$ T cells by IFN-γ ELISpot (Figure 6.4). Interestingly, pLuc-G9V-specific $CD8^+$ T cells elicited by the bivalent MVA (Double x 1) was statistically higher than mixture and separate groups ($p < 0.05$ by one way ANoVA). However, the frequencies of Pb9-specific $CD8^+$ T cell were indistinguishable in all groups.

These data shows that after a single-shot MVA vaccination, the bivalent MVA elicits pLuc-G9V-specific $CD8^+$ T cell frequencies higher than that generated when the single recombinants were co-administered as a mixture or given at separate site, but frequency of Pb9-specific $CD8^+$ T cells were otherwise indistinguishable in all groups. It also suggest that pLuc might be poorly immunogenic in comparison to Pb9 (compare legend of pLuc and Pb9 in Figure 6.3 and 6.4), which might have abrogated competition for CD8 T cell between pLuc-G9V and viral peptide epitopes.

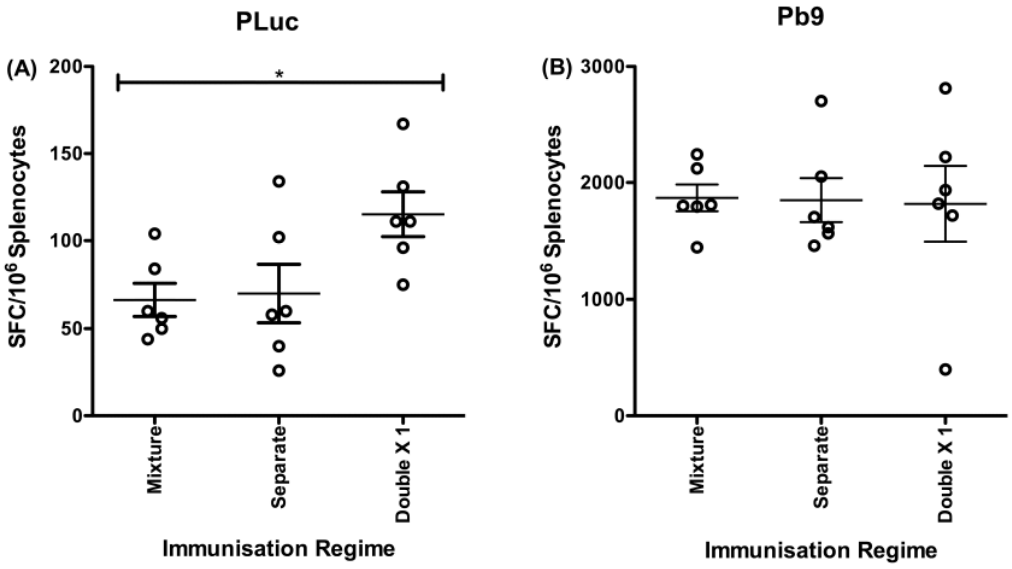


Figure 6.4: Cellular immunogenicity in mice immunised with bivalent MVA compared with two rMVAs given as a mixture or at separate sites.

4 groups of female Balb/c mice (n = 6) were immunised intramuscularly (i.m) with the indicated rMVA doses as shown in (Table 6.1 – groups 3, 4, 5 and 6). Seven days post immunisation, splenic $CD8^+$ T cell responses to pLuc-G9V (A) or Pb9 (B) were determined by IFN-γ ELISpot assay. Circles represent responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is a representative of two independent experiments.

6.2.5 Effect of virus-specific $CD8^+$ T cells on immunogenicity of recombinant MVA vaccines

To investigate whether $CD8^+$ T cells generated by viral antigens interfered with immunogenicity of the recombinant antigens (tPA-Pb9-rLuc8PV and pLuc), rMVAs expressing pLuc or tPA-Pb9-rLuc8PV were each coadministered with a non-recombinant MVA (nrMVA) in comparison with single recombinants expressing either pLuc or Pb9 administered alone. Seven days after vaccination of mice with

rMVAs (Table 6.1 – groups 1, 2, 6 and 7) mouse splenocytes were assayed for the frequencies of Pb9 and pLuc-G9V-specific $CD8^+$ T cells by IFN-γ ELISpot. The frequency of pLuc-G9V-specific $CD8^+$ T cells induced by single-pLuc co-administered with a nrMVA was comparable to those induced by the single-pLuc recombinant administered alone. (Figure 6.5A). However, The frequency of Pb9-specific $CD8^+$ T cells induced by single-Pb9 co-administered with a nrMVA was significantly lower (Figure 6.5B) than those induced by the single-Pb9 recombinant administered alone ($p = 0.008$ by Mann Whitney test). These data confirms that pLuc is poorly immunogenic and is suggestive of minimal antigenic competition for $CD8^+$ T cells between pLuc-G9V and viral antigens but high peptide epitope competition for $CD8^+$ T cells between Pb9 and viral antigens.

Having established that pLuc-G9V is less immunogenic compared to Pb9, the next step was to investigate whether viral antigens interfered with the immunogenicity of the bivalent MVA vaccine. To address this question, the bivalent MVA (Double x 1) was co-administered with a nrMVA as a mixture or at separate sites. As before, pLuc-G9V-specific $CD8^+$ T cells elicited by the bivalent MVA was unaffected when co-administered with a nrMVA (Figure 6.5C). The frequency of Pb9-specific CD8+ T cells elicited by the bivalent MVA was significantly lower ($p < 0.05$ by one way ANoVA) when co-administered with a nrMVA as a mixture or at separate sites (Figure 6.5D).

These data supports previous observations (207) that competition for $CD8^+$ T cell is complex and antigen dependent, so we reasoned that because pLuc epitope (pLuc-G9V) might be subdominant to Pb9, there was minimal competition for $CD8^+$ T cell between the recombinant pLuc and viral antigens. Though the mechanism underlying

competition between pLuc-G9V, Pb9 and viral antigens is unclear, the following mechanisms (not investigated in this study) may have contributed to CD8 T cell competition: (i) competition for physical access to APCs (compartment size) (ii) antigen processing (iii) binding of peptide to mHC (affinity and avidity) (iv) competition for cytokines for activation (iv) (v) epitope abundance on the priming APCs (vi) suppressing effect of one T cell population on others (clonal expansion).

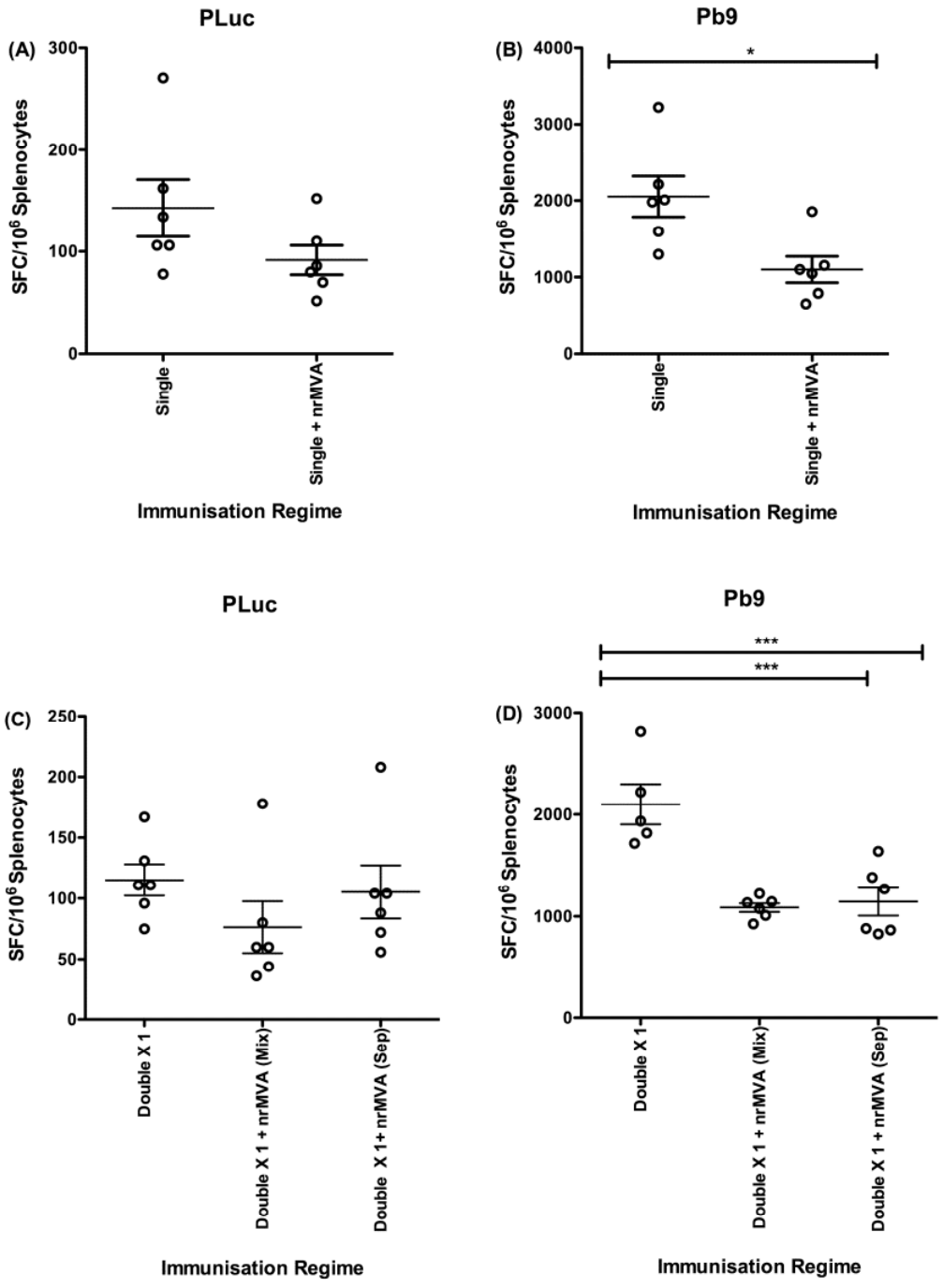


Figure 6.5: Cellular immunogenicity in mice to determine virus interference on pLuc and Pb9-specific CD8$^+$ T cells.

Female Balb/c mice (n = 6) were immunised intramuscularly (i.m) with the indicated rMVA doses as shown in (Table 6.1 – groups 1, 2, 5, 6, 7, 8 and 9). Seven days post immunisation, splenic CD8$^+$ T cell responses to pLuc-G9V (A and C) or Pb9 (B and

D) were determined by IFN-γ ELISpot assay. Circles represent responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is a representative of two independent experiments.

6.2.6 Immunogenicity of monovalent and bivalent MVA vaccines after prime-boost immunisation

Since the frequencies of pLuc-G9V and Pb9 specific $CD8^+$ T cells were indistinguishable in both single recombinants and bivalent MVA (double-pLuc-Pb9) after a single dose of MVA, the next step was to assess whether the immunogenicity of the single and double-pLuc-Pb9 were similar after prime-boost immunisation. It has been shown previously in this work (Figure 5.5 – chapter 5) that priming with an adenovirus expressing (TIP – containing Pb9) and boosting with rMVAs expressing the tPA-Pb9-rLuc8PV under the control of four endogenous poxviral promoters (pB8R, pF11L, pA44L and pC11R) were capable expanding the frequencies of Pb9 specific $CD8^+$ T cells. In addition, a previous work by Limberis *et al* (157) has shown in murine models that a single dose adenovirus vector (HAdV5 at $5x10^{10}$ vp/mice) expressing pLuc was capable of eliciting potent pLuc-G9V-specific $CD8^+$ T cells in Balb/c mice. To assess the frequencies of pLuc-G9V and Pb9-specific $CD8^+$ T cells after prime-boost vaccination, mice were primed by mixing two adenoviruses (HAdV5), each at 10^8 vp/mouse either expressing pLuc or TIP (Table 6.2), two weeks post HAdV5 prime, mouse PMBCs were stimulated by pLuc-G9V and Pb9 peptides and analyzed by IFN-γ ELISpot. pLuc-G9V and Pb9 specific $CD8^+$ T cells were detectable in all animals (Figure not shown). The two recombinant HAdV5 were mixed because the vaccination regimen is clinically relevant in humans. Eight week (56 days) after prime, mice were boosted with rMVAs at 10^6 pfu/mouse (single-pLuc,

single-Pb9 or double-pLuc-Pb9). Two weeks (14 days) after the boost, mouse PBMCs and splenocytes were assayed for the frequencies of pLuc-G9V and Pb9 specific $CD8^+$ T cells by IFN-γ ELISpot. The double-pLuc-Pb9 was capable of boosting pLuc-G9V to levels equivalent to the single-pLuc in the blood and spleen (Figure 6.6), though the trend of single-pLuc in the blood (Figure 6.6A) was higher than the double-pLuc-Pb9 but was not found to be statistically significant ($p = 0.1797$ by Mann Whitney Test).

However, the Pb9-specific $CD8^+$ T cell elicited by the double-pLuc-Pb9 was significantly lower than the single-Pb9 both in the blood (Figure 6.6B) and spleen (Figure 6.6D), ($p = 0.04$ and $p = 0.002$ respectively by Mann Whitney Test). These data show that at the level of single-shot MVA immunisation and for both pLuc-G9V and Pb9, the double-pLuc-Pb9 was capable of eliciting potent pLuc-G9V and Pb9 specific $CD8^+$ T cells comparable to the single recombinants, but after the boost the single-Pb9 was capable of boosting memory $CD8^+$ T cells specific for immunodominant peptide epitope (Pb9) to higher levels than the double-pLuc-Pb9. A plausible explanation for this is immunocompetetion being that the double-pLuc-Pb9 expresses two recombinant antigens (pLuc and tPA-Pb9-rLuc8PV) competing for presentation on APCs as against one recombinant antigen by the single expresser.

S/N	Groups	Adenovirus Prime (vp)	MVA boost (pfu/mouse)
1	Single-pLuc	1×10^8 pLuc + 1×10^8 TIP	1×10^6
2	Single-Pb9	1×10^8 pLuc + 1×10^8 TIP	1×10^6
3	Mixture A+B	1×10^8 pLuc + 1×10^8 TIP	2×10^6
4	Separate A+B	1×10^8 pLuc + 1×10^8 TIP	2×10^6
5	Double A+B (Double x 1)	1×10^8 pLuc + 1×10^8 TIP	1×10^6
6	Double A+B (Double x 2)	1×10^8 pLuc + 1×10^8 TIP	2×10^6

Table 6.2: Prime-boost immunisation regime.

Mice were immunised intramuscularly (i.m) by mixing two adenoviruses (HAdV5), each expressing pLuc or TIP in a total dose of 2×10^8 viral particles (vp), each at 1×10^8vp. Mice were immunised in a total volume 50μl divided into 25μl/lower limb.

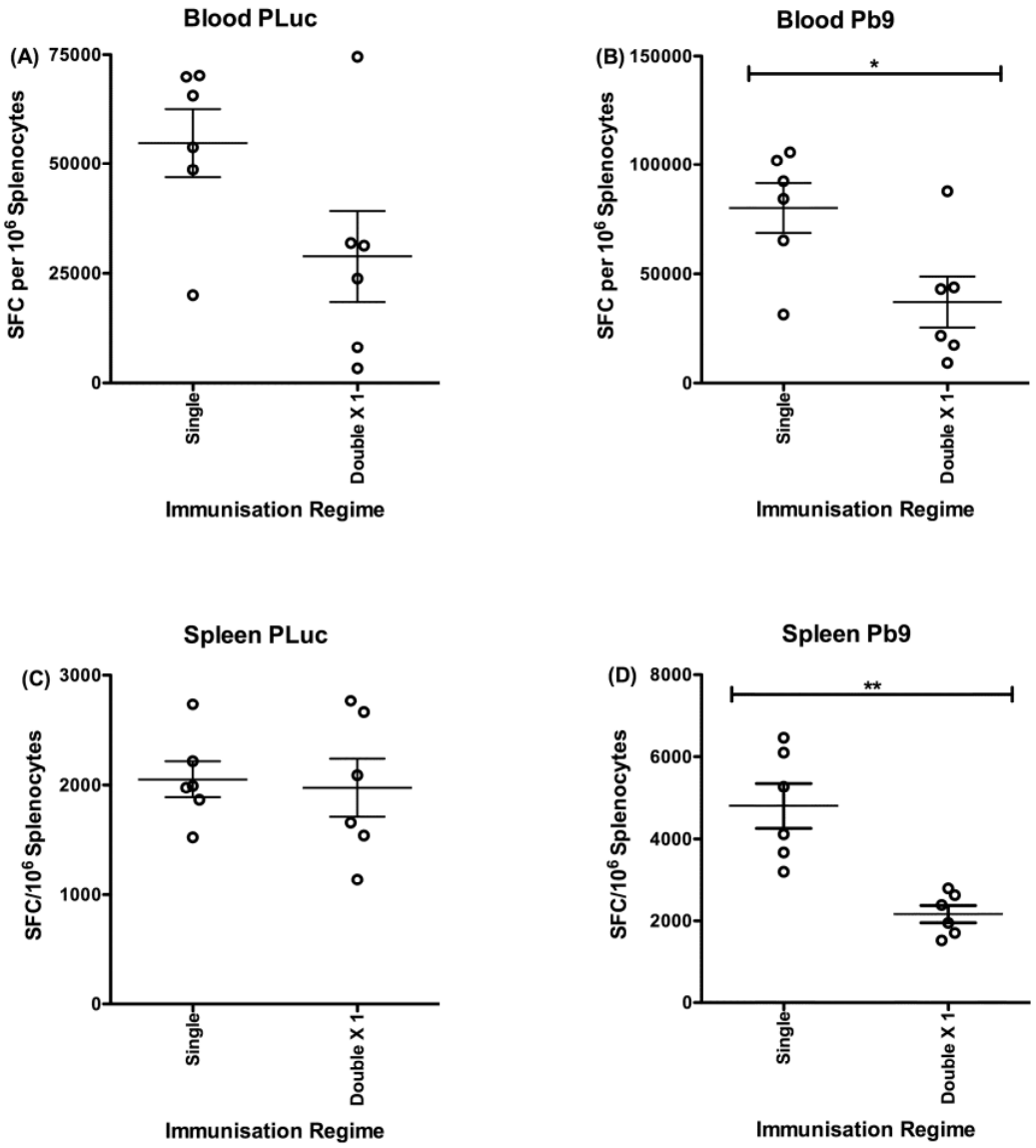


Figure 6.6: Cellular immunogenicity of monovalent and bivalent MVA vaccines in prime-boost immunisation regime.

3 groups (n = 6) of mice were immunised by mixing two adenoviruses (HAdV5), each expressing pLuc or Pb9 in a total dose of 2 x 10^8 viral particles (vp), each at 1 x 10^8vp. 56 days (8 weeks) later mice were boosted with 10^6 pfu/mouse rMVA (Table 6.2 – groups 1, 2 and 5). At day 70 (2 weeks post-boost) post immunisation, mouse PBMCs responses to pLuc-G9V and Pb9 (A and B) or splenic $CD8^+$ T cell responses to pLuc-G9V and Pb9 (C and D) were determined by IFN-γ ELISpot assay. Circles

represent responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is a representative of two independent experiments.

6.2.7 Immunogenicity of bivalent MVA equivalent to mixture and separate immunisation regimes in the blood

Having found comparable pLuc-G9V and Pb9 specific $CD8^+$ T cells between the single recombinants and double-pLuc-Pb9 expressors in single dose MVA vaccination, further investigation was carried out whether responses elicited by the double-pLuc-Pb9 would be comparable to the two single recombinants co-administered as a mixture or at separate sites following adenovirus prime, MVA boost. This is because previous studies (207) employing two recombinant MVA vaccines each expressing PyCSP or $PyMSP_{142}$ showed competition for $CD8^+$ T cells when the two vectors were co-administered as a boost in a mixture vaccination regimen but the competition eliminated when administered at separate sites.

Mice were immunised by mixing two adenoviruses as shown in (Table 6.2) and boosted 2 weeks later with single rMVAs expressing pLuc or tPA-Pb9-rLuc8PV in a mixture or separate vaccination regimen, in comparison with immunogenicity of the double-pLuc-Pb9. Two weeks (14 days) after the boost, mouse PBMCs and splenocytes were assayed for the frequencies of pLuc-G9V and Pb9 specific $CD8^+$ T cells by IFN-γ ELISpot. Interestingly, the immunogenicity of the double-pLuc-Pb9 was equivalent to those of the mixture or separates groups for both pLuc-G9V and Pb9-specific $CD8^+$ T cells in the blood (Figure 6.7). The minimal competition observed in the blood might be due to a mixture of circulating and emigrant T cell population not primed in the blood. However, there appears to be competition (we

assume) regardless of whether the recombinant antigens are expressed in one or two viruses.

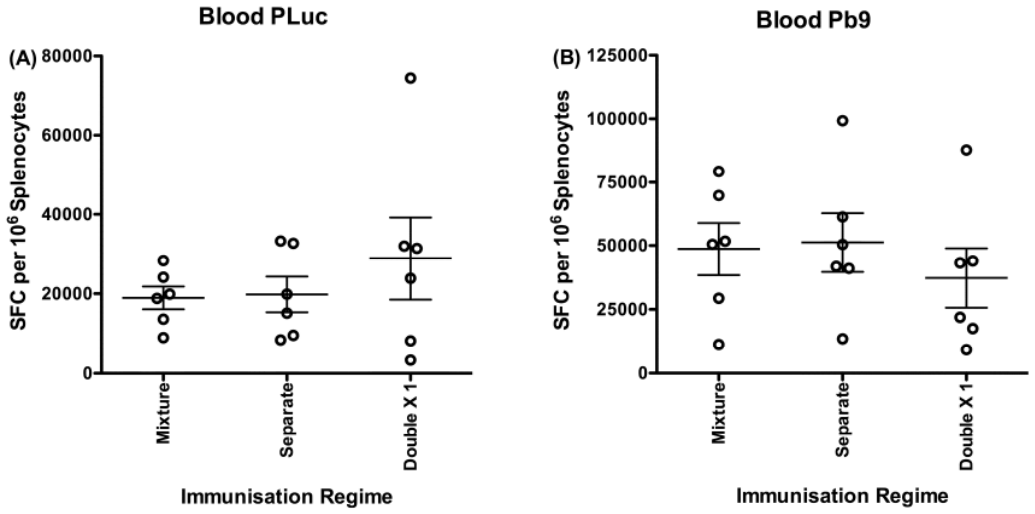


Figure 6.7: Cellular immunogenicity in the blood after vaccination with bivalent MVA in comparison to mixture and separate immunisation regime.

3 groups (n = 6) of mice were immunised by mixing two adenoviruses (HAdV5), each expressing pLuc or TIP in a total dose of 2 x 10^8 viral particles (vp), each at 1 x 10^8vp. 56 days (8 weeks) later mice were boosted with either 2 x 10^6 pfu/mouse (mixture and separate) or 10^6 pfu/mouse (Double x 1) and as shown in (Table 6.2 – groups 3, 4 and 5). At day 70 (2 weeks post-boost), mouse PBMCs responses to pLuc-G9V (A) or Pb9 (B) were determined by IFN-γ ELISpot assay. Circles represent responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is a representative of two separate experiments.

6.2.8 Immunisation at separate sites eliminated competition and enhanced Pb9 specific CD8$^+$ T cells in the spleen

Next was to investigate immunogenicity of the mixture, separate and double-pLuc-Pb9 groups in the spleen because it has been reported in the paper by Forbes *et al*. (207), that CD8$^+$ T cell interference is a local effect occurring at secondary lymphoid organs such as lymph nodes and spleen. To investigate this, mice were primed by mixing two adenoviruses as before (Table 6.2) and boosted with double-pLuc-Pb9 in comparison to single-pLuc and single-Pb9 co-administered as mixture or at separate sites. Two weeks (14 days post-boost) splenocytes were stimulated for the frequencies of pLuc-G9V and Pb9-specific CD8$^+$ T cells by IFN-γ ELISpot.

Interestingly, the double-pLuc-Pb9 elicited potent pLuc-G9V-specific CD8$^+$ T cells that were indistinguishable from the mixture and separate groups (Figure 6.8A), however immunisation at separate sites enhanced immunogenicity of Pb9 and eliminated Pb9-specific CD8$^+$ T cell competition. The frequency of Pb9-specific CD8+ T cells was significantly higher than the mixture or double-pLuc-Pb9 groups ($p < 0.05$ by one way ANoVA), (Figure 6.8B). Importantly, the frequency of Pb9-specific CD8$^+$ T cells elicited by the separate vaccination regimen was similar to that seen previously when the single-Pb9 rMVA was administered alone (Figure 6.6D). These data is in agreement with reports from Forbes *et al*. (207), and confirms that competition for CD8$^+$ T cells could be eliminated by immunising vaccine vectors at physically separate sites and that competition is antigen dependent and vaccination regime chosen. It is also suggestive that competition for CD8$^+$ T cells by vectored vaccines might be a local effect occurring in secondary lymphoid organs such as

spleen as demonstrated here by enhanced Pb9-specific $CD8^+$ T cells after vaccination at separate sites.

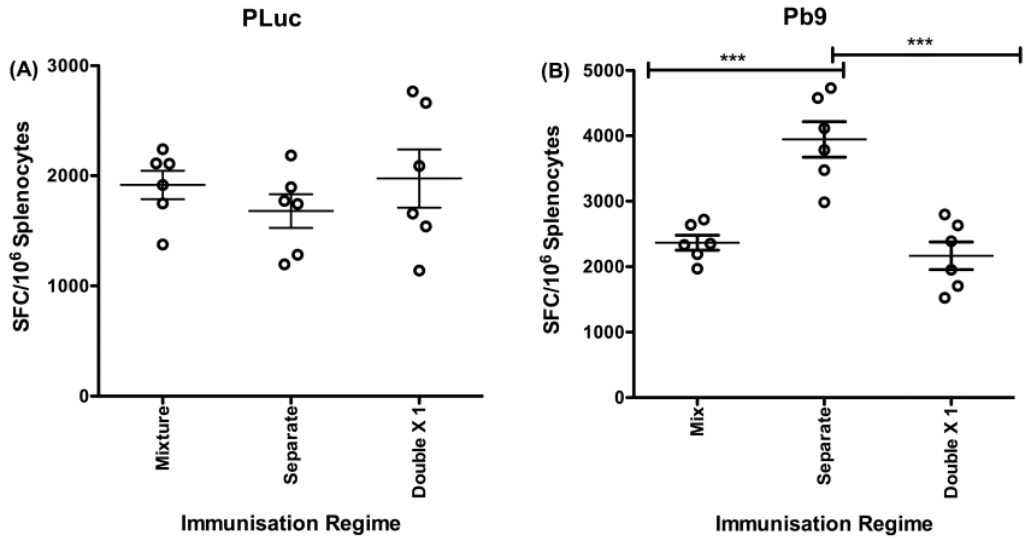


Figure 6.8: Cellular immunogenicity in the spleen after vaccination with bivalent MVA in comparison to mixture and separate immunisation regime.

3 groups (n = 6) of mice were immunised by mixing two adenoviruses (HAdV5), each expressing pLuc or TIP in a total dose of 2 x 10^8 viral particles (vp), each at 1 x 10^8vp. 56 days (8 weeks) later mice were boosted with either 2 x 10^6 pfu/mouse (mixture and separate) or 10^6 pfu/mouse (Double x 1) and as shown in (Table 6.2 – groups 3, 4 and 5). At day 70 (2 weeks post-boost), post immunisation, splenic responses to pLuc-G9V (A) or Pb9 (B) were determined by IFN-γ ELISpot assay. Circles represent responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is a representative of two separate experiments.

6.2.9 Immunogenicity of bivalent MVA not enhanced by increasing vaccine dose

Upon confirmation that immunisation at separate sites enhanced the frequencies of Pb9-specific $CD8^+$ T cells than mixing two antigens or the bivalent MVA, it was hypothesized that increasing the dose of the bivalent MVA might increase the responses of pLuc and Pb9-specific $CD8^+$ T cells. In addition, previous studies have shown that $CD8^+$ T cells responses induced by vectored vaccines follow a dose-response curve (214); therefore it was hypothesized that a similar dose-response pattern might be observed with the bivalent MVA.

Mice were immunised by mixing two adenoviruses as before (Table 6.2) and boosted eight weeks later with an increased dose of the bivalent MVA – from 1 x 10^6 pfu/mouse (Double x 1) to 2 x10^6 pfu/mouse (Double x 2). Two weeks after the boost splenocytes were stimulated with pLuc-G9V and Pb9 peptides and assayed by IFN-γ ELISpot for the frequencies of pLuc-G9V and Pb9-specific $CD8^+$ T cells. Surprisingly, increasing the dose of the bivalent MVA did not improve immunogenicity of the vaccine both for pLuc-G9V and Pb9-specific $CD8^+$ T cells, as observed in the blood (Figure 6.7A and B) and spleen (Figure 6.8C and D). The rationale for this may partly be that a two-fold increase was too small to show any appreciable increase in immunogenicity. Secondly, the amount of viral antigens of the double x 2 is twice more than double x 1, so the data is suggestive that $CD8^+$ T cells elicited by viral antigens may outcompete those of the recombinant pLuc and tPA-Pb9-rLuc8PV antigens for access to APCs. Importantly, the frequencies of Pb9-specific $CD8^+$ T after immunisation at separate sites and in the spleen were statistically significantly higher than those induced by the double x 2 even though the

double x 2 contained twice the antigen in the separate group (p = 0.007 by Newman Keuls Test following One-way ANoVA).

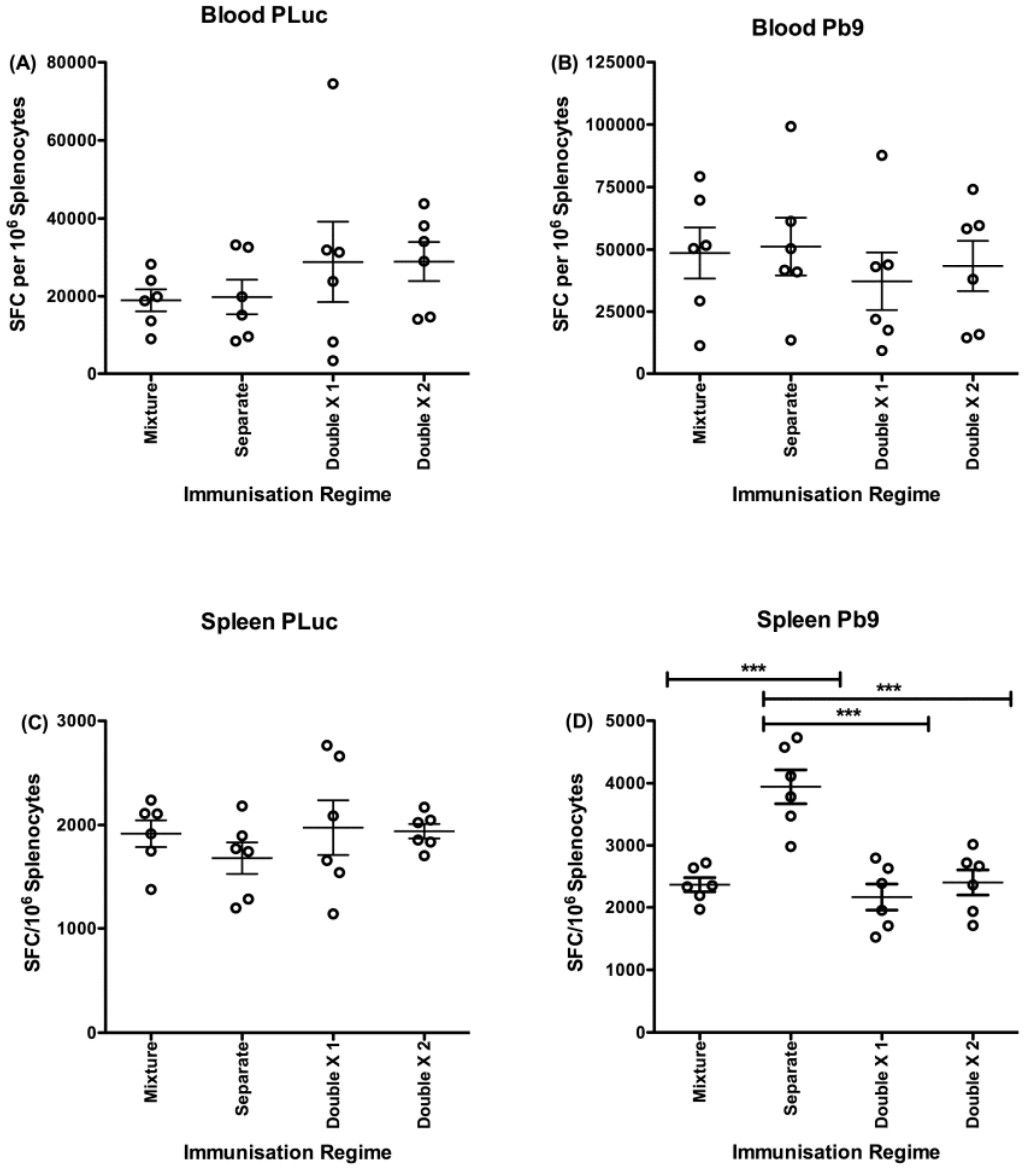


Figure 6.9: Cellular immunogenicity of bivalent MVA after increasing dose.

4 groups (n = 6) of mice were immunised by mixing two adenoviruses (HAdV5), each expressing pLuc or TIP in a total dose of 2 x 10^8 viral particles (vp), each at 1 x 10^8vp. 56 days (8 weeks) later mice were boosted with as shown in (Table 6.2 – groups 3, 4 and 5). At day 70 (2 weeks post-boost), post immunisation, mouse PBMCs responses to pLuc-G9V and Pb9 (A and B) or splenic $CD8^+$ T cells responses

to pLuc-G9V and Pb9 (C and D) were determined by IFN-γ ELISpot assay. Circles represent responses of individual mice, with line indicating the mean and the error bars showing SEM. Data is a representative of two separate experiments.

6.3 Discussion

In this chapter, a multivalent MVA expressing two recombinant antigens under the control of two-poxviral insertion driven loci was developed by two BAC recombineering technologies. Firstly, the genome of MVA bacterial artificial chromosome (MVA-BAC) in *E.coli* was engineered using a *GalK* positive selection scheme (153, 155) to introduce a model antigen (tPA-Pb9-rLuc8PV) into the insertion driven locus of *C11R*. Secondly, the MVA-BAC carrying the tPA-Pb9-rLuc8PV under the control of the *C11R* endogenous poxviral promoter was engineered using an *AphAI* kanamycin recombineering (158) to introduce a second model antigen (pLuc) into the insertion driven loci of *B8R*. The resulting MVA-BAC carrying the two model antigens, tPA-Pb9-rLuc8PV and pLuc under the control of the *C11R* and *B8R* endogenous promoters respectively (Figure 6.1), was rescued back to infectious virus, plaque purified, propagated and titred to values which fell within acceptable range of rMVA vaccines generated in the Jenner laboratories. Two comparator rMVAs were also generated, each expressing either the tPA-Pb9-rLuc8PV or pLuc under the control of the *C11R* and *B8R* endogenous poxviral promoter respectively.

The recombinant MVAs expressing the two model antigens (double-pLuc-Pb9 (bivalent MVA)) or single antigens (single-pLuc or single-Pb9) were assessed for their ability to elicit cellular immune responses against the recombinant pLuc or tPA-Pb9-rLuc8PV antigens. To determine the frequencies of pLuc-G9V and Pb9 specific $CD8^+$ T cells, mice were either vaccinated in a single-dose MVA or prime-boost vaccination regimen. Mouse PBMCs and splenocytes were assessed for the production of pLuc or Pb9 specific $CD8^+$ T cells by IFN-γ ELISpot.

It was first hypothesized that rMVA expressing the two model antigens (double-pLuc-Pb9) might elicit potent pLuc-G9V and Pb9 specific $CD8^+$ T cells as well as the single recombinants. Interestingly, indistinguishable pLuc-G9V and Pb9 specific $CD8^+$ T cells elicited by the double-pLuc-Pb9 were observed in comparison to the single recombinants (Figure 6.2). However, we observed that the pLuc-G9V-specific $CD8^+$ T cells were ~100 spots forming cells/million (SFC/million) in both single-pLuc and double-pLuc-Pb9 (Double x 1) groups, while Pb9-specific $CD8^+$ T cells were ~1500 SFC/million (Pb9 $CD8^+$ T cell epitope in Balb/c is known to be immunodominant). The frequency of antigen specific $CD8^+$ T cells obtained with Pb9 was not surprising as it fell within the range obtained previously with single recombinants (see legends in chapter 5) but the frequency of pLuc was unexpected because a previously study reported ~1700 SFC/million, though this was after a single dose of recombinant adenovirus expressing pLuc in Balb/c mice (157).

These results are suggestive that pLuc-G9V $CD8^+$ T cell epitope might be sub-dominant to Pb9 in Balb/c mice. One could also argue that the poorly immunogenic pLuc after a single dose rMVA immunisation might be partly due to amount of viral antigens in MVA that normally competes for $CD8^+$ T cells with recombinant antigens while the high pLuc-G9V-specific $CD8^+$ T cell after single-dose adenovirus in the paper by Limberis *et al* (157) might be attributable to fewer amounts of viral antigens in adenoviral vector vaccines. However, up to 2500 SFC/million were observed after adenovirus prime and MVA boost in our study with pLuc-G9V in the spleen (Figure 6.7A).

Since pLuc-G9V and Pb9 specific $CD8^+$ T cells elicited by the double-pLuc-Pb9 were indistinguishable from those induced by the single recombinants after single dose

MVA, it was next hypothesized that the magnitude of the responses elicited by the double-pLuc-Pb9 might be equivalent to that obtained when the two single recombinants are co-administered as a mixture or at separate sites. After the single dose MVA immunisation regimen, the frequency of pLuc-G9V-specific $CD8^+$ T cells elicited by the double-pLuc-Pb9 were unexpectedly found to be significantly higher than that induced by the single recombinants co-administered as a mixture or at separate sites (Figure 6.3A), but the frequencies of Pb9-specific $CD8^+$ T cells were nevertheless indistinguishable in all groups. An important finding from this experiment is that the bivalent MVA (expressing two foreign antigens from different endogenous poxviral insertion driven) might have an advantage over combining rMVA vaccines (either co-administered as a mixture or given at separate sites). This advantage might be due to lesser amounts of viral antigens competing with the recombinant antigens in the bivalent MVA. It is also possible that there is a threshold for immunocompetition between recombinant antigens and viral antigens and once the limit was exceeded, an increase in viral antigens as in the mixture or separate groups, suppresses peptide epitope presentation on mHC class 1 molecule and more evidently if the recombinant antigen has sub-dominant peptide epitope like pLuc-G9V.

Following these findings, and to investigate whether $CD8^+$ T cells elicited by viral antigens interfered with pLuc-G9V and Pb9-specific $CD8^+$ T cells, single pLuc and singl-Pb9 recombinants were co-administered with an empty vector (non-recombinant MVA (nrMVA)) at separate sites. Interestingly, there was minimal viral-specific $CD8^+$ T cells interference with pLuc-G9V-specific $CD8^+$ T cells (Figure 6.4 A), though the trend was more evident for single-pLuc recombinant administered alone. However, it was observed that viral-specific $CD8^+$ T cells interfered with Pb9-specific

$CD8^+$ T cells (Figure 6.4B), confirming competition for $CD8^+$ T cells is epitope specific. This finding is in agreement with data from previous work that competition between antigens of high epitope specificity (Scherer, 2006) reduces the breadth of the immune response, which may have contributed to the reduction of Pb9-specific $CD8^+$ T cells.

Upon confirmation that $CD8^+$ T cells induced by viral antigens interfered with Pb9-specific $CD8^+$ T cells, it was subsequently hypothesized that the amount of viral antigens in the double-pLuc-Pb9 in comparison to mixture and separate groups might be responsible for the robust antigen specific $CD8^+$ T cells generated by the double-pLuc-Pb9. To address this question, the double-pLuc-Pb9 was co-administered with nrMVA, as a mixture or at separate sites. Co-administration of double-pLuc-Pb9 with the nrMVA did not affect the frequency of pLuc-G9V-specific $CD8^+$ T cells in the mixture or separate groups (Figure 6.4C). However there was interference with Pb9-specific $CD8^+$ T cells when the double-pLuc-Pb9 was co-administered with the nrMVA as a mixture or at separate sites (Figure 6.4D). This finding confirmed that the robust antigen specific $CD8^+$ T cells generated by the double-pLuc-Pb9 is directly related to the amount of viral antigens in the double-pLuc-Pb9 and dependent on peptide epitope "strength" (subdominant or immunodominant).

It is well documented that the resource that T cells compete for is access to APCs, in addition to interleukins, cytokines and survival factors. However, experimental data regarding competition for access to APCs are controversial (207). From data presented here it appears that different principles govern competition: (i) vaccination regime chosen – single dose, mixture or separate vaccination regimen and bivalent MVA (ii) peptide epitope expression – sub-dominant versus immunodominant.

Following Ad-MVA immunisation, the pLuc-specific $CD8^+$ T cells generated by the double-pLuc-Pb9 were indistinguishable from single-pLuc, mixture and separate groups in the blood which might be attributable to the priming regime employed (mixing adenoviruses). Though we only looked at priming by mixing two adenoviruses, but whether a difference would be seen after priming with adenoviruses at physically separate site is yet to be demonstrated. The study by Forbes *et al*. (207) reported that co-administering vaccines at separate sites eliminated $CD8^+$ T cells competition in the spleen. In agreement with this finding, co-administration of the single-pLuc and single-Pb9 recombinants at physically separate sites and after the MVA boost eliminated competition and enhanced Pb9-specific $CD8^+$ T cell responses in the spleen. One plausible explanation for this is that competition for $CD8^+$ T cells is a local effect occurring in secondary lymphoid organs like spleen (where there are more localized APCs) and minimally evident in the circulatory system (blood). This is because, after immunisation at separates site and at high epitope competition, one could argue that immunodominant peptide epitopes are distinctively presented on different APCs, which abrogated competition.

These findings have strong implications for rationale design of $CD8^+$ T cell-based vaccine and are suggestive that careful consideration should be taken when selecting two or more antigens for the construction of recombinant vectored vaccines such as MVA. Though we have only looked at competition between a "weak" (pLuc) and "strong" (Pb9) epitope, it appears that competition for $CD8^+$ T cells would be apparent when two "strong" antigens are co-administered or two "strong" peptide epitopes are investigated because data presented here demonstrate that an immunodominant peptide epitope (Pb9) outcompeted a sub-dominant (pLuc) presumably for access and presentation by APC. However, the pLuc-G9V peptide

epitope is classified here as being poorly immunogenic "weak" in comparison to Pb9, but in the study by Limberis *et al*. (157), the pLuc-G9V peptide epitope was immunodominant in Balb/c compared to the two others identified, so it is possible that these other two pLuc peptide epitopes not explored in our study might induce different peptide specific $CD8^+$ T cell responses. Future studies combining two antigens should investigate $CD8^+$ T cell responses to more than one peptide epitope per antigen, as this would provide more insight into $CD8^+$ T cell competition.

Secondly, data presented here shows that the choice of immunisation regime is important for responses generated. While there was a strong correlation between vaccination responses and immunisation regime chosen, one could argue that the bivalent MVA is a suitable model for expression of two or more antigens. This is because following a single dose MVA vaccination regimen; the immunogenicity of the double-pLuc-Pb9 was comparable to that observed in the single, mixture or separate groups. We also found that following Ad-MVA immunisation regime; the frequencies of pLuc-G9V-specific $CD8^+$ T cells were also comparable to those of single, mixture groups in the blood and spleen. We were also able to establish that lesser amounts of viral antigens in the double-pLuc-Pb9 may be partially responsible for the overall immunogenicity of the bivalent MVA vaccine.

In conclusion, data presented here shows the construction, generation and immunogenicity of a bivalent MVA vaccine expressing two recombinant antigens under the control of two poxviral promoters, *B8R* and *C11R*. We also showed that the bivalent MVA vaccine was capable of eliciting potent $CD8^+$ T cell to the two recombinant antigens *in vivo* in comparison to single recombinants or when the single recombinants were co-administered as a mixture or at separate sites. In addition,

because of reactogenicity and economic gains, development of a multivalent MVA vaccines seems scientifically and financially viable for diseases of global health importance such malaria, HIV, tuberculosis and influenza where single or sub-unit vaccines have not been able to confer sterile protection.

CHAPTER 7

CONCLUSION AND FUTURE DIRECTIONS

7 Conclusions and Future Directions

7.1 Summary

The design, construction and generation of rMVA vaccines have focused on insertion of the foreign gene of interest linked to a small number of early/late promoters (p7.5, mH5 and SSP) prior to insertion into the viral genome at a suitable insertion locus such as (TK) by homologous recombination in infected cells. The work in this thesis has focused on the design, construction and generation of rMVA by the robust bacterial artificial chromosome (BAC) recombineering technology that allowed manipulation of the MVA genome in *E. coli* for insertion of an epitope tagged luciferase (tPA-Pb9-rLuc8PV) into seven-poxviral endogenous promoter driven insertion loci (*B8R, F11L, A44L, K6L, E5R, C11R and B2R*). These BAC derived MVA were rescued back to infectious viruses, plaque purified, propagated and titrated to values that fell within the acceptable range of rMVAs generated in the Jenner Laboratories. Four out of the seven BAC derived MVAs employing the *B8R, F11L, A44L, and C11R* promoters regulated the tPA-Pb9-rLuc8PV for protein expression and Pb9-specific $CD8^+$ T cells to levels equal to or exceeding those elicited by p7.5 and SSP recombinants expressing the same antigen. Deletion with concomitant insertion of the model antigen did not impair viral growth. Genetic stability of these endogenous promoter driven insertion loci (*B8R, F11L, A44L,* and *C11R*) was demonstrated after 10 serial passages as they were found to be genetically stable for transgene expression.

The genome of MVA was further manipulated for the generation of a bivalent MVA for the expression of two antigens from two of the newly identified poxviral endogenous promoter driven insertion loci. The *GalK* positive selection and *Apha1*

kanamycin recombineering were employed for insertion of tPA-Pb9-rLuc8PV and photinus luciferase (pLuc) into the promoter driven insertion loci of *C11R* and *B8R* respectively. The bivalent MVA was rescued back to infectious virus, plaque purified, propagated and titrated to values that fell within the acceptable range of rMVAs generated previously. The bivalent MVA was capable of eliciting potent Pb9 and pLucG9V-specific $CD8^+$ T cells comparable to single-pLuc and single-Pb9 recombinants in single dose and Ad-MVA prime-boost immunisation regimes. When the bivalent MVA was compared to co-administration of the two single recombinants as mixture or at separate sites, the frequency of Pb9-specific $CD8^+$ T cells was enhanced following vaccination at separate sites only after Ad-MVA immunisation regime.

7.2 Conclusions

7.2.1 Recombineering

The application of recombinant mediated genetic engineering (Recombineering) the generation of rMVA was efficient in all cases. The design, construction and generation of BAC derived MVAs via this technology was straightforward and required limited cloning steps often employed in conventional methods. BAC derived MVAs were generated between 10 – 12 weeks which is less than the time, 14 – 20 weeks required for conventional methods. Although in some cases, BAC derived MVAs took longer than the estimated time because mutation was found on the insert after the recombinant virus was made. This could have been avoided if the insert was sequenced at specific times during the development stage. For example, mutation was found on the insert in viruses employing the *A44L, F11L, E5R and C11R* promoters after propagation in T150 flask (refer to section 2.7.3.2 in M+M) and because of this the whole progress had to be repeated, but this time two additional sequencing steps (after generating the insert and after the BAC Maxi prep) were incorporated into the protocol (see recombineering work plan in section 2.6 in M+M).

The efficiency of the BAC technology for the generation of BAC derived MVAs described in this work demonstrates how important the technology is for the generation of rMVA vaccines against infectious diseases. Experience gained from the BAC technology is now being employed for the generation of BAC derived adenoviruses and rMVA for pre-clinical vaccines against malaria, TB and influenza in the Jenner Laboratories.

7.2.2 Endogenous poxviral promoters

A panel of seven non-essential or non-functional genes in MVA (*B8R, F11L, A44L, K6L, E5R, C11R and B2R*) was selected based on the studies carried out by Assarson *et al*. and Yang *et al*. (136, 166). These genes were selected based on the strong early or immediate-early responses demonstrated for protein expression so we reasoned that their endogenous promoters might have an advantage over traditional early/late p7.5 and SSP promoters for eliciting antigen-specific $CD8^+$ T cells. It was demonstrated that viruses employing four of the seven promoters, *B8R, F11L, A44L and C11R* induced potent Pb9-specific $CD8^+$ T cells equal to or exceeding that induced by p7.5 and SSP promoters, in single-dose MVA and Ad-MVA immunisation regimes. It was show that application of inferior ATG start codons of *K6L* and *B2R* presumably led to inefficient translation initiation, which resulted in poor expression of the foreign protein. Future design and construction of poxviral endogenous driven insertion loci should take careful inspection of MVA annotations when selecting poxviral genes as antigenic insertion driven loci. This is because some genes in MVA are either fragmented or mutated and could be overlooked during the design stage.

7.2.3 Viral growth and genetic stability

Since this is the first time these endogenous promoter driven insertion loci were used for the expression of a foreign antigen, it was important to characterise whether deletion with concomitant insertion affected viral growth. It was also important to test the genetic stability of the promoter driven insertion loci for expression of a foreign antigen. The growth patterns of viruses employing the *B8R, F11L, pA44L* and *C11R* promoter were equivalent to the parent MVA and the ORF of these four genes were

genetically stable for the expression of the model antigen. Though we have only looked at expression of a model antigen, 2.4kb, from these insertion loci, it will be important for future studies to take this into consideration that a larger insert or different antigen might affect the stability of the virus. In addition, the stability of these insertion loci was only demonstrated in terms of foreign gene expression after 10 passages, it would have been more informative to sequence regions surrounding the insert to further guarantee stability of these insertion loci. Overall, it appears that endogenous insertion loci like those investigated here might be more stable than TK locus because the insertion site has its own promoter intact in its natural position, while for TK, an exogenous promoter is inserted which might result in the insert falling off during propagation of the recombinant virus.

7.2.4 Protein in viral preparations

A sucrose-purification technology is required for the development of rMVA vaccines but it is not clear whether these viral preparations carry over unbound recombinant proteins. We investigated this by employing a spinoculation and washing protocol and found soluble luciferase proteins in viral preparations. This was an unexpected find, though might be peculiar to rLuc because studies have shown that some proteins could be incorporated within compartments of vaccinia virions (192, 193). Data from the ELISA and LIPS showed that viruses employing the four endogenous promoters *B8R, F11L, pA44L* and *C11R* were capable of inducing antibodies but we could not establish whether the presence of proteins in viral preparations had any significant contributions. Though informative, it will be worthwhile for future experiments to investigate this further. This is because administration of a mixture of proteins and

rMVA vaccines might improve the immunogenicity of the rMVA vaccine in comparison to rMVA vaccine administered alone.

7.2.5 $CD8^+$ T cells

One principal aim of this project was to investigate if poxviral endogenous promoters such as those studied here could transcribe foreign antigens for the induction of antigen-specific $CD8^+$ T cells. Following single dose MVA and Ad-MVA immunisations regime, viruses employing the endogenous promoters *B8R, F11L, A44L* and *C11R* were capable of inducing Pb9-specific $CD8^+$ T cells equal to or exceeding those induced by p7.5 and SSP recombinants. In addition, after three shots of rMVA four weeks apart, the virus employing the *B8R* endogenous promoter elicited the highest frequencies of Pb9-specific $CD8^+$ T cells in comparison to the other three endogenous promoters. It would have been interesting to see if after 3 doses of MVA immunisation, $CD8^+$ T cells induced by the promoter surpassed that of the viral antigens as demonstrated with the pHyb promoter in the paper by Baur *et al*. (178).

Based on the data presented by Assarson *et al*. (136) and the more recent vaccinia virus deep sequencing mRNA data by Yang *et al*. (166) it was initially hypothesized that the endogenous promoters being investigated might have immediate early advantage for transgene expression over early promoters for the induction of $CD8^+$ T cells. Based on the data presented on the four endogenous viral promoters, it is difficult to correlate the protein expression data in Figure 4.5 with those of the vaccinia virus microarray data (136) and the vaccinia virus deep RNA sequencing study (166). The endogenous poxviral promoters were selected based on the microarray data but careful analysis of the protein expression data from Figure 4.5

shows some discrepancies with these published studies. In addition, there was also disagreement between these two published work in the clustering of *B8R, F11L, A44L* and *C11R* into the immediate early (IE.1) and early (E1.2) clusters in Table 4.1. So it is possible that the reason while an immediate early response was not detected from this work was because (i) the two published worked used vaccinia while this work employed MVA (ii) the two published work quantified mRNA while protein expression from this work was quantified by rLuc expression (iii) both studies used HeLa cells while rLuc expression was quantified in BHK cell lines. A plausible approach to detect the immediate early kinetics of these promoters would be the application of real-time PCR (qPCR), which was not taken advantage of in this work.

7.2.6 Bivalent MVA

Another important aim of this project was to develop a bivalent MVA employing two of the endogenous promoter driven insertion loci for the expression of two model antigens, tPA-Pb9-rLuc8PV and pLuc. The *C11R* and *B8R* endogenous driven insertion loci were taken forward for this purpose because they are well characterized in MVA, found to be genetically stable for transgene expression and viruses employing these promoters were capable of inducing potent Pb9-specific $CD8^+$ T cells as well and antibodies (as shown in chapter 5). The bivalent MVA was capable of inducing potent pLuc and Pb9 specific $CD8^+$ T cells similar to single-pLuc and single-Pb9 recombinants. Further assessment of administration of the multivalent MVA in comparison to the two single recombinants administered as a mixture or at separates sites also showed indistinguishable responses following single-dose MVA and Ad-MVA in the blood. Further assessment of these regimes in the spleen showed that immunisation at physical separate sites enhanced Pb9-specific $CD8^+$ T cells. We also demonstrated that a two-fold dose increase of the bivalent MVA did not improve immunogenicity. Since $CD8^+$ T cells responses induced by vectored vaccine follow a dose-response curve (214) a dose escalation study is required to assess characteristic $CD8^+$ T cells induced by the bivalent MVA. The lack of a definitive antibody assay did not allow assessment of the antibody responses induced by the multivalent MVA. Therefore, future studies employing bivalent or multivalent MVA should take into consideration the choice of foreign antigens and the availability of proteins to enable assessment of antibodies.

7.3 Future directions

7.3.1 Improving expression of endogenous promoters

The data presented in this thesis showed that viruses employing the endogenous promoters of *B8R, F11L, A44L* and *C11R* were capable of inducing potent antigen-specific $CD8^+$ T cells, though the results were rather disappointing as they were expected to elicit unprecedented antigen-specific $CD8^+$ T cells in comparison to the traditional p7.5 and SSP promoters. Based on previous modification techniques applied to the mH5 and pHyb (140, 178) it will be important to modify these promoters for optimal expression of foreign antigens. Previous work has shown that substitution of one nucleotide sequence in the early promoter core region of the natural promoter that drives the H5 gene enhanced the promoter activity (140). This promoter was renamed mH5 and is now considered as the strongest early/late promoter for generating recombinant poxviruses (177). In addition, previous studies have demonstrated that poxviral early promoter elements are A-T rich with a consensus G sequence found at position 6 of the promoter core region. The promoter core region of the *B8R* endogenous promoter show >90% homology with predicted poxviral early promoters from the literature (137, 140). In the B8R promoter core region, there is C nucleotide residue in position 6 which could be modified to G to enhance its activities. A proof of principle study exploring this approach with the *B8R* endogenous promoter is currently ongoing at the Jenner Laboratories.

In addition to this, the paper by Baur *et al*. (178) demonstrated enhanced activity of the p7.5 promoter by arranging five copies the early core element in tandem repeats plus one copy of the late ATI promoter element. They showed that this new promoter, pHyb, induced immediate-early promoter activities that generated high OVA-specific

$CD8^+$ T cells, which surpassed the immunodominant activities of MVA. This approach could also be employed to the newly identified endogenous promoters to enhance their activities.

Another aim of this project was to investigate the ability of strong early or immediate-early poxviral promoters in their authentic position for the expression of foreign antigens to induce $CD8^+$ T cells. While this was achieved it will be important to investigate the ability of strong early/late promoters for the expression of foreign antigens to induce potent $CD8^+$ T cells as well as antibodies. This is because malaria antigens like CSP or ME-TRAP can induce protective $CD8^+$ T cells as well as antibodies, so a strong early/late poxviral endogenous promoter might favor this antigen. To explore this concept, there are several non-essential and non-functional strong early/late promoter-driving genes in MVA such as *F5L, F8L* and *C1*2L that could be harnessed as poxviral endogenous promoter driven insertion loci. Furthermore, for some malaria candidate antigens such as transmission blocking antigens (Pfs25, Pfs27 and Pfs230), strong antigen-specific antibodies are required to achieve transmission-blocking activities. Late promoter driven insertion loci such as the ORF of *A45R* might be a good candidate promoter for the induction of antibodies because studies have shown that antibodies are exclusively targeted against late viral proteins (148, 149). Though it is unclear whether this approach would be able to generate high antibodies comparable to those achieved with the RTS,S and ASO2 adjuvant series.

7.3.2 Development of multivalent MVA vaccines

The development of a bivalent MVA vaccine expressing two foreign antigens from two strong early endogenous promoter driven insertion loci has opened an area in the

field of vectored vaccine research. It will be important to further investigate the application of this approach for diseases of global health important where sub-unit rMVA vaccines have not been able to confer sterile protection such as malaria, HIV, TB and influenza.

7.3.2.1 Malaria

Two sub-unit malaria vaccines developed at the Jenner Laboratories, ME-TRAP and MSP-1 (O'Hara et al submitted, (12)) showed vaccine induced $CD8^+$ T cells as well as antibodies in malaria naïve volunteers and delay in time to patent parasitaemia but were unable to confer sterile protection in all vaccinees. Data represented in this thesis are suggestive that a bivalent MVA approach could be employed to combine the ME-TRAP and MSP-1 to achieve greater efficacy, though there is still the possibility of antigenic competition. The availability of other strong early poxviral endogenous driven insertion loci offers an opportunity to express the AMA-1 malaria antigen in addition to the antigens above. AMA-1 has shown vaccine-induced responses in murine models that were protective upon challenge with sporozoites (60), though no protective efficacy has been achieved with this antigen in field trials (215, 216).

The development of a bivalent MVA vaccine has also opened an avenue for the polyprotein malaria vaccine developed by A. V. S. Hill and colleagues to be revisited. The vaccine was designed based on six malaria antigens (liver stage antigen 1 (LSA), liver stage antigen 3 (LSA3), sporozoite threonine and asparigine rich protein (STRARP), exported protein (Exp1), Pfs16 and the thrombospodin related adhesion protein (TRAP). A sequence coding the six antigens, L3SEPTL, was expressed by a plasmid DNA vector and by two attenuated poxviral vectors, FP9 and MVA under the

control of the early/late p7.5 promoter. The vaccines failed to induce responses to the full-length protein in mice (204) and in naïve malaria volunteers (217) partly due to poor expression from a single promoter. However, responses against individual components of the L3SEPTL polyprotein were generated so it will be important to revisit this vaccine and investigate the applicability of a multivalent MVA approach.

7.3.2.2 Influenza

Preliminary data from a rMVA vaccine expressing NP+M1 developed by Gilbert *et al*. (11) showed vaccine induced $CD8^+$ T cells in healthy adult volunteers that were protective against influenza A virus challenge. Experience gained from this thesis has now been applied for the development of a pre-clinical multivalent MVA expressing NP and M1 from two of the recently identified endogenous driven insertion loci – *B8R* and *C11R*. It is anticipated that the multivalent MVA-NP+M1 influenza vaccine would improve the immunogenicity of the individual antigens and overall efficacy of the vaccine.

7.3.2.3 Tuberculosis

The MVA85A TB vaccine developed by Mcshane *et al*. (218, 219) is the most advanced TB vaccine and is currently being assessed in safety and immunogenicity clinical trial in African children. Although, priming with the BCG followed by boosting with MVA85A has shown vaccine induced protective responses, it will be important to investigate whether expression of other mycobacterial antigens in addition to the 85A employing the multivalent MVA approach would improve overall efficacy of the vaccine.

7.3.2.4 HIV

The HIV_{CONSV} vaccine developed by Hanke *et al*. (101) contains a sequence encoding T cell epitopes from 14 conserved regions of the HIV-1 proteome. The sequence coding the T cell epitopes was expressed in three vaccine vectors, DNA, HAdV-5 and MVA. Heterologous DNA-HAdV-5-MVA in mice showed that the vaccine induced $CD8^+$ T cells as well as $CD4^+$ T cells to individual T cell epitopes but not to the entire polyprotein. The design of the HIV_{CONSV} vaccine should be revisited because it is possible that expressing individual T cell epitope segments (Clade A, B, C and D) from two or more poxviral endogenous promoter driven insertion loci as described in the multivalent MVA approach would improve immunogenicity and vaccine efficacy.

7.3.3 Vaccine towards greater efficacy

Despite the success achieved in pre-clinical and clinical development of individual rMVA vaccines mentioned above, it is important for vaccine researchers to consider the immunodominance effects of these vectored vaccines. This is because administration of three or more rMVAs vaccines in routine immunisation could possibly direct immune responses to the vector rather than the target antigen of interest. To overcome this challenge, a multivalent, multi-disease MVA could be employed to express antigens from malaria, TB, HIV. For example a multivalent MVA expressing ME-TRAP, TB antigen 85A and conserved HIV epitopes might be capable of boosting natural immunity where these diseases are endemic. Alternatively, priming with mixtures of adenoviral vectors followed by boosting with a multivalent, multi-disease MVA might be capable of inducing protective responses against all diseases being targeted. In addition to overcoming reactogenicity and anti-

vector immunity, a multivalent, multi-disease MVA vaccine would be cost-effective as well as increase vaccine compliance.

7.4 Final Remarks

The attenuated poxviral modified vaccinia Ankara (MVA) is a promising viral vectored vaccine candidate and is capable of inducing antigen-specific $CD8^+$ T cells when administered alone or as a boost in heterologous prime-boost with adenoviral vectors as seen malaria, TB, HIV and influenza studies. Most of these rMVA vaccines are based on single antigens from pathogens that cause these infectious diseases but there is the urgent need to develop rMVA vaccines that express more than one antigen. This is because for some pathogens such as malaria, there is the need to express more than one antigen to achieve protective immunity. The work in this thesis described the manipulation of the MVA genome by a recombinant mediated bacterial artificial chromosome technology for the creation of novel poxviral endogenous promoter driven insertion loci. Viruses employing these poxviral endogenous promoters were capable of inducing antigen-specific $CD8^+$ T cells equal or exceeding those elicited by p7.5 and SSP recombinants. Most importantly, these endogenous promoter driven insertion loci were used for the generation of a multivalent MVA vaccine that expressed two model antigens from different endogenous promoter driven insertion loci. The multivalent MVA vaccine was capable of inducing antigen-specific $CD8^+$ T cells comparable to single recombinants as well as administering the single recombinants as a mixture or separately. The work described in this thesis has opened new insights into the design, construction and generation of rMVA vaccines employing poxviral endogenous promoter driven insertion loci for the expression of a foreign antigen. It has also identified new strong early or immediate-early promoters

that should be investigated further for transgene expression. The applicability of the multivalent MVA vaccine for the expression of multiple antigens for malaria, TB, HIV and influenza also needs further investigation.

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Appendix



1.0 Jenner Protocol Number: J211

2.0 Version Number: 2.0 – this procedure remains exploratory and protocol is subject to change.

3.0 Title: Luciferase Immunoprecipitation System

This protocol uses GMOs. All users MUST be registered with the University Occupational Health Service as set out in the University's health and safety regulations. Health surveillance is a legal requirement under COSHH.

If you are not currently registered with the Occupational Health Service for work GMOs, or need to check your registration, please contact the ORCRB NDM Personnel team.

4.0 Other documents

J 211 Appendix IIIS:\PROTOCOLS\Immunology\Pierce TechNote 34 IgG binding protein characteristics.pdf

J211 Appendix I S:\PROTOCOLS\Immunology\SkanIt For Varioskan Flash User Manual.pdf

J211 Appendix II S:\PROTOCOLS\Immunology\Varioskan Flash User Manual.pdf

R001 DNA vaccines and shuttle vectors GMO RA

R004 GMO appendix

J159 v3 S:\PROTOCOLS\Tissue Culture\J159 v3 Use of Leica DMI3000 B microscope

5.0 Definitions

Luciferase: Unless otherwise specified, the luciferase used in this assay is 'rLuc8'- this is a renilla luciferase with 8 point mutations which is active when secreted (though these mutations are not necessary for this assay).

6.0 Objective

Detection and quantification of antigen-specific antibody, usually when antigen suitable for ELISA (e.g. purified recombinant protein) is not available.

Up to July 2011, this method has been used for mouse, rabbit, human and macaque antibodies in the Jenner. However it should be transferable to other species, as long as protein A/G is known to bind that species' IgG. See S:\PROTOCOLS\Immunology\Pierce TechNote 34 IgG binding protein characteristics.pdf

7.0 Materials

Transfection

- Complete media (DMEM + 10% FBS + Pen/Strep + L-Glu)
- Serum-free medium- OptiMEM with no additions (800ul per well to be transfected)
- Lipofectamine 2000 (10ul per well to be transfected)
- HEK293 cells (70% confluent in 6 well plates x3)
- DNA plasmids (with rLuc8)
- Renilla luciferase assay system (Promega: 1000 assays: E2820 £461.00)
    - Renilla luciferase assay buffer (100ml) can be aliquoted e.g. into 7ml Bijous.
    - The lysis buffer supplied with the kit is not needed.
- Halt Protease Inhibitor (Thermo Scientific cat no 78430, also available from Fisher cat no PN78430 £116.67) (x100 conc. 100ul aliq.)
- **Cell lysis buffer** (reference Burbelo et al. 2009 LIPS assay protocol)
    - 50mM Tris
    - 100mM NaCl
    - 1% Triton X-100
    - 50% glycerol
    - EDTA (x100, provided in Halt protease inhibitor kit)
    - For 100ml, mix 5ml Tris, 0.58g NaCl, 1ml Triton-X, 50ml glycerol, 1ml EDTA and 43ml dH_20
    - Add protease inhibitor to lysis buffer just before use
- Solutions for quenching and luminometer washing
    - 2M hydrochloric acid
    - 0.1M hydrochloric acid
    - 0.1M sodium hydroxide
    - Deionized water

LIPS assay reagents

- Renilla luciferase assay system (Promega: 1000 assays: E2820 £461.00)

- Protein A/G UltraLink Resin (ThermoScientific, Cat # 53132, 2ml settled resin, also available from Fisher, cat no PN53132, £241.60)
- **Buffer A**
    - 50mM Tris, 100mM NaCl, 5mM $MgCl_2$, 1% Triton X-100
    - For 100ml, mix 5ml Tris, 0.58g NaCl, 0.102g $MgCl_2$, 1ml Triton X100 and ~94ml dH_2O
    - For 500ml, mix 25ml Tris, 2.9g NaCl, 0.51g $MgCl_2$, 5ml Triton X100 and 470ml dH_2O
    - Store at room temperature.
- 96 well non-sterile V bottom plate (Jenner stores)
- 96 well filter plate MultiScreen HTS with hydrophilic Durapore PVDF membrane Barex/TiO2 non sterile opaque 0.45μm pore size (Millipore, Cat # MSHVN4B50, £599 for 50 plates i.e. £12/plate)
- Serum samples
- Transfected cell lysates containing target antigen.rLuc products (with known luminescence light unit/ml)

8.0 Method

Please read Sections 9 and 10 for notes on how to use the luminometer, luminescence measurement and antigen suitability and cloning before beginning this assay. You will also need to be shown how to use the luminometer dispensers.

Transfection

Advance preparation

1. Make sure you have at least 3ug of all antigen.rLuc plasmids, as well as positive control (luciferase expressing, e.g. #2502) and negative control (non-luciferase expressing, e.g. #2437) plasmids. You will also need 1ug of GFP-expressing plasmid #1733 per transfection.
2. Book cell line TC hood
3. Order 6-well plates with HEK293 cells to be 70% confluent on day of transfection (one well per antigen, plus 2 wells for controls); need to give 3 days notice
4. Prepare complete media (optional) and Burbelo lysis buffer (do not add protease inhibitor yet).

Day 1

1. Mix 3ug of antigen-rLuc plasmid with 1ug of GFP-expressing plasmid 1733 in Eppendorfs or a 24 well plate
2. Add 250ul of Opti-MEM to each DNA and mix
3. Prepare lipofectamine mix: 10ul lipofectamine plus 250ul OptiMEM per sample. Incubate 5mins RT.
4. Mix DNA and lipofectamine solutions and incubate 20min at RT
5. Add 300ul Opti-MEM to each DNA mix to make up final volume 800ul
6. Aspirate media from the 6 well plates completely. Do so carefully, as 293 cells are not very strongly adherent and can be sucked off easily.
7. Add 800ul of the mix to cells gently – pipette very slowly down the side and swirl gently. Do not pipette on aggressively, or the cells will all fall off. Check that all cells are covered.
    a. If they are not, add additional Opti-MEM media (no lipofectamine) to all wells until the cells are covered, mixing by swirling. Record any excess added in this way.
8. Incubate for 2-4hrs at 37°C, then aspirate Opti-MEM and add 1ml 37C complete medium
   This step is optional; good results have been achieved without it.
9. Incubate overnight at 37°C

Day 2

1. Get Renilla assay buffer out to thaw.
2. *Check of transfection efficiency:* Measure fluorescence with BMG machine or view with fluorescent microscope in TC2

-Measure fluorescence with BMG machine

a. OPTIMA software: password = "bmg"
b. Protocol used: 6 Well GFP Scan
c. Excitation filter: 485-12, Emission filter 520, Bottom optic
d. Gain adjusted to 50-70%
e. For result, use data analysis software. Raw value should be around 10 000.

-View with fluorescent microscope

a. View under filter 2 to see green fluorescence (see protocol J159 v3)

Harvest cell lysate (on bench)

3. Carry out this procedure on ice; pre-cool the epp tubes
4. Add protease inhibitor (x100 conc) to the Burbelo lysis buffer
    a. For 1 6 well plate, 85ul protease inhibitor + 8.5ml lysis buffer
5. Carefully aspirate all medium without disturbing cells
6. Add 1.4ml of lysis buffer to each well. Ensure all cells are mobilized + lysed- use cell scrapers if nec.
7. Transfer the lysate to 1.5ml epp tubes (pool if multiple wells of same construct)
8. Sonicate cells (15 sec) e.g. in cleaning waterbath in immunology lab.
9. Centrifuge the cell lysate at 12,500rpm for 4 min spin (ideally at 4°C)

Measurement of antigen-luciferase expression levels

10. Add 20ul of each cell lysate to each of 2 wells of a white luminometer plate
11. Prepare a 1/10 dilution of cell lysate in Buffer A (or cell lysis buffer?); take 20ul into each of 2 wells of the luminometer plate.
12. Add 80ul Buffer A (or PBS?) to all sample-containing wells of the luminometer plate. Allow plate and samples to come up to room temperature.
13. Prepare Renilla Luciferase assay reagent by adding 1 volume of Renilla Luciferase assay substrate to 100 volume of Renilla Luciferase assay buffer in a tube. Allow 50ul per sample plus 1ml for luminometer dead volume if using dispensers. Allow substrate solution to come up to room temperature.
14. Set up the luminometer and prime the dispensers if being used (See Section 9)
15. Place the plate in luminometer and press 'execute session' ('play' button). Make sure it starts to run- it won't do so until you have completed the 'Run Name' dialogue which doesn't always pop up visibly- check the taskbar if nothing's happening.
16. 'Empty' and then 'wash' dispensers, shutdown luminometer and computer. Allow 15-30 min for cleaning procedure. The dispenser heads should be

replaced in their holders, and the blank plugs should be inserted in the dispensing positions. See Section 9 for washing protocol.

Analysis of data

17. Locate the Excel sheet containing the exported data. Using the four wells of data for each sample, calculate the LU/ml in the cell lysate. This must be >$2x10^8$ to permit the assay to be performed.
18. Dilute working aliquots at $2x10^8$ LU/ml and store in -80°C. Check the luminescence each time an aliquot is to be used in the LIPS assay.

LIPS assay

1. Make buffer A- you will need ~1ml per well.
2. Design plate layout, e.g. as below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
B	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	Internal Control	
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22		
G	1	2	3	4	5	6	7	8	9	10	Blank	
H	1	2	3	4	5	6	7	8	9	10		

This is based on the malaria trial ELISA SOP (ML023).

Blue wells= test samples. E.g. 1:100 or 1:300 for unboosted samples, 1:1000 or 1:3000 for boosted samples.
Diluted in naive serum or Buffer A.

Pink wells= standard curve, e.g. 2-fold dilutions from an initial dilution of 1:100 or 1:300 of pooled Ad-MVA immunized serum

Blank: try to include 6 wells with naïve or vector-control-immunized serum at the same dilution as the most concentrated test sample, to set the positive cut-off level

Internal control: a second sample known to be positive could be used to check that consistent titers are obtained when assays are performed on different days. This may not be necessary.

It may be possible to use 2 rather than 3 replicates of each sample, in which case up to 12 samples and a standard curve could be fitted in rows A-D, i.e. use only half a plate to perform an assay.

3. Set up vacuum manifold- ensure the tubes are connected as specified in the manufacturer's handbook- in particular that there is a trap (conical flask) between the manifold and the pump, that the pressure gauge is attached to the longer of the two cross-piece arms, and that the pressure-adjusting gauge is loosened to the minimum-pressure setting (turned fully anticlockwise, at first- pressure can subsequently be increased).

V-bottom plate stage

4. Prepare dilutions of serum **in buffer A** as required for plate layout.
5. Prepare/thaw solution of antigen-containing lysate at 2 x 10^8 LU/ml
6. Add 50ul of lysate (i.e. 1x10^7 LU) per well to a non-sterile V-bottom plate, as accurately as possible.
7. Add 50ul of each serum sample to the appropriate wells of the plate.
8. Incubate for 1hr at room temperature on a rotary shaker *Filter plate stage*
9. Prepare 30% suspension of protein A/G slurry in PBS (5ul per well; this will contain ~15% settled vol of resin)

It is possible to dilute this suspension 1/10 to make a 3% suspension of the slurry and pipette 50 ul/well using a multichannel (keep mixing), then aspirate liquid on vacuum manifold.

10. Add 5ul of beads to the bottom of filter plate wells, using a P200-tip electronic multidispensing pipette. Because the resin settles rapidly, it will be important to keep it well suspended- e.g. shake/VORTEX tube of suspension between adding to every few wells.
11. Transfer 100ul Ag-Ab reaction mixture to the filter plate
12. Incubate for 1hr at room temp on a rotary shaker

NB. It is possible to store your plates at 4°C o/n (or longer) at this point. (After 3 days the results were still good!)

13. Meanwhile, thaw Renilla assay buffer, turn on and configure luminometer and software.

    On opening SkanIt RE software, select 'New session' → User defined protocol →'LIPS assay protocol template'

    Double-check the following (see Section 9 for more details):

    -incubation
    -**plate layout**
    -**report settings**

    Wells can be read immediately after addition of substrate.
14. Prepare Renilla luciferase assay reagent by adding 1 volume of Renilla luciferase assay substrate to 100 volume Renilla luciferase assay buffer. Allow 50ul per well plus 1ml for dispenser dead volume. Add to a conical-bottom universal bottle and insert the aspirating head for dispenser 1.
15. Prime the substrate dispenser (dispenser 1) with the assay reagent and the quenching dispenser (dispenser 2) with 2M hydrochloric acid
16. Insert both dispensers into their correct positions (L1 or L2)

17. Wash the plate x8 with 100ul buffer A, using vacuum manifold (avoid wetting unused wells of the plate- these can be re-used for another assay, another day).
18. Wash the plate x2 with 100ul PBS, using vacuum manifold
19. Fill wells with 50ul PBS so the membrane doesn't dry out

20. Place the plate in luminometer and press ‘execute session’ (‘play’ button). Make sure it starts to run- it won’t do so until you have completed the ‘Run Name’ dialogue which doesn’t always pop up visibly- check the taskbar if nothing’s happening.
21. ‘Empty’ and then ‘wash’ dispensers, shutdown luminometer and computer. Allow 15-30 min for cleaning procedure. The dispenser heads should be replaced in their holders, and the blank plugs should be inserted in the dispensing positions.

Data analysis

No consensus has yet been reached on methods of data analysis.

Positive cut-off should be the average of 6x blank (i.e. naïve control) + 2xSD.

In principle, a similar analysis to that performed for the standard curve ELISA performed for malaria vaccine trials should be appropriate. See SOP ML023. Data can be imported (by a somewhat laborious route) into Gen5 software to allow this analysis to be performed.

9.0 Notes on Luminometer Use

Luminometer

Configure luminometer and software

1. Open SkanIT RE software, username and password = admin
2. Select 'New Session'→User defined protocol→ 'LIPS assay protocol template 110120'→give it a session name→choose 96 well NUNC plate→save it in your folder
3. The following parameters require attention in the software:

   -**incubation temperature** ('Settings' menu → Options → Temperature)- uncheck 'plate start up temperature' so plates are read at RT. Otherwise sample temp will change during course of plate reading.

   -**Plate layout**: specify which wells are to be read. It is not necessary to annotate which wells contain unknowns, controls etc. unless the data will be analysed using the SkanIt software. However, it is necessary to specify which wells contain samples and which don't to avoid adding substrate to empty wells.

   -'**Results' save/ export settings**. Select 'Results' under 'Session' and then 'Report1' under 'Results'. In the main window, check that the report items column includes the readings of interest (e.g. includes 'luminometric', not just 'General Info and 'Layout'). Check under 'after execution' that the report will be exported to an appropriate folder with an appropriate name: e.g. H:\[your desired folder]\LIPS_{run name}_{timestamp}.xls Using a filename which automatically includes the name of the SkanIt 'run' and a timestamp should make exported data identifiable and avoid any risk of files over-writing each other.

   - **Lag time** between substrate addition and reading. Luminescence in a 20% lysis buffer 80% PBS mix are fairly stable from 30s to 2mins. However, to avoid cross-talk, substrate addition/ quenching of individual wells is necessary. Readings at ~30s after substrate addition are optimal. As long as you have not performed a very large number of transfections, a lag-time of 30s can be inserted prior to the luminometric measurement. If large numbers of wells must be read and this would be too slow, the lag time can be shortened e.g. to 10s (luminescence should be at ~90% of peak within this time).

   -**Use of dispensers:** to quench individual wells it is best to use the dispensers. You must initially prime both dispensers (see below). If only measuring a few wells, it is a waste of reagent to use the dispensers as up to 1ml is lost in dead space.
4. For general use, the protocol should be as follows:

   -plate in

   - well loop 1 (shows execution order)

   - **dispense 1**: 50ul from dispenser 1 into position L1

   - **luminometric1:** optics normal, lag time 5 sec, measurement time 1000ms, dynamic range-auto range

- **dispense2:** 100ul from dispenser 2 into position L2

Priming: must be done before use of dispensers

5. Prime both the substrate dispenser (1) and the quencher dispenser (2)
6. Put the substrate reagent mix in dispenser 1 and 2M hydrochloric acid for quenching in dispenser 2.
7. First click 'connect' to connect the luminometer to the computer
8. Go to execute→prime→choose dispenser 1 or 2 and prime with 800ul
   - make sure the dispensers are pointing into a waste beaker
   - as they dispense, check the fluid is going straight down
   - if it is dispensing at an angle, unscrew the outside of the dispenser and replace the black tip (spares taped onto machine)
9. Once primed, carefully insert the dispenser heads into their appropriate positions (**ask someone to show you this the first time)** and remove the waste beakers

Washing the dispensers (also see instructions on side of luminometer)

10. Make sure the dispenser heads are pointing into waste beakers; a plate also needs to be in
11. Go to execute→wash→choose only dispensers 1 and 2
12. Put the aspirate tubing into washing liquid 1 (0.1M HCL), choose 1 cycle and select 'flush'
13. Then choose 'soak' and define the time, i.e. 5 minutes
14. Put the aspirate tubing into distilled water and 'flush' 5 times
15. Put the aspirate tubing into washing liquid 2 (0.1M NaOH), choose 1 cycle and select 'flush'
16. Then choose 'soak' and define the time, i.e. 5 minutes
17. Put the aspirate tubing into distilled water, 'flush' 5 times and then choose 'dispose' to empty the tubing

10.0 Notes on the assay

Notes on luminescence measurement

The Thermo VarioSkan instrument has the capability to measure luminescence, and to perform programmed protocols with a variety of additional steps including substrate and quencher addition to individual wells. The machine and software are relatively complex. **New users should seek the assistance of an experienced supervisor, particularly when using the dispenser options.**

Before using the dispenser options, users should be familiar with the relevant sections of the instrument manual (see links under '4.0 Other Documents') and the software help section: as well as dispensing, those concerning priming, emptying and washing the dispensers.

Of relevance to the LIPS assay, cross-talk of luminescence between wells has been observed when using this machine without quenching: up to 1% of the signal in a sample-containing well can be detected when reading empty adjacent wells. The following measures have been taken to reduce/remove this problem:

-alignment of the plate reader

-development of read protocols involving substrate addition to individual wells, followed by reading, followed by quenching with 100ul 2M HCl. The latter reduces luminescence by 3-5logs within a few seconds of addition.

-modification of reading aperture by Thermo has been requested.

Luminescence kinetics (changing LU readings over time) is an important factor in this assay. Kinetics appear to differ between samples of neat cell lysate (in which readings may not peak within 2 minutes), 20% cell lysate mixed with PBS (in which readings peak ~90s but are within 2% of peak within 30s), and samples of protein A/G-IgG-antigen.rLuc on filter plates (for which readings appear to peak immediately after substrate addition).

Further variables of known or potential importance include the following:

Temperature (effect of which can be avoided if all reagents and luminometer are at RT),

Shaking of sample (which does not appear to increase peak readings when substrate is added to antigen on protein A/G beads, but may affect other samples)

Drying of protein A/G bead samples- further work is needed to define whether this affects LU over a time period of ~10 minutes; **drying can be avoided by adding 50ul PBS to all wells after wash steps.**

Viscosity of sample (cell lysis buffer is relatively viscous)

Chemical constituents of sample buffer: measured LU appears to be up to 10-fold lower for samples in cell lysis buffer than for the same samples in 20% lysis buffer/ 80% PBS.

Antigen suitability and cloning

Antigens should be cloned into Jenner plasmid #2501 (pMono2.FlicV3.rLuc8) between Acc65I and BamHI sites. The antigen should *not* have a secretory signal, nor a stop codon (this plasmid adds luciferase as a C-terminal fusion to the antigen).

Of the antigens tested to date, good results have been obtained with FliC, the FLAG epitope, TRIP (i.e. PfTRAP codon optimised), PfMSP1-19, PfCSN (codon optimised) and PfRH5.

No useful results have been obtained with PfRH1 and PfRAP2, due to very high levels of background in all wells (up to 10^6 LU even without serum addition). This may relate to aggregation e.g. of hydrophobic proteins. No useful results have been obtained so far with PfTRAP either, but this has been a problem of low levels of luminescence.

Intermediate results (ability to detect antigen-specific antibody but relatively poor quantitation due to moderately high levels of background) have been obtained with PfRH4.9 and PfRAP3.