New Approaches for Improving the Immunogenicity of Modified Vaccinia Virus Ankara as a Recombinant Vaccine Vector

Thesis Submitted For the Degree of Doctor of Philosophy
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Abstract

The $CD8^+$ T cell response to a transgenic antigen expressed in Modified Vaccinia virus Ankara (MVA) can be augmented with enhanced transgene expression. This thesis presents different approaches to increase transgene expression in MVA. The transgene used was the sequence of the pb9 murine malaria epitope, fused to *Renilla* luciferase gene, and then fused to a secretion leader sequence (tPA) at the N terminus. The insertion of Internal Ribosomal Entry Site (IRES) upstream of the transgene, driven by the pB8 promoter and inserted at the *B8R* locus, or the modification of the pB8 promoter by nucleotide substitution both failed to enhance the transgene expression or immunogenicity. However, utilising endogenous promoters such as the pB8, pE3, and pF11 at their natural loci to drive the transgene resulted in lower transgene expression *in vitro*, but stronger immune responses to the pb9 epitope in BALB/c mice, as compared to the conventional p7.5 or mH5 promoters. Utilising these promoters at the TK locus (ectopic promoters) revealed similar patterns of transgene expression and immunogenicity, indicating that the improved immunogenicity is linked to promoters despite the low transgene expression measured *in vitro.* Therefore, the pF11 promoter was further studied to determine its activity *in vivo* and showed higher transgene expression, as compared to the mH5, which correlated with *in vivo* immunogenicity. Inactivation of the TK gene in MVAs with endogenous promoters showed reduced *in vivo* transgene expression and immunogenicity. Finally, in a related attempt to enhance MVA immunogenicity, deleting multiple immunomodulatory genes from the MVA genome did not affect MVA cellular immunogenicity in a range of vaccination regimens, testing either the pb9 epitope or the *M. tuberculosis* antigen 85A.

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Dedication

This thesis is lovingly dedicated to my mother, Meznah Alharbi, and to my late father, Khalaf Obied Alharbi who passed away on the 9th December 2014, for their support, encouragement, patient, and constant love that sustained me throughout my life.

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Publication from this thesis

1. **Expression and Cellular Immunogenicity of a Transgenic Antigen Driven by Endogenous Poxviral Early Promoters at Their Authentic Loci in MVA**
   Toritse Orubu, Naif Khalaf Alharbi, Teresa Lambe, Sarah C. Gilbert, Matthew G. Cottingham*
   PLoS ONE | June 2012 | Volume 7 | Issue 6 | e40167

Potential publications from this thesis

1. **Insertion of an Internal Ribosome Entry Site into the Genome of Recombinant MVA Fails to Prevent Decapping of Early Transcript**
   Naif Khalaf Alharbi, Senthil Chinnakannan, Sarah C. Gilbert, and Simon J. Draper.

   Re-Submitted to PLoS ONE after minor revision, March 2015.

2. **The Cellular Immunogenicity of MVA is not Improved with a Deletion Mutant Lacking Fifteen Genes in BALB/c Mice**
   Naif Khalaf Alharbi, Alexandra J. Spencer, Adrian V. S. Hill, and Sarah C. Gilbert.

   Re-submitted to PLoS ONE after minor revision, March 2015.

3. **Enhancing Cellular Immunogenicity of MVA Delivered Vaccines by Utilising the *F11L* Endogenous Promoter**
   Naif Khalaf Alharbi and colleagues.

   Submitted to Molecular Therapy, March 2015.

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β-gal: Beta-galactosidase.

85A: The 85A antigen of *M. tuberculosis*.

aa: amino acids.

A549: Lung adenocarcinoma epithelial cell lines (Human).

ACAM2000: Second generation smallpox vaccine.

AraC: Cytosine arabinoside, a DNA replication blocking agent.

BHK-21: Syrian Baby Hamster Kidney fibroblasts cell line.

BrdU: Bromodeoxyuridine, a thymidine analogue.

bp: Nucleotide base-pair.

CEF: Chick embryo fibroblast.

CMC: Carboxymethyl cellulose.

CPE: Cytopathic effects.

CPXV: Cowpox virus.

Ct, ΔCt, and ΔΔCt: Cycle of threshold in qPCR.

CTL: Cytotoxic CD8+ T cell.

cGMP: current Good Manufacturing Practice.

DCs, iDC and mDC: Dendritic cells, i: immature, and m: mature.

DelIII: The deletion 3 region in the MVA genome.

Dryvax: The first generation smallpox vaccine.

eIFs: Eukaryotic initiation factors.

ELISpot: Enzyme linked immune spot.

EMCV IRES: The encephalomyocarditis virus's internal ribosome entry site.

FBS: Heated-inactivated foetal bovine serum.

GalK: Galactokinase gene as a bacterial selectable marker.

GFP: Green fluorescent protein.

h.p.i: hours post infection.

HEK 293: Human embryonic kidney epithelial cell lines.

i.d.: intradermal injection.

i.m.: intramuscular injection.

i.p. Intraperitoneal injection.

i.v. Intravenous injection.

ICS: Intracellular cytokine staining.

KanR: kanamycin resistance gene as a bacterial selectable marker.

kb: kilo base pair of nucleotide.

kDa: Kilo Dalton of protein molecular weight.

LB: Lysogeny broth media.

MDCK: Madin-Darby Canine Kidney cells.

MDEM: Dulbecco's modified Eagle's medium.

mH5: The modified promoter of VACV H5R ORF.

MOI: Multiplicity of infection.

MVA, nrMVA, and rMVA: Modified Vaccinia virus Ankara, r: recombinant, and nr: non-recombinant.

NP-M1: Fused protein of Influenza nucleoprotein (NP) and Matrix 1 protein (M1).

nt: Nucleotide(s).

Opti-MEM: Reduced serum modified Eagle's medium.

ORF: Open reading frame.

pb9: a murine malaria immunodominant H2d-restricted peptide from *Plasmodium Behrgie* circumsporozoite protein.

pfu: Plaque forming unit.

qPCR: Quantitative real time PCR.

rLuc: Chemoluminescence luciferase protein from Renilla reniformis.

SSP: Short synthetic promoter combined from VACV early and late promoter consensus sequences.

TK: Thymidine kinase.

tPA: The leader sequence of the human tissue plasminogen activator.

VACV CPN: VACV Copenhagen strain.

VACV CVA: Chorioallantois vaccinia virus Ankara strain.

VACV WR: VACV Western Reserve.

VACV: Vaccinia virus.

VARV BSH: VARV Bangladesh strain.

VARV: Variola virus, causative of smallpox.

VERO: African green monkey's kidney epithelial cells.

Chapter 1

Introduction

1. Introduction

1.1. Modified Vaccinia virus Ankara: The virus

1.1.1. History and origin of Vaccinia virus and Modified Vaccinia virus Ankara

On the 14thof May 1796, Edward Jenner used a cowpox pustule from the hand of a milkmaid in Gloucestershire, England to vaccinate an eight year old boy (James Phipps). Jenner later challenged the famous boy with live smallpox, caused by variola virus (VARV), but the boy remained healthy. Although the practice of vaccination could arguably have preceded Jenner, he was the first to document it and communicate it to the scientific community at the time. Hence, Jenner is known as the father of immunology and often called the father of vaccinology too. Two centuries later, the world health organisation (WHO) succeeded in the eradication of smallpox from the planet using Vaccinia virus (VACV), which is distinct from cowpox virus (CPXV) [1]. Therefore, Jenner's vaccine could not be CPXV although it came from an infected cow. Jenner himself believed the vaccine he used was originally from horse heel infection that was passaged through cows and became suitable for human vaccination[2].Many hypotheses have been suggested to define the origin of VACV. For example, the presence of cowpox host range genes in the genome of some strains of VACV, such as western reserve (VACV WR) strain and Modified Vaccinia virus Ankara (MVA) could suggest that VACV and CPXV have a shared ancestor [3]. Horsepox virus has also been suggested as the origin of VACV[1], supporting Jenner's belief. However, the origin is very difficult to identify because there is no natural host for VACV [2,4].

Modified Vaccinia virus Ankara (MVA) was attenuated during the 1960svia passaging a VACV strain more than 570 times in chick embryo fibroblast primary cells (CEF). The strain Chorioallantois Vaccinia virus Ankara (CVA), which was obtained from a pox lesion from a horse in Ankara, Turkey [5,6], was used for the attenuation by Anton Mayr in the Institute for Infectious Diseases and Zoonosis at the Ludwig-Maximilians-University of Munich, Germany. MVA proved safe in animal models, including neonatal and irradiated mice, and primates[5,6]. It was then used in more than 120 000 people in southern Germany and Turkey, during the Smallpox eradication campaign (1966-1980)[6,7].There was no adverse side effect, no viral excretion from vaccinated humans, and MVA produced small white pocks unlike the large ulcerated pocks by VACV[6-8]. This was the first documentation of MVA safety in humans.

Although MVA was used in humans during the WHO programme, it did not replace the Vaccinia virus during the campaign. MVA is considered the third generation of smallpox vaccines, which proved efficacious in animal models challenged with WR VACV and CPX[9]. IMVAMUNE vaccine, a MVA strain developed by Baverian Nordic, is under the late stages of licensure [10], and the USA suggests it as a vaccine of choice against any potential smallpox threat for immunocompromised individuals [11].

1.1.2. Virus morphology and entry

MVA, similar to other poxviruses, has two distinct forms of mature virion: intracellular mature virion (IMV) and extracellular enveloped virion (EEV), which has a double membrane. Both forms can be released from infected cells by cell lysis or budding, respectively. The EEV form has six membrane proteins, whereas the IMV form has twenty proteins. These proteins facilitate the cell entry of both forms by binding to glycosaminoglycans or laminin on the host cell surface, although the EEV has never been studied properly due to its fragile nature. The IMV is more stable and much abundant form of the virus and responsible for host-to-host transmission. In terms of entry, the EEV is understood to lose its outer membrane before binding to the plasma membrane. Then, the EEV with now a single membrane, located on the cell surface behaves in a similar way to the IMV that has naturally a single membrane. Both forms are, then, fused with either the plasma or the endosomal membrane, allowing the core virion to enter the cytoplasm.

The uncoating takes place to release the genomic DNA as well as the early viral transcription machinery although some early transcription is believed to occur within the core virion before the uncoating. The expression of early genes occurs before DNA replication, which are both followed by the intermediate and late gene expression. DNA replication occurs in defined foci in the cytoplasm called the “virus factory”, and synthesised proteins assemble in a crescent-like form, which is then changed into a rounded immature virion (IV). The IV subsequently loses its main surface honeycomb-like D13 protein, gains other proteins on the surface, and converts into a barrel-shape intracellular mature virion (IMV), which has the poxvirus envelope around it. This IMV

either exits by cell lysis, or goes into the trans-Golgi membrane and gains a second double membrane, resulting in a wrapped virion (WV) with a triple membrane. The WV then loses one membrane by fusing with the cell plasma membrane and buds out of the cell as the EEV form, with a double membrane. The EEV can be also located on the cell surface, on the tip of actin-tail (protrusions),and then moves between cells, when it is called the cell-associated enveloped virion (CEV) [12]. The life cycle of vaccinia virus is illustrated in figure 1.1. MVA life cycle in non-permissive cells is blocked after the formation of IV forms, with no production of IMV forms.

The attenuation of MVA did not affect its morphology very much and the majority of MVA structural, membrane, and core proteins are conserved, apart from only three mutated proteins: A36, D3, and A4. Antoine *et al* (1998) suggested that replacing these proteins with their wild type counterpart could improve MVA morphology and optimise the physical properties of the virus [3]; however, this has not been tested.

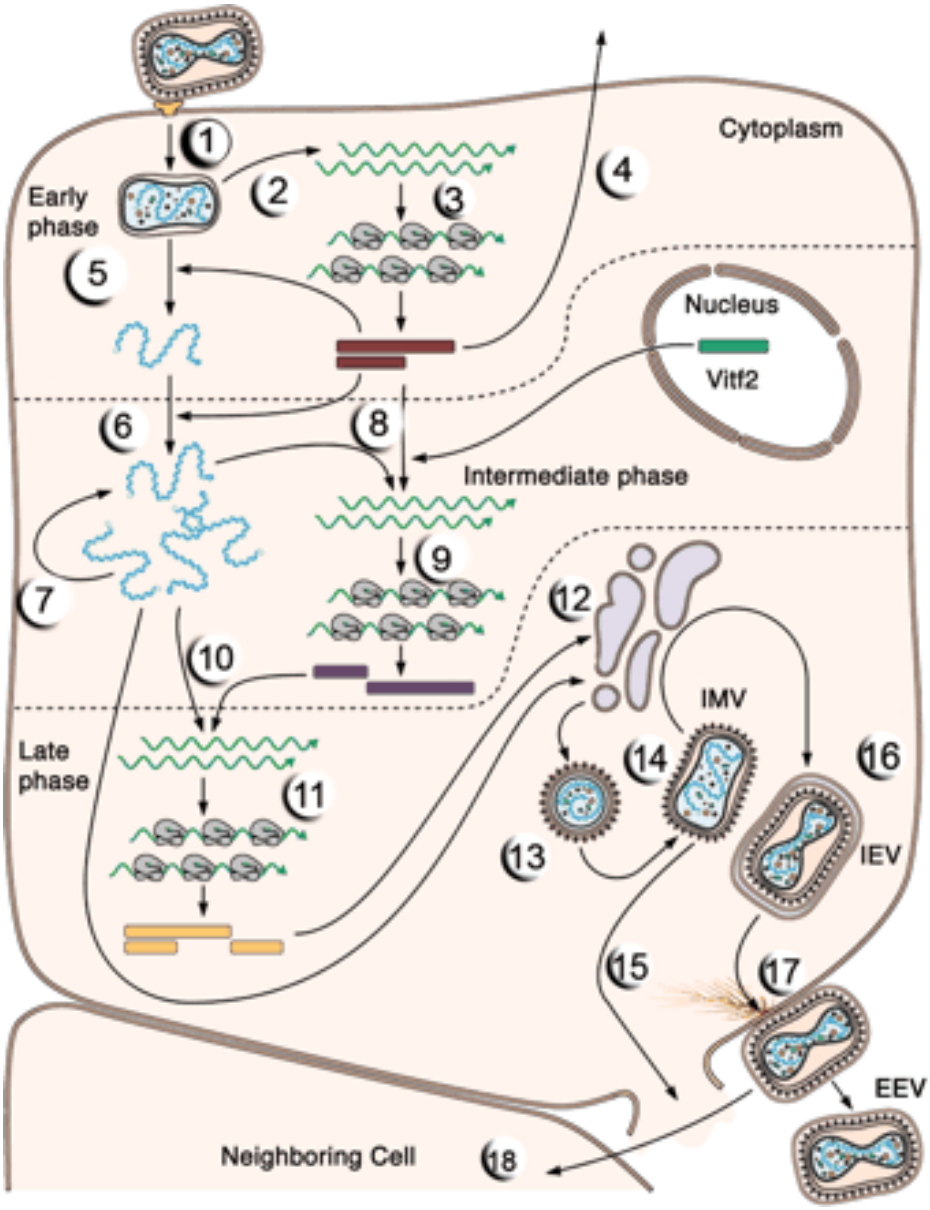


Figure 1.1: Vaccinia virus life cycle

Different stages of VACV life cycle, which is also similar to MVA infection in permissive cells, are illustrated. In non-permissive cells, MVA infection halts after the formation of IV forms (i.e. between steps 13 and 14). Dotted lines show gene expression stages, which occurs in a sequential manner (early, intermediate, and late). Early gene expression occurs within the virion and directly after the uncoating stage using poxviral transcription machinery that recognises early promoters, which are thought to enhance cell-mediated immunity in contrast to late gene expression and late promoters as discussed throughout the thesis. The figure is adopted from Harrison *et al* [13].

1.1.3. Genome, DNA replication, and gene expression of MVA

MVA, in common with all poxviruses, has a double-stranded linear DNA genome with closed hairpin structures at the termini. The MVA genome was sequenced in 1998 and was not compared to its parent CVA, which was sequenced ten years later[14], but rather to VACV Copenhagen strain (CPN)[3]. This comparison revealed numerous point mutations and internal deletions as well as seven large deletions, relative to VACVCPN (table 1.1). However, it is known that MVA has only six large deletions when compared to its parental CVA virus[15].The MVA genome is 178 kb (kilo base-pair) in length and encodes 177 genes. Twenty five genes are mutated in MVA, resulting in truncated proteins[3].

The nomenclature of genes in MVA follows that of VACV CPN. For historical reasons, it is based on resultant fragments from a HindIII restriction map where each fragment is given an alphabetical label(A being the biggest fragment), and then a serial number, from left to right, for each ORF within a fragment. ORFs have L (left) or R (right) letter indicating the expression direction, whereas proteins are distinguished by omitting the L or R letters of their genes. For example, MVA ORF 3 in the fragment E that is expressed towards the left terminus is the *E3L* that encodes the E3 protein.

The genome consists of three regions. The central region contains many conserved genes, which are involved in essential activities of the virus such as DNA replication, gene expression, and virion morphogenesis. This central region has only three mutated ORFs:*F5L*, *F11L* and *O1L*, resulting in truncated proteins. The left terminal region,

which is the most affected by the attenuation, has four large deletions relative to VACV CPN, or three deletions relative to CVA[15] as well as a large insertion that matches cowpox virus and has cowpox virus host range genes. The majority of genes in the left region of the genome are mutated or fragmented by internal deletions, leaving only 8 intact genes that could be functional. The right terminal region has three large deletions and many fragmented genes[3]; see the summary in table 1.1 for more details.

The boundaries of the three regions are as follow: the left region extends from the left hairpin loop to the *K6L* ORF, the central region from *K7R* to *A24L* ORF, followed by the right region, from *A25R* to the end of right hairpin loop. The inverted terminal repeats (ITR), found in all poxviruses, are covalently closed, identical but inverted at each end of the genome with a hairpin loop structure. In MVA, the ITRs are around 9.8 kb in length and have three repetitive regions, two of them (called RII and RIII) have repeats similar to one another, which is clearly a result of duplications, whereas repeats in the third repetitive region of the ITRs (the RI) are slightly different [3].

Poxvirus genome replication takes place in the cytoplasm, starting with a nick close to the end of the genome. A single strand is then used as a template to start the replication by covalently linked primers forming a lagging strand. Because the two strands of poxviral DNA are linked together with a hairpin loop at each end, the DNA replication results in a long concatemer that is then resolved by a viral endonuclease into individual linear genomes, before DNA packaging. Almost half of the newly

synthesised DNA, which is about 10000 copies per cell, is packaged into progeny virions. DNA replication, DNA packaging, gene expression, and virion assembly take place in the virus factory [16].

The expression of vaccinia genes (as in all poxviruses) occurs in a cascade-like temporal manner, starting in the core virion with the early gene expression that also continues after the uncoating stage (Figure 1.1). Within the virion, there are some non-structural proteins that are involved in the early transcription like the RNA polymerase and capping enzyme. This early phase produces proteins involved in DNA replication and intermediate gene expression as well as immune modulation. After the DNA replication occurs, the intermediate genes are expressed with help from intermediate transcription factors that are produced from the early gene expression. This intermediate expression then produces transcription factors required for the late expression, which in turn, provides early transcription factors that are packaged into new virions. Post-DNA replicative gene expression, intermediate and late, produces structural proteins involved in virion morphogenesis and assembly[17]. Different promoters that are divided into the same classes (early, intermediate, and late) control this sequential expression. The required transcription factors for each class of these promoters are products of the preceding stage. However, some genes have tandem early and intermediate or early and late promoters while some have a promoter with two active core regions (early and intermediate or early and late)[18,19].Poxviruses do not possess introns or splicing sites, which could be related to their evolution as cytoplasmic-replicating viruses[20]. Moreover, the sequence required for eukaryotic

translation initiation (a.k.a. Kozak sequence) is not required for vaccinia virus translation [20].

Attenuated ORFs	Details
Split ORFs *	• **Left**: *C17L*, *C9L*, *K5L*, *K6L* • **Central**: *F5L, F11L,* and *O1L* • **Right**: *A39R, B2R, B4R, B13R, B14R,* and *B23R*
Deleted ORFs	**Left: 4 deletions **** • d1771[deleting *C22L*to*C19* ORFs] in the left ITR. • d3585[deleting *C16L* to *C12L* ORFs] still in the left ITR. • d4817[deleting *C5L* to *N1L* ORFs]. • d2763[deleting *M1L* to *K1L* ORFs] **Right: 3 deletions** • d3501[deleting *A51R-A55R*], a.k.a. DelIII • d809[*B20R*] • d1771[deleting *B25R-B28R*] in the right ITR
Intact ORFs	• **Left:** 8 intact (out of 27 ORFs) • **Central:** All, except *F5L*, *F11L* and *O1L* • **Right:**8 intact ORFs

Table 1.1: The attenuated ORFs in MVA, adapted from ref. Antoine *et al*[3].

* Relative to VACV Copenhagen (CPN) because there are some fragmentations relative to VARV Bangladesh strain (VARV BSH). ** When MVA was compared to its parental CVA, only six deletions were reportedMeyer et al. [15]. Left, central, and right indicate the location within the MVA genome.

1.1.4. Viral replication of MVA

MVA was originally passaged in CEFs, and was reported to undergo abortive infection in most non-avian cells[6].However, it can grow efficiently in one mammalian cell line, the baby hamster kidney fibroblast (BHK-21). Interestingly, MVA grows and produces higher yield than the parental Vaccinia virus in the CEF and BHK-21 [21]. There are a few mammalian cells that can allow for some degree of MVA replication (1-2 log lower than that observed in CEFs), these cells are referred to as semi-permissive, such as the African green monkey cells CV-1 and BS-C-1 [21,22].In non-permissive mammalian cells, like HEK293, HeLa, and CHO cells, MVA can infect and cause a rapid and severe cytopathogenic effect (CPE), causing complete detachment of the cells after 24 hours, but it does not complete its replication cycle. MVA replication in non-permissive cells ceases at the virion assembly stage after it replicates its DNA genome and expresses all early and late genes [23].

Like other mammalian cells, all tested human cells are non-permissive to MVA, which was reported to produce lower than 10 pfu per 1 pfu input in human cells[21]. Some studies reported MVA replication in human cell lines, such as HeLa, but not in MRC-5 primary human cells[24], whereas others reported the opposite [25]. Drexler *et al* also showed a very limited replication of MVA in HeLa and HEK 293 cell lines [26]. However, Cottingham and Carroll warned that these studies never showed more than 10 pfu per 1 pfu input, which is clearly a limited replication that is likely to be due to a laboratory contamination with vaccinia virus[27]. They pointed out that MVA replication in human cells (including HeLa, HEK 293 and TK-143B cells) did not occur when a defined clonal MVA, identical to the five deposited genomic sequencing data, was used

[28].Clearly, MVA is replication-deficient in human cells, and any growth study should be careful about the clone purity of the inoculum.

With regards to non-mammalian cells, beside CEF, MVA was reported to replicate efficiently in other avian cell lines like the QT35 quail embryo fibroblast [21].In the last decade, quail cell lines, permissive to MVA, were developed using specific free serum media to support large-scale MVA-based vaccine manufacturing. These cells include the Avian EB66® (from Vivalis, France)[29], the AGE1.CR (ProBioGen, Germany)[30,31], and theQOR2/2E11 quail cell line (from Baxter, Austria) [32]. Currently, MVA-based vaccines are produced in primary CEF cells that are both time and cost consuming; it also requires a large number of eggs and a rigid quality control screening to avoid any contamination. However, validating any of these cell lines for current Good Manufacturing Practice (cGMP) standards should enable large-scale MVA-based vaccine manufacturing at a lower cost and faster time, enabling much greater development in the use of MVA vectors.

On the other side of this issue, manipulating MVA to grow in cell lines that are already validated and used for clinical trials, like the VERO (African green monkey's kidney epithelial cells) and MDCK cells (Madin-Darby Canine Kidney cells) would accelerate the manufacturing process. MVA does not replicate in either of those cell lines, but Melamed *et al*[33]reported a MVA recombinant that grows in VERO cells. This recombinant was made using cosmids to provide around 20 kb of the parental CVA genome that matches the left region of MVA genome. It was not characterised as to

which gene is responsible for the extended host range recombinant[33]. If a single clone of such a recombinant with a defined genetic property is produced and used as a viral vector, it could also make a great impact on manufacturing MVA-based vaccine, provided that the safety profile of the vector is not altered.

1.1.5. Host-virus interaction: MVA Immune evasion

Vaccinia virus has evolved to express immunomodulatory proteins that are either intracellular or secreted from infected cells. The intracellular proteins interfere with some pathways, whereas the secreted proteins function as soluble receptors to counteract immune mediators, allowing VACV to evade immune responses and spread within the infected host. Between a third and half of the VACV genome encodes immunomodulatory proteins, summarised in table 1.2 with their functions and genetic status in MVA. Many of these proteins co-target a specific pathway while some proteins have multiple functions in different pathways like the *E3L* product[4]. VACV can counteract the anti-viral interferon (IFN) system by blocking the induction of IFN in many ways, which include blocking the intracellular pathways of NFκB or IRF3 by different VACV proteins (see table 1.2), leading to the inhibition of interferon expression, especially IFN-β. It could also express extracellular soluble IFN receptors that can neutralise the effect of IFNs (type I, II, and III) on neighbouring cells. VACV also expresses soluble receptors that bind and block CC-chemokines and complement proteins. Moreover, it expresses decapping enzymes (encoded by *D9R* and *D10R*) that decap the cellular mRNA, reducing the cellular protein expression[4]. These decapping proteins are also involved in regulating the viral sequential expression by decapping viral mRNA (described in depth in the introduction of chapter 3). The multi-functional immunosuppressive E3 protein binds to ds-RNA and prevents the activation of dsRNA-dependent protein kinase R (PKR), blocking interferon expression as well as blocking apoptosis induction. The PKR activation is also prevented by another VACV protein (coded by *K3L*), which can act in the absence of E3 protein, allowing for redundancy in this immune evasion interaction[34]. However, VACV is still immunogenic despite all these immunosuppressive proteins.

However, immune evasion by MVA is greatly reduced since it has lost many of VACV immunomodulatory genes due to complete deletion, or in most of the cases, due to gene inactivation by point mutations or internal deletions (table 1.2). Therefore, MVA is unable to express functional receptors, for some immune mediators, such as IFNαβR, IFNγR, CC chemokine binding proteins, or TNFαR[24], but it has retained some immunomodulatory genes [3,24]. This could explain the strong immunogenicity of MVA despite its replication deficiency. The completely intact immunomodulatory genes in MVA include: *K3L, E3L, A41, A46, A49, K7R, C6L, C7L, H1, B16R*, and 008L ORF, which is VACV WR *C12L*but not present in VACVCPN[3]. However, mutated genes could still function as was the case with *C12L* (MVA008 ORF) that increased MVA immunogenicity when deleted, suggesting that the *C12L* gene could be active [35]. In contrast, only a small deletion in the retained ORFs might inhibit their function as was demonstrated with MVA *B14R* ORF, which was non-functional due to a 6 amino acid deletion [36].Many of the retained genes have been experimentally deleted to improve MVA immunogenicity, discussed elsewhere in this introduction and chapter 7.

1.1.6. Host-virus interaction: Host range factors and other interactions of MVA

MVA attenuation and inability to replicate in most cells could be a result of lost host range genes, which are located in the left terminus of the genome, the most affected region [3]. Host range genes are possessed by poxviruses and determine their viral replications in a given host (tropism). The human host range genes include *K1L* and *C7L* genes. The *K1L* ORF is partially deleted in MVA[3] and the reinsertion of a DNA fragment containing *K1L* ORF into a host range VACV mutant, which lacks 18kb of the left genomic region[37], allowed this host range mutant to grow in human HepII cells. Thisidentified*K1L* as a human host range gene in VACV[38,39].However, in MVA, Carroll and Moss have tested a MVA mutant with a restored *K1L* in ten non-permissive mammalian cells and reported that the reinsertion enabled MVA to grow only in the rabbit kidney RK13 cells[21]. This was also confirmed later by Wyatt *et al* [25], suggesting that MVA needs more than the *K1L* to replicate in human cells. The *C7L* gene was also shown as a human host range gene, which has 100% identity to VACV WR and is identical across abroad range of VACV strains [39,40]. In both VACV and MVA, the *K1L* is deleted but *C7L* is intact [3]. However, the deletion of *C7L* alone is not enough to render VACV replication-deficient in human cells [40].

In total there are around 12 host range genes (not necessarily human host range) identified in VACV that include *K1L*, *K3L*, *E3L*, *B5R*, and the gene families of *C7L*, TNFR II, serpins (cellular serine protease inhibitors) [41,42], some of which are intact in MVA. Some functions of host range genes have been identified, such as the E3 protein as discussed above. Products of these genes play important roles in defining the tropism

of Vaccinia virus (and poxviruses in general).Poxvirus tropism is not defined by cellular surface receptor used for virus entry, but rather by interactions with (and manipulations of) intracellular targets of the host range factors. These targets include cellular kinases and phosphatases, apoptosis, and various antiviral pathways [43].Nevertheless, MVA's inability to replicate in human cells is not only dependent on the affected host range genes, but also on the genetic make-up of MVA as suggested by some reports[3,15].

Other MVA genes that could be involved in host interaction include the *A40R* ORF, which has a natural killer cell (NK) receptor homolog [3]. The*K4L*ORF is also an intact gene that encodes a protein with a homology to human phospholipase D[3]although there was no phospholipase activity of MVA K4 protein[44]. Ankyrin repeat-containing proteins are found in poxviruses and could also be involved in host-virus interaction [45]. However, all Ankyrin-like genes are fragmented in MVA, except *B18R*that encodes a 68kDa protein [3].

1.2. Modified Vaccinia virus Ankara: The recombinant vector

Viruses have been used as vaccines, either inactivated (killed) or attenuated to vaccinate against diseases that are caused by the same virus such as vaccines against influenza, which can be inactivated influenza vaccines (IIVs) or live attenuated influenza vaccine (LAIV). Although attenuated viruses can induce strong immune responses, they are able to replicate in humans and may not be safe vaccines. In contrast, replication-defective viruses or recombinant vaccines are safe but could fail in inducing sufficient immunogenicity to confer protection (Figure 1.2).A number of viral vectors, encoding recombinant heterogeneous antigens, have been developed for human and veterinary infectious diseases as well as cancer; and showed improved cellular and antibody-mediated immunity. These viral vectors include: adenoviruses, bacteriophages, Newcastle disease virus (NDV), Turkey herpes virus (HVT), Yellow fever virus (YFV-17D), lentivirus, Sendai virus, measles virus, attenuated canarypox virus, fowlpox virus (FPV), and Vaccinia virus (VACV).Some of these vectors have been tested in human clinical trials, including human adenovirus type5, 6, 26 and 35, chimpanzee adenovirus subtype 3, type 63, and type Y25 (called ChAdOX1 as well), lentivirus, YFV-17D, ALVAC (canarypox virus), VACV (for cancer only), and attenuated strains of VACV such as MVA and NYVAC (attenuated VACV CPN strain). Flavivirus YFV-17D (live chimeric virus) as a vaccine against Japanese encephalitis virus (developed by Sanofi Pasteur) is the first and the only viral vector-based vaccine licensed for human use. However, some vaccines have been licensed for veterinary use based on other viral vectors like ALVAC,FPV, NDV, HVT, and VACV, in addition to flavivirus YFV-17D.

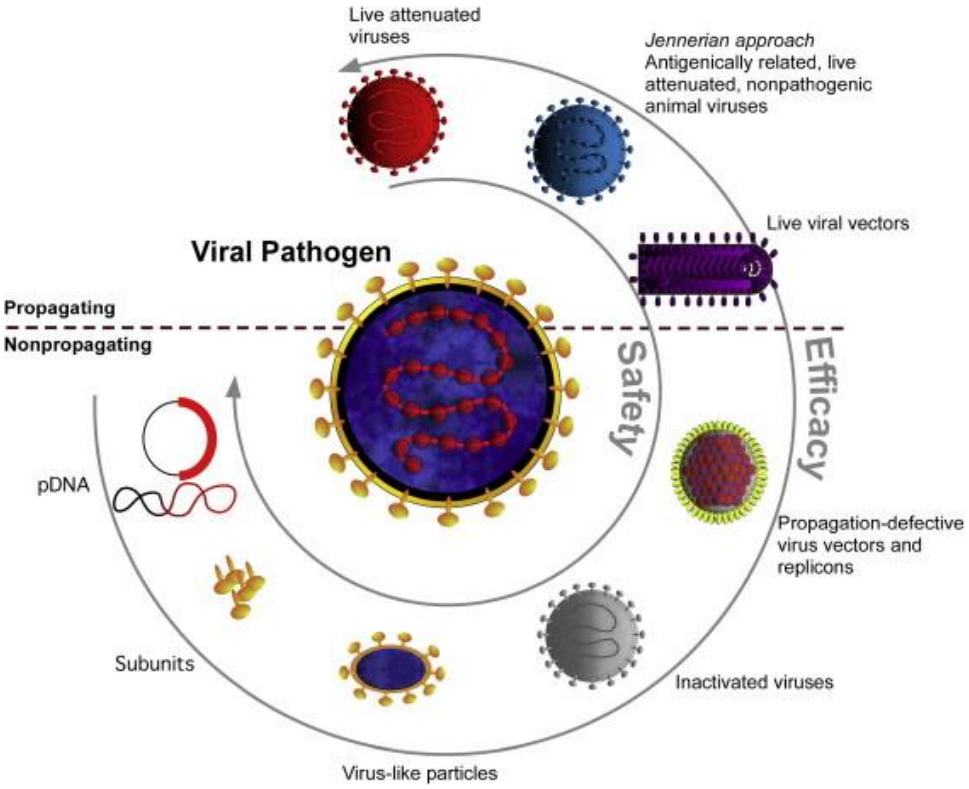


Figure 2.2: Vaccine platforms.

Platforms used to make vaccines are arranged in a circular diagram to illustrate their unique characteristics that influence both safety and efficacy. The figure is subdivided by the dashed line into viral vaccines that replicate in human and those that do not. From Koff *et al* [46].

1.2.1. Poxvirus vectors

Poxviruses are among the most studied and tested viral vectors, especially FPV, ALVAC, VACV, and VACV attenuated strains like NYVAC and MVA. VACV is the first and most extensively studied poxvirus vector. Non-essential ORFs in VACV were used for the insertion site to deliver foreign antigens in the early 1980s [47]. Because of its large genome, VACV can accommodate large DNA and still replicate in a variety of cells and cell lines, whereas small viruses may have limited host range and their infectivity could be reduced due to foreign gene insertion [20]. There is no need for deleting some of the vector genes to accommodate transgenes, an approach applied to adenovirus, SV40 and other retroviral vectors[20].

VACV was first used to express foreign antigen in 1982 by Mackett, Smith, and Moss [48], and by Panicali and Paoletti [49].It was then used by the same research groups to raise antibody against glycoproteins and surface antigens of different viruses, including hepatitis B virus[50]and Influenza virus [51,52].By 1984, these groups and others had moved to test VACV-based vaccines against influenza [51]hepatitis B virus [53], HSV [54], and rabies virus [55].A polyvalent vaccinia vaccine was also constructed, encompassing the herpes simplex virus glycoprotein D, hepatitis B virus surface antigen, and the influenza virus haemagglutinin, inserted at three different sites, showing an advantage of VACV that rivals many viral vectors. It induced reactive antibodies against all three antigens in rabbits, vaccinated either intravenously (i.v.) or intradermally (i.d.)[56].

Veterinary rabies vaccine is the only VACV-based licensed vaccine so far. In human, recombinant VACV (rVACV) has been tested only in cancer vaccine trials because the safety concerns over VACV replication ability is not a major issue in cancer patients. For prophylactic vaccines against infectious disease, more attenuated, but immunogenic strains of VACV, like NYVAC and MVA, have been tested in humans[57].In addition, other attenuated non-VACV poxviral vectors like FPV and ALVAC are also being developed and tested in humans[58].NYVAC is a replication-deficient VACV CPN strain that has 18 deliberately deleted genes and has a different genetic make-up from MVA; however, it shares some deletion similarities with MVA, including d4817deletion (*C5L-N1L*), *B13R and B14R* (ICE inhibitor), remnant of *A26L* (ATI), and *K1L*[59]. But, unlike NYVAC, MVA has intact *C7L, C6L, A56* (Haemagglutinin), *I4L,* and TK ORFs[3]. Some of the deletion in NYVAC improved its immunogenicity, such as type I IFN receptor gene (*B18R* WR (*B19R* in MVA)) and type II IFN receptor gene (*B8R*), but these are already deleted in MVA [58].

1.2.2. MVA as a vector

Recombinant MVA (rMVA) has been used since 1994 to deliver vaccine against infectious diseases and cancer. It was first used for protein expression when Sutter and Moss showed that rMVA expressed a recombinant protein to a level similar to that of the same protein expressed by VACV WR, using the same promoter (p11)[23]. The recombinant protein level expressed by rMVA was even similar in non-permissive cells, which made MVA a highly effective expression vector. MVA was also used to express T7 polymerase under the control of p7.5 promoter (a.k.a. T7/MVA expression system) that resulted in a recombinant protein expression level similar to that achieved with T7/VACV system [60,61].

Subsequently, MVA was used to deliver antigens to vaccinate against infectious diseases; first against influenza by Sutter *et al* in 1994, [62], and many vaccines were then tested using the MVA vector in the following years[63-67]. It was also tested in monkeys as a trivalent recombinant vaccine vector[66]. MVA was then tested as a vector for a cancer vaccine by Carroll *et al* in 1997[68]. In the last decade, MVA has gone into a boom as a viral vector and tested in mice, rabbits, dogs, horses, sheep, goats, cows, camels, and humans. Although, its first clinical use in humans was as a smallpox vaccine in the 1970s, a recombinant MVA encoding a malaria vaccine was first tested by Oxford University in 2000 [69].

The choice of a poxviral vector is very often made between the avipox viruses such as FPV or ALVAC, or non-replicating Vaccinia virus strains such as MVA and NYVAC. Avipox

viruses are host-limited to birds and do not replicate in humans and other mammals; therefore they are considered safe vectors. ALVAC was dominantly used in HIV vaccine trials even though other vectors have also been tested[58],whereas MVA was used mainly as a boosting vector in prime-boost vaccination regimens in HIV [70], Influenza [71], TB, and malaria [72,73]vaccine trials.

1.3. Modified Vaccinia virus Ankara vector: The advantages

Using MVA as a delivery vehicle for vaccines can be advantageous in many aspects. First, MVA can replicate its DNA genome in human non-permissive cells and the expression of recombinant proteins is not affected, unlike NYVAC or avipox viruses, which are naturally host-restricted and cannot replicate their DNA in mammalian cells [23]. Second, MVA is a very safe virus and showed no adverse events when tested in immune deficient non-human primates, even with high doses[5,6]. In immune deficient mice, MVA was safe even though it was given 1000 times more than the lethal dose of VACV Wyeth strain[74]. In man, MVA was first used during the 1970s as a smallpox vaccine with an excellent safety profile[7]. It was used again in humans as a recombinant vaccine to deliver a malaria antigen and showed acceptable safety with no serious adverse events or severe non-serious adverse events. Its safety was also confirmed by showing undetectable levels of viral DNA, both systematically and in the inoculation site [69]. In immuncompromised HIV-infected humans, MVA was also a safe vector [75,76]. Third, VACV vectors can accommodate large quantities of transgenic DNA, this could be up to at least 25kb without any effect on the viral growth or infectivity and with no compensatory deletions [77]. Considering that MVA has already lost around 30kb of its genome, this should increase its capacity to take up to at least 55kb as has been suggested [20]. Fourth, MVA is a highly immunogenic viral vector. It can induce potent innate, adaptive humoral, and adaptive cellular immune responses, despite its inability to replicate in animals or humans. Evidence showed that MVA immunogenicity sometimes rivalled that achieved with VACV, as discussed below.

3.2.1. Immune responses to MVA

Studies on MVA immunogenicity are either comparing it to the replication efficient VACV as a third generation smallpox vaccine or as a viral vector compared to other viral vectors like recombinant VACV and NYVAC. In most cases, MVA is compared to the licenced smallpox vaccines; Dryvax (VACV Wyeth strain) and ACAM2000 (VACV Sanofi Pasteur strain). Although these are mainly data on adaptive immunity, there are some studies on the innate immunity elicited by MVA in comparison to VACV WR strain.

1.3.1.1. Activation of innate immune responses

MVA can infect human monocytes, B cells, and dendritic cells (DCs) and to lesser extent NK cells *in vitro*; this leads to the activation of monocytes with up-regulation of co-stimulatory molecules and MHC-II, and to a partial activation of immature DCs, where B cells remained inactivated[78]. MVA can efficiently infect mature and immature dendritic cells (iDCs and mDCs) [79], both of these populations can process and present high level of recombinant antigens delivered by MVA and this stimulates peptide-specific T cells, priming the T cell immune responses. Furthermore, in human monocyte-derived DCs experiments *in vitro*, iDCs are more susceptible to apoptosis upon MVA infection[79]. MVA (as well as VACV) infection halts the maturation of human monocytes-derived iDCs even though these cells could still process and present recombinant peptides[79,80]. This finding was also observed in mouse bone marrow-derived iDCs (*in vitro*); however, it could be different *in vivo* since MVA infection induced DC maturation in mice [81] and the strong activation of immune responses suggests that DCs were activated *in vivo*. Nonetheless, the activation of DCs *in vitro* is

still unclear due to many variables in this research; for example, mouse versus human DCs, bone marrow derived versus splenic DCs or monocytes-derived DC (for human), incubation time, markers used for read-out, and MOIs. The latter was shown to be important in activating DC *in vitro* and showed up-regulation of MHC-I, MHC-II, and co-stimulatory molecules like CD80 and CD86 [80], whereas in another study, only low MOI was able to activate DCs [82]. On the other hand, MVA-infected mDCs were resistant to apoptosis, expressing the surface marker CD83 (maturation marker); and CD86 and CD80 (co-stimulatory molecules) unlike VACV-infected mDCs, so the living infected mDCs were able to stimulate T cell responses[79]. Beside the co-stimulatory molecules, mDC cells infected with MVA express MHC-I, MHC-II[83].

However, while the infected APC (including iDC and mDC) would eventually die, the uninfected DC (called bystander DCs) could mature by engulfing viral antigens, processing and presenting viral peptides on MHC class I in cross-presentation manner, which leads to stimulation of $CD4^+$ and $CD8^+$ T cells. When they are activated, bystander DCs exhibit more maturation and activation markers than infected DCs. Their maturation is induced by mediators released from MVA infected DCs, partially type I IFN [80], which triggers DCs via IFN-α receptors (IFNAR)[84,85]. Maturation of bystander DC could also result from other infected cells [80]. MVA infected human leukocytes led to the maturation and activation of uninfected immature DCs (bystanders), although partial activation, and enabled them to stimulate the antigen-specific T cells [78]. So, MVA activates immune responses, such as the activation of CTL (cytotoxic $CD8^+$ T cell), against recombinant antigens through cross-presentation [78,83]. The cross-presentation is strikingly unusual because MVA can efficiently infect

APC that are able to process and present antigens with the expression of co-stimulatory molecules, yet cross-priming is the dominant mechanism behind MVA-induced CTL responses. This is unlike the VACV vector that is able to induce cellular responses via direct priming.[83].

MVA can stimulate innate immunity via activation of TLR9 and TLR2-TLR6, MDA-5, and NALP3 inflammasome that leads to the expression of type I IFN [86-88]. MVA could induce the expression of a large number of chemokines that induce the migration of monocytes, neutrophils, and $CD4^+$ T cells to the site of inoculation (e.g. lung), whereas infection with VACV strains WR, Wyeth, and Lister (Elstree) cannot induce the same range of chemokine expression[89,90]. This could be due to VACV expression of immunomodulatory proteins that block intracellular signalling (see table 1.2). Upon MVA infection, the expression of CCL2 chemokine recruits monocytes, T cells, and NK cells. Interestingly, the production of CCR1 chemokine enables the migration of neutrophil cells to bone marrow, priming immune cells, including $CD8^+$ T cells, in the bone marrow. Moreover, using Luminex technology for simultaneous detection of multiple cytokines and chemokines, a study reported that MVA could induce 13 out of 40 immune mediators upon infection of human monocyte derived DCs. These include chemokines such as IP-10/CXCL10, CXCL8, and MIG/CXCL9 and proinflammatory cytokines such asIL-8, IL-6, IL-17, IL-9, IL-16 and TNF-α[80]. In addition to human monocyte derived DCs, MVA infection of monocytes and B cells also enhanced the expression of CXC10, TNFα, and IL-6 [78].

The induction of type I IFN by MVA has been well reported by a number of studies. MVA induces strong IFN-α/β in human primary cells[24] or human derived DCs[80]. It also induces direct expression of type I IFN from infected DCs in mouse model[85]. Type I IFN strong expression in mice might explain the lack of IFN-γ and IL-12 as they can be inhibited by IFN-α/β [91]. Furthermore, in bone marrow derived mouse DCs, induced type I IFN was mainly IFN-α, produced by mainly plasmacytoid DCs. This induction was elicited via IFNAR, and was independent from MVA productive infection or its viral genome replication in DCs[85], supporting the finding on bystanders DC activation and cross-priming. Interestingly, IFN induction was increased upon UV-treatment for MVA, suggesting there is an anti-type IIFN modulator retained by the virus [85].Such an inhibitor could be the E3 protein, which confers resistance to type I IFN, at least in CEF [92].

Other aspects related to MVA's strong innate immune activation include; first, its inability to express soluble receptors for IFN-γ, IFN-α/β, tumour necrosis factor and CC chemokines [24]. Second, MVA activation of NF-κB pathway via inhibition of PKR by MVA E3 and K3proteins, even though MVA has lost NF-κB inhibitors (table 1.2) [93]. Third, MVA, similar to all VACV strains, does not seem to manipulate MHC antigen presentation unlike other viruses [90].

When rMVA was compared to rVACV in a mouse model, rMVA could induce a persistent expression of IL-6 and TNFα, but not IL-12 or IFN-γ, whereas VACV induced the expression of these four innate cytokines only for one day post inoculation, and did

not persist [91].The expression of TNFαand IL-6by APC upon rMVA infection stimulated Th1-biased immune responses[78,82,91]. The Th1-biased $CD8^+$ T cell responses remained similar in different tested doses, whereas responses to VACV WR shifted from Th1-bias to balanced Th1 and Th2 T cell responses when doses were increased. Moreover, increasing the dose reduced the overall $CD8^+$ T cell responses to rVACV but not to rMVA. On the other hand, non-recombinant MVA (nrMVA) showed mixed Th1/Th2 responses. Interestingly, rMVA elicited low level of anti-vector $CD8^+$T cell responses, lower than that induced by rVACV, but the level of $CD8^+$ specific to recombinant antigens was high, higher than that induced by rVACV[82,91]. This gives another advantage of rMVA in overcoming anti-vector immunity, as compared to rVACV (discussed in anti-vector immunity section).

1.3.1.2. Adaptive immune responses to MVA

Regarding smallpox vaccines, VACV was the main vaccine used to eradicate smallpox, and has been shown to elicit humoral and cellular immune responses in mice [94] and in humans[95]. Currently, the immunogenicity and protective capacity of MVA is being tested and compared to the conventional smallpox vaccines, Dryvax and ACAM2000, to develop a much safer vaccine. Such vaccines often called the third generation smallpox vaccine[96]and aimed for the increasing population of immunocompromised people who could develop adverse events to the current VACV-based smallpox vaccine. For example, IMVAMUNE, which is currently being stockpiled for the U.S.A., is MVA virus produced from a defined single clone by Bavarian Nordic[10].

Compared to Dryvax VACV, MVA was found to share the same protection mechanisms as this conventional smallpox vaccine. Both MVA and Dryvax protected mice through induction of neutralising antibodies[97]against many antigens in the virus IMV form, EEV form, and core proteins, especially antibodies against the B5, D8, and H3 antigens[98]. This protection was not due to T cells since passive administration of combined anti-vaccinia antibodies protected naïve mice from lethal challenge[99]. Similarly, passive immunisations with anti-vaccinia sera protected naïve monkeys[97]. Moreover, the transfer of human neutralising anti-vaccinia immunoglobulin protected naïve rhesus macaque from lethal monkeypox challenge[100]. Microarray data also revealed that vaccination of humans and macaques with MVA or Dryvax induced similar antibody specificities, despite the genetic deletions and mutations in MVA [101].Thus, MVA shares this antibody-mediated protection mechanism with VACV,

which is a different mechanism from other viruses where both arms of immunity are required.

$CD8^+$ and $CD4^+$ T cell responses were not sufficient to protect mice from VACV WR challenge after vaccinating with either MVA or VACV T cell immunity however was important in preventing the dissemination of the VACV challenge. It was also important in providing natural resistance in unimmunised mice to VACV WR challenge given at low dose, lower than that used for protection experiments[94,97]. The cellular responses to this low dose prevented the death of unimmunised mice, but did not prevent the disease. A similar finding was observed in monkeys where T cell responses prevented the spread of VACV WR challenge, but neutralising antibodies conferred protection[102]. The T cell and memory T cell responses required a lower dose and longer time to react in mice or monkeys studies, unlike antibody responses [97,102]. Nevertheless, the T cell responses are still important for the protective memory immunity[4,103].

Two vaccinations of MVA induced a log higher neutralising antibody responses as well as higher magnitude of T cells than one vaccination of Dryvax that protected cynomolgus monkeys from monkeypox. MVA also require a higher dose, which could be related to its inability to replicate *in vivo*. This higher dose was safer than VACV, even 1000000 times more than the established safe Dryvax dose, and 1000 times more than the lethal dose of Dryvax. This clearly showed the superiority of MVA in terms of safety[97,102].

Despite being an immunogenic vector, no one yet has complete understanding of the basic mechanism underlying MVA immunogenicity; however, it has been accepted that the loss of the majority of VACV immunomodulatory proteins resulted in the more potent and immunogenic MVA vector[90].

1.3.1.3. Immune responses to MVA delivered recombinant antigens

Recombinant MVA has been developed and deployed in many preclinical studies that showed its ability to induce neutralizing antibody responses against many viral diseases such as HIV/AIDS, influenza, measles, Japanese encephalitis, dengue, SARS and hCMV [90,104]. Not only that, but MVA has also been evaluated as a better choice of T cell-inducing vaccines against some viral internal structural antigens like influenza NP and M1 proteins [1,105]. Moreover, MVA has been used to activate $CD8^+$ and $CD4^+$ T cell responses against intracellular parasitic and bacterial infections as well as against cancer [90,104]. The immunogenicity and protective capacity induced by recombinant MVA vectors has been assessed in preclinical and clinical studies for many different vaccines[104]. Although animal models should represent vaccine protection in humans, allowing for quick and rational vaccines testing, this depends on targeted diseases. For example, testing HIV/AIDS and malaria vaccines in animals is very difficult to predict immune responses in humans, whereas orthopoxvirus and influenza virus infections in animal models are better translated to that of humans and to assess vaccines more rationally [9].

In clinical studies, recombinant MVA-based vaccines showed strong immunogenicity in different trials. For instance, MVA-METRAP, a malaria T cell-inducing vaccine, was found to efficiently boost $CD8^+$ T cell magnitudes after priming with a viral vector (adenovirus (Ad) or FPV) or $CD4^+$T cell responses after priming with DNA delivering the same antigen. This showed the strong cellular immunogenicity of MVA, especially that MVA expands the primed T cell subpopulations in human [11]without skewing the T cell subtypes. However, assessing rMVA immunogenicity is antigen-specific and based

on different immunological readouts for different vaccines, in the absence of strong immune correlate of protection. For example, malaria MVA-METRAP as a liver stage vaccine would be assessed by T cell responses, whereas the blood stage malaria MVA-MSP1 vaccine would require serum antibody evaluation.

Interestingly, rMVA could induce antigen-specific cellular immune responses (in mice) higher than those induced by rVACV, but with lower anti-vector cellular immune responses than that induced by rVACV. This low anti-VACV immune responses induced by rMVA are, in fact, lower than responses to recombinant antigens (unlike rVACV),particularly at higher doses such 5x10^7 pfu. This gives MVA an exquisite advantage as a T cell-inducing vaccine viral vector [91].

With regard to antibody responses, rMVA could induce antigen-specific antibody responses similar to that induced by rVACV vector; however, at low doses rMVA was not able to induce antibody responses [91].The use of rMVA for antibody-inducing vaccines has been evaluated in many animal models like mouse, rabbit and macaque as well as in clinical trials and showed its ability to induce high antibody titres that are sometimes linked to protection. For example, MVA encoding the spike protein of SARS induced protective level of antibodies in mice, rabbits, and macaques. Recombinant MVA with Influenza HA antigen has been tested, in different studies in mice, ferrets, and cynomolgus macaques, inducing strong neutralising and protective antibodies that were better or comparable to that achieved with rVACV vectors[98]. However, not all antigens can induce antibodies when delivered by rMVA, for example, MSP1 antigen (a

malaria merozoite surface protein 1)delivered to mice via DNA-prime MVA-boost, FPV-prime MVA-boost, or MVA-prime alone vaccination regimens showed no detectable antibody responses. In contrast, human adenovirus type 5 vector (AdHu5), encoding the same antigen, was able to prime antibody induction; and AdHu5-prime MVA-boost induced higher neutralising antibodies that protected mice from murine malaria challenge. This latter regimen was tested in mice, rabbit and rhesus macaques and showed similar results in terms of antibody induction[98]. The DNA-prime MVA-boost regimen, which did not protect mice in malaria studies, and AdHu5-prime MVA-boost regimen were tested to deliver HIV antigen in monkeys. Both regimens induced antibody responses that protected rhesus macaques from SIV challenge. Therefore, it is clear that the induction of antibody responses by rMVA is dependent on the targeted disease, recombinant antigens, animal models, dose and route of administration, and vaccination regimens[91,98].

3.2.2. MVA in prime-boost vaccinations

Recombinant MVA-based vaccines have been shown to benefit from utilising MVA as a boosting vector in heterologous prime boost vaccinations as mentioned above. Boosting with rMVA could expand the adenoviral vector-primed responses to a greater T cell magnitude superior to that achieved with either DNA-prime MVA boost, or FPV-prime MVA boost; also higher than any tested prime only regimen[1]. In contrast, priming with rMVA and boosting with any other vectors, such as DNA, adenovirus, or FPV, showed very weak T cell responses[11]. The superiority of MVA as a booster rather than a primer in heterologous prime boost vaccination regimens is now well-established as a result of many preclinical studies as well as clinical trials testing malaria liver-stage[73]malaria blood-stage [98], TB[72], HIV/AIDS [70], hepatitis C virus [106] and Influenza [71] vaccines.

The proposed mechanism of heterologous vaccination regimens is that the boosting vector would expand the recombinant antigen-specific T cells whilst priming a lower number of naïve T cells to its own antigens, resulting in low anti-vector T cell responses to the boosting vector[1].However, the mechanism underlying the unique boosting, but not priming, ability of MVA is poorly understood. Some speculate that MVA expands the pre-existing immune responses specific to recombinant antigens without activating strong anti-vector immunity[1]. This is also because rMVA elicits low level of anti-vector neutralising antibody and T cell responses while eliciting higher anti-recombinant antigen immunity, in agreement with its high expression of recombinant antigens[82,91]. Another possibility is that rMVA could not evade the immune system due to its loss of many immunosuppressive proteins (see table 1.2), eliciting a potent

anti-viral state that could clear the virus but expands the pre-existing recombinant antigen-specific immunity. Moreover, MVA triggers CXCL9 and CXCL10 expression that would allow memory $CD8^+$ T cells, concentrated in lymph nodes, to expand and respond quickly upon rMVA boost[93].The antigen-specific memory T cells, primed previously by another vector, should then expand with rMVA encoding the same antigen. This possibility could also fit with the weak priming ability of rMVA; first, the anti-viral immune status would clear the virus with its recombinant antigen cargo when used for priming. Second, when MVA is used for priming, there are no antigen-specific memory T cells that could benefit from rMVA strong immune activation of memory $CD8^+$ T cells.

The immunogenicity of a given antigen, induced by rMVA in prime-boost regimens could vary depending on dosage, priming agents, route and interval of applications, vaccine formulations, and the targeted immunity arm. All of that also depend on the targeted disease [72,73,107,108]. For example, prolonging the prime boost interval from 2 to 8 weeks or even to 14 weeks allowed for a better boost of primed antibody responses[109].

3.2.3. Routes and doses of administering rMVA

MVA's strong immunogenicity has been proved as route-dependent and to less extent dose-dependent. Regarding the route of administration, MVA was used as a boosting vector in a DNA-prime MVA-boost vaccination regimen and achieved 100% protection against malaria challenge when delivered i.v., 80% when delivered i.d., and 50% after i.m. injection in mice. This shows the role of injection route in MVA immunogenicity and protection, but it does not necessarily mean that i.m. injection is not immunogenic. Other studies on chimpanzees showed strong MVA immunogenicity when delivered intramuscularly; so it may depend on the antigen and the targeted disease as well [8]. Even for replication-competent VACV-vectored vaccines, the immunogenicity depends on the route of administration[20]. However, in many clinical trials, MVA was given i.m. due to the volume restriction and the recorded increased severity of i.d. route.

For humans, the dose of 2x10^8 pfu was shown to be the cut-off for the safety and tolerability of MVA-based prophylactic vaccines. At higher doses severe adverse events we rerecorded, but there were no serious adverse events. However, for MVA-based therapeutic vaccines for cancer, a higher dose has been assessed because the limit of acceptable severity is different[11]. Although escalating MVA dose in mice, increased the recombinant antigen-specific immune responses [91],the protection achieved with MVA against VACV WR challenge needed lower doses when MVA was given i.n. than that given i.m. Therefore, the dose could also be route-dependent.

3.2.4. MVA as an adjuvant

Non-recombinant MVA (nrMVA) has been used as an adjuvant *in vitro* to assess its effect on DCs. Only at low MOI, nrMVA was found to activate DC (as mentioned earlier) and enhance antigen presentation on MHC-I and MHC-II that resulted in improved antigen-specific $CD4^+$ and $CD8^+$ T cell responses. In mouse model, the co-administration of nrMVA with a model recombinant protein resulted in higher and long-lasting antigen-specific CTL responses; and enhanced and durable antibody responses that were even higher than encoding the same antigen in a rMVA[82].Enhanced cellular and humoral responses were also obtained with HBsAg co-administered with a number of non-recombinant poxviral vectors, e.g. MVA, FPV, ALVAC, and NYVAC, in homologous prime boost vaccinations[110]. In the latter study, non-recombinant MVA also showed higher level of cellular responses (against HBsAg) when co-administered with the recombinant protein (HBsAg) than encoding it in recombinant MVA although this was not in all organs(it was only in lymph nodes). In future, this exquisite feature of MVA should allow for optimising other suboptimal vaccines by co-administering them with MVA-based vaccines.

1.4. Modified Vaccinia virus Ankara vector: The limitations

With the increasing number of viral vectors being deployed in clinical trials, anti-vector immunity could be a problem. This immunity is very important to be understood in order to develop safe and effective MVA-based vaccines; and it can be divided into pre-existing and concurrent anti-vector immunity. Although it is possible to study anti-vector T cell immunity, assessing anti-Vaccinia vector T-cell immunity in a more specific manner was made possible with the published immunodominant HLA-A*0201-restricted VACV-specific $CD8^+$ T-cell epitopes in humans[111] and $H2^d$ or $H2^b$-restricted VACV-specific $CD8^+$ T-cell epitopes in different strains of mice[112,113].

Pre-existing anti-vector immunity elicited to a given vector used in the past, in the same individual, could be a problem when the same vector is used again, especially with more MVA-based vaccines being tested in humans[90]. Moreover, it could result from priming and boosting with rMVA in homologous vaccination, but it could also occur when a close relative vector is used for priming in heterologous prime-boost vaccination. For, example, when MVA was used to boost immune responses in vaccinated adult humans, primed with a FPV-based vaccine; the pre-existing anti-poxvirus immunity prevented MVA from boosting immunogenicity[11], whereas MVA boosted immune responses to higher levels when a different viral vector (adenovirus) was used for priming. However, a recombinant MVA encoding soluble glycoprotein B of human CMV, delivered to mice that were primed with wild type MVA or VACV viruses, induced neutralising antibodies against the hCMV antigen with titres that were similar to non-primed mice [114]. This suggests that the pre-existing anti-MVA or anti-VACV immunity is more evident in T cell responses and does not affect antibody-

induced responses. The pre-existing immunity could also be a result of natural exposure to the vector. An example is the well-known incident of adenovirus pre-existing immunity that was noted in the HIV vaccine STEP clinical trial after it had been stopped for reasons of futility and increased susptibility to contracting HIV infection [115,116]. Therefore, the heterologous prime boost vaccination regimen, discussed above, was effective in circumventing the pre-existing anti-vector immunity.

On the other hand, recombinant vectors that induce recombinant antigen (rAg)-specific immunity, could also induce concurrent vector-specific immunity. Such immunity could compete with, or might reduce, rAg-specific responses and become immunodominant[117,118].MVA induces lower anti-vector immunity than VACV; also rMVA induces even lower anti-vector immunity than non-recombinant MVA[119], which makes it an elite viral vector. However, treating rVACV with psoralen and UV light, *in vitro*, to destroy the expression of the majority of VACV genes, resulted in 66% reduction in VACV-specific cellular immunogenicity while enhancing recombinant peptide-specific $CD8^+$ T cell responses [117]. This clearly demonstrated the effect of the immunodominant anti-vector immunity on rAg-specific immune responses and showed a room for vector improvement. However, it seems impossible to do this *in vivo*, besides, the immunodominance would vary in the outbred human population. An innovative approach was reported, in which the essential Uracil DNA glycosylase (*udg)* gene that is required for viral late expression was deleted from rVACV [120] or rMVA [121], the mutants were then grown via genetic complementation. This approach reduced the complex antigen repertoire of MVA *in vivo*, enhancing higher $CD8^+$ and $CD4^+$T cell responses to recombinant antigens in macaques, but there was no

difference in mice[121]. Surprisingly, the antibody responses, which are normally against late antigens [101], was not affected or enhanced by the *udg* deletion, suggesting the antibody induction is a result of MVA-delivered antigens rather than *in vivo* MVA-synthesised antigens[121].

In addition to reducing vector antigen complexity, research approaches have also focused on deleting MVA immunomodulatory genes to reduce anti-vector immunity and improve MVA immunogenicity. On the other hand, approaches to increase expression of recombinant antigens are the aim of many studies, to reset the balance with this anti-vector immunity. These include the use of different insertion sites, fusing transgene to VACV transmembrane domain, and encoding immune stimulatory molecules. Moreover, a continuing approach to enhance rAg expression focuses on utilising a better promoter that could potentially enhance rAg-immune responses.

1.5. Improving MVA immunogenicity

Despite the strong immunogenicity of MVA, there are still some opportunities for enhancing it to an even stronger level that could benefit various vaccines. In fact, sometimes there is a need to improve such replication-deficient vector [96]. Some poxvirologists (e.g. G.Smith [4] and B.Moss [103]) believe that we do not yet have a clear understanding of how MVA protects against smallpox, and how the immune response to MVA works. Therefore, research focusing on improving MVA immunogenicity could also elucidate some mechanisms concerning MVA immunogenicity and protection, as suggested [88].

1.5.1. Deleting immunomodulatory genes to enhance MVA immunogenicity

A growing piece of research has focused on deleting a number of immunomodulatory genes from rMVA-based vaccines to enhance their immunogenicity. The deleted genes include *A41L*[122], *B15R*[123], *A35R*[124], *C12L[35], C6L*[125], *K7R*[126], *N2L*[127], and *F1L*[128] and each showed stronger adaptive cellular immune responses, especially at the memory phase, with sometimes improved antibody responses as well. However, some reports showed no improvement upon the deletion of one or a few immunomodulatory genes such as *C12L*, *B15R* and *A41L*[129]. It is important to note that all reports, with either positive or negative outcomes, have different details, such as route of administration, dose, and recombinant antigens. Therefore, this approach should be vaccine-specific and might not be a generic application for MVA improvement. Chapter 7 of this thesis focuses on the deletion of 15 immunomodulatory and unknown genes from rMVA to improve its immunogenicity. The relevant literature will be reviewed in more depth and discussed in that chapter.

1.5.2. Encoding Molecular adjuvants

Recombinant vectors that encode molecular adjuvants such as IFN, cytokines, or immune stimulatory molecules, improved the immunogenicity and protection capacity of rVACV. This type of research opened a new area for improving antigenicity and immunogenicity of MVA-based vaccines. For, example, IL-15 was inserted into VACV or MVA. Both elicited higher level of cellular immunogenicity and protected both immunocompetent and immunocompromised mice from lethal VACV WR challenge [130].To generate a safer VACV-based smallpox vaccine, IL-2 [131] IFN-γ [132], or IFN-λ [133] were inserted in rVACV, resulting in enhanced immune responses that also made VACV avirulent and protected immunocompromised mice. Although these were performed to attenuate VACV, it illustrated the feasibility of inserting these cytokines into MVA as well. A more relevant example is that MVA expressing stimulatory cytokines and chemokines like CCL20 and mGM-CSF recruited more DCs to the inoculation site and enhanced the priming of naive T cells against a recombinant antigen with more titres of MVA-specific antibody responses[134]. Furthermore, MVA could also accommodate adjuvants that improve the antigenicity of recombinant antigens rather than the immunogenicity of MVA itself. For instance, fusing a transgene to the IMX313 molecular adjuvant, which is the α-chain of C4b-binding protein oligomerisation domain, augmented cellular immunogenicity in mice and macaques [135] as well as mice humoral immunogenicity in another report [109].

1.5.3. Fusing transmembrane domains to recombinant antigens in MVA

The humoral immunogenicity of MVA-vectored vaccines was also enhanced by fusing the recombinant antigens to the transmembrane domain of some of VACV surface proteins. For, example fusion of the transmembrane domain of the vaccinia D8 protein to a *Y. pestis* antigen directed the antigen to the surface of VACV IMV form and subsequently enhanced both humoral immunogenicity and protection in mouse model [136]. In addition, the fusion of the transmembrane domain of vaccinia B5 protein to a HIV-1 antigen targeted the antigen to the surface of VACV EEV form and improved the antibody-mediated immunity and protection[137]. This approach of using poxviral transmembrane domain was also attempted with that of the A9 surface protein, and therefore should be applicable to any VACV surface protein[138].

1.5.4. Enhancing recombinant antigens expression in MVA by utilising a stronger promoter

MVA infects the same tissues as VACV WR *in vivo* (mouse model), and expresses recombinant antigens to a higher level than VACV WR, up to 6 hours post-infection [91]. However, in spite of its strong immunogenicity, MVA gene expression declines to a very low level just after 24 hours post-infection, with no expression by 48 hours post infection, likely because the virus does not replicate *in vivo*[91,139]. Early MVA transgene expression, which occurs immediately after uncoating of virions (see MVA life cycle in Figure 1.1), was reported to persist in infected human macrophage-derived DCs [79]. Therefore, if this occurred *in vivo*, it would be important to utilise an early promoter to drive recombinant antigens in order to ensure maximum and persistent expression. The level of transgene expression *in vitro* was shown to correlate with the

level of cellular immunogenicity *in vivo*[140,141], especially when the p7.5 and mH5 promoters were compared. However, when the mH5 and SSP (short synthetic promoter) were compared, the SSP showed higher activity *in vitro*, but *in vivo* immunogenicity was higher with the mH5. The *in vitro* strong expression of the SSP promoter with the relatively low *in vivo* immunogenicity was interpreted as high expression toxicity on cells[141], but it could also be due to its weak early expression. The SSP has a very strong late activity, with a weak early activity, whereas the mH5 has strong early activity [67,142]. This could explain the improved cellular immunogenicity achieved with mH5 due to its stronger early expression. An early report by Coupar *et al* (1986) showed the importance of using an early promoter in VACV-based vaccine for priming and re-stimulating T cells[143]. Another study by Bronte *et al* [144] has also showed that early transgene expression in professional antigen presenting cells is critical in inducing strong cellular immune responses. Therefore, promoters with early activity should be chosen for targeting cell-mediated immune responses.

Regarding antibody-mediated immunity, there was no clear study focusing on the promoter influence [98], Coupar *et al* did not find a difference on induced antibody titres after using different promoters to drive Influenza haemagglutinin (HA).Observation, made in the Jenner institute, suggests that the promoter type (i.e. early vs. late) does not impact on the induced antibody titres. This is despite the fact that in VACV or MVA infections, induced antibodies mainly targeted late antigens [101].

Non-poxviral promoters cannot be used for transgene expression because poxviruses replicate in the cytoplasm and require their poxviral transcription machinery[17].Poxviral promoters have a unique sequence that is distinct from eukaryotic or prokaryotic sequences [145], and cannot be transcribed by eukaryotic RNA Pol II [20].Even when a transgene, driven by a poxviral promoter, was deliberately sent to the nucleus of infected cells, it was not expressed by the cellular machinery [146].

A large number of poxviral promoters have been tested and used in rVACV and rMVA, aimed at increasing rAg expression and potentially inducing more immune responses. For example, the P11 promoter[20] was used in rVACV. This is a strong late promoter that naturally drives the11K protein [147] encoded by the *F17R* ORF [3,20].Another described promoter was the I1L promoter, a strong late promoter[148]that could rival other poxviral intermediate and late promoters, like the promoter of *A1L*, *J3R*, and*E4L*ORFs [149]. The I1L promoter is also short (29 nucleotides) and can be stronger than the bacteriophage T7 promoter, in the hybrid T7/VACV expression system, which is considered a very strong promoter. However, the I1L promoter has one disadvantage as it has to include a G residue at the 3' of the ATG initiation codon [149]. The common early and late p7.5 promoter is being extensively used for its ability to express antigens during both early and late stages of VACV infection. It was originally described as two promoters, a promoter that drives the expression throughout the replicative VACV cycle via two different transcriptional start sites, 55nt apart [150].The native p7.5 promoter is found in two copies that drive the expression of*C23L* and *B29R* ORFs in the ITR of VACV genome. The use of this promoter involves insertion of a third

copy driving transgenes at a different insertion site. The homologous recombination between these three copies of the p7.5 promoter has never been a concern or addressed, and to our knowledge, all vaccines produced in the Jenner Institute, are stable despite using three copies of p7.5. Moreover, recombination between two copies of the p7.5 would result in a very small genome that is unable to produce infectious virions and would be selected against (Personal observations on MVA-BAC recombineering system). Other early and late promoters have also been characterised such as the promoter of the TK gene[151], the F7 promoter [152], or the H5 promoter [153,154]. However, the p7.5 promoter is often used as a conventional comparator with any newly optimised or described promoter[67,141,155-157].

The search for new promoters has continued despite the availability of strong promoters like the p7.5. Some studies reported the replacement of non-essential ORFs with a transgene using the adjacent endogenous promoters to control the transgene expression[49,56]although this method could result in unpredicted protein translation. However, we managed to utilise endogenous promoters of fragmented ORFs, with a more precise method where a transgene is expressed from the authentic start codon of these ORFs [156]. A naturally tandem promoter was also studied and showed increased immunogenicity to the expressed recombinant antigen although the improvement was observed only after four homologous immunisations[158] making this unlikely to be of use in clinical vaccine development.

Some other studies have, instead, focused on optimising existing promoters. For instance combining the consensus sequences of early and late elements of VACV promoters, resulted in the optimised early/late SSP[159,160], which was based on earlier studies by Davison and Moss [161-163]. Another method of promoter optimisation is achieved by mutating promoter core sequences. For example, the intermediate element in the early/intermediate I3 promoter was mutated to make it active after DNA replication (i.e. early/late) [164]. An eminent example in this field is the modified early/late H5 promoter (mH5) which was optimised by two nucleotide substitutions in its core early and late sequences[67]. Moreover, combining four copies of the p7.5 early element and one copy of its late element resulted in a hybrid tandem promoter (called pHyb) that enhanced cellular immunogenicity although this was only apparent after four homologous immunisations [155], and that is unlikely to be applicable in vaccine clinical trials. In addition, another optimisation was achieved by swapping early and late elements of the SSP promoter with an adjustment for the spacer region between the early element and the transcription start site (called LEO promoter). The LEO promoter enhanced GFP (green fluorescent protein) expression and cellular immunogenicity[165]. Overall, the p7.5, mH5, and SSP are very frequently used in recombinant MVA vectors, and will be presented in much more depth in this thesis.

1.5.5. Enhancing MVA early expression

As illustrated above, early expression of transgenes could improve cellular immunogenic profiles of MVA-based vaccines. Therefore, blocking MVA late gene expression should halt the infection at the early stage and allow for persistent early expression. When MVA late gene expression was blocked *in vivo*, the cellular immune responses against MVA were elicited to a limited range of vector antigens whereas recombinant antigen-specific $CD8^+$ and $CD4^+$ T cell responses were enhanced by 2-fold, in macaques[121]. Although this study was designed to reduce the antigen complexity and immunodominance of the vector antigens, it could mean that early antigen expression, when enhanced, or allowed to persist, increases cellular immunogenicity.

1.5.6. Using different insertion sites in rMVA

Thymidine Kinase (TK) locus was traditionally used as an insertion site, which helped in purifying VACV and MVA recombinants in TK-negative cells in the presence of bromodeoxyuridine (BrdU)[166,167]. Deletion III region of MVA genome is frequently used to insert transgenes and any of the six deletion regions can be an insertion site. Other insertion sites include the internal position within the HindIII F fragment, non-essential genes, which were linked to the use of their adjacent promoters[49,56], the haemagglutinin locus (*A56R*), and Intergenic regions (IGR). IGR-3, between the*I3L* and *I4L* ORFs, was shown as a stable region for transgene expression[168]. Antoine *et al* suggested that split genes in the central region such as *O1L*, *F5L*, and *F11L* could also be used as stable insertion sites. The *F11L* as well as other fragmented genes, like *B8R*and *C11R*, were recently used as insertion sites, taking advantage of their endogenous early promoters to express transgenes [156].

The effect of insertion sites on MVA expression profiles or immunogenicity is rarely addressed. The co-insertion of HBsAg and HBcAg of hepatitis B virus at either the TK or the *K1L* ORFs did not affect their expression level *in vitro*[169]. The TK versus the *F7L*insertion loci were also compared to express either IL-2 or IL-4 using the same promoter[170]. The expression of IL-2 or IL-4 at either site was not different *in vitro*, but the *in vivo* effect of IL-2 in protecting immunocompromised mice from VACV WR lethal challenge was more efficient when expressed from the *F7L* site. However, other studies did not support the effect of insertion sites on *in vivo* immunogenicity. For example, insertion of human IL-2 at either of three separate sites (HindIII C, VACV HA (*A56R*), and TK), using the same promoter, was not critical for attenuating effects observed in mice[131].

Although insertion loci do not seem to affect *in vitro* expression, there are discrepancies on whether the insertion site of a given antigen would reduce or enhance its function *in vivo*. Therefore, the effect of using TK versus other endogenous loci on MVA immunogenicity is further discussed in chapter 4 and 5.

1.6. Novel approaches to enhance transgene expression and immunogenicity in MVA

This DPhil project was carried out to implement novel approaches to improve transgene expression and immunogenicity of MVA. These approaches include; first, insertion of internal ribosome entry site from endomyocarditis virus (EMCV IRES) into MVA genome to enhance transgene expression and immunogenicity (chapter 3). Second, utilising MVA endogenous promoters to enhance transgene expression and immunogenicity as well as creating new insertion sites since these promoters are linked to non-essential genes (chapter 4). Third, examining the effect of the thymidine kinase gene on transgene expression and immunogenicity (chapter 5). Fourth, optimising the MVA *B8R* promoter by nucleotide substitution to increase transgene expression and immunogenicity (chapter 6). Fifth, deleting immunomodulatory and unknown genes from MVA to enhance its immunogenicity (chapter 7). Each approach will be discussed further in the relevant chapters.

All of these approaches were tested using MVA-BAC recombineering technique (explained in materials and methods) to derive rMVAs. This technique was shown to be effective, quicker, and does not alter immune responses to MVA vector or recombinant antigens when compared to conventionally derived rMVA[129].This technique was used to derive more than 15 rMVAs, for this DPhil study, which were then propagated in BHK-21 cells. These rMVA, unless otherwise stated, encode a reporter transgene (tPA-pb9-rLuc) consisting of tPA (leader sequence), fused to pb9 (a murine malaria immunodominant $H2^{d}$-restricted peptide from *Plasmodium berghei* circumsporozoite protein), and then fused to rLuc (chemoluminescence luciferase

protein from *Renilla reniformis*. For cellular immunogenicity, MVA E3 peptide-specific, MVA F2(G) peptide-specific, recombinant pb9 peptide-specific and/or rLuc antigen-specific immune responses were measured to assess the immunogenicity elicited by rMVAs after each optimising approach. Immune responses were always compared to non-mutated (non-modified) rMVA control. For *in vitro* studies, although many enzymatic reporter antigens have been used in vaccinia vectors (reviewed in [20]), rLuc was used in this project for its strong sensitivity to assess transgene expression. The GFP gene was originally inserted into MVA-BAC, and the GFP expression was therefore used for viral replication, titration and plaque assays.

Immunomodulatory gene†	Function	Status in MVA	Notes on functions, mainly in VACV
VCP	**VACV Complement C3b and C4b binding protein**		
***C3L*(VCP)**	Complement C3b and C4b binding protein	Affected by the deletion d4817	
B15R, CrmC, CrmE, E3L, A46R, A5R2, K7R, B14R, N1L, C4L, A49R, K1L*and *M2L.	**IFN inhibitors (Anti-NFκB)** NF-κB can be activated by: IL-1, TNF, and TLR-mediated pathways.A46, A52 and K7 proteins inhibit NFκB activation by acting on IL-1- and TLR-mediated pathways. In contrast, all other VACV NFκB inhibitors act on IL-1 and TNF-mediated pathways.		
B15R		Mutated	
E3L		Conserved	• Inhibits NFκB activation in both PKR-dependent and independent manners • Inhibit NFκB activation by antagonizing the RNA polymerase III–dsDNA sensing pathway. • Inhibit IFN-induced antiviral protein (see below).
A46R	TLR signalling inhibitor	Conserved	• Binds to several molecules that called TIR-domain adaptors (e.g. MyD88, MAL, TRIF, TRAM). • These TIR adaptors bind to PAMP-activated TRL3/TLR4 and result inactivation of NFκB, IRF3, MAP Kinase, leading to inhibition of IFN-β. • TIR-domain is found in molecules that are able to interact with TLR and IL-1R.
A52R	TLR signalling inhibitor	Deleted, part of DelIII region	• Structurally, it has B-cell lymphoma-2 (Bcl-2) fold. • Binds to IRAK and TRAF6 → (1) inactivating NFκB and (2) inhibition of IL-1. - IRAK2 (IL-1R-associated kinase 2) - TRAF6 (receptor-associated factor 6)
K7R	NFκB and IRF3 inhibitor	Conserved	• Also has Bcl-2. • Binds to IRAK and TRAF6

			→inactivating NFκB. • Functions in IRF3 pathway (see below)
B14R	NFκB inhibitor	Fragmented (Split ORF)	• Also has Bcl-2. • Binds to IKKβ → prevents its phosphorylation by upstream kinases, and prevent it from phosphorylating its target (which is IκBα, an inhibitor of NFκB). - IκBα when phosphorylated, it allows for the continuity of the signal.
N1L	NFκB inhibitor	Mutated, partially deleted at the end of d4817 region	• Also has Bcl-2. • Bind to IKK complex (inhibitors of κB Kinase), but exact mechanism is **unknown**.
C4L	NFκB inhibitor	Deleted, part of d4817 region	• Inhibits activation of NFκB at, or downstream of, the IKK complex by an **unknown** mechanism
A49R	NFκB inhibitor	Conserved	• Near the N terminus of A49, there is a short sequence containing conserved serine residues that are present in IκBα. • IκBα is phosphorylated by IKKβ. Normally, once IκBα is phosphorylated by IKKβ, p-IκBα is recognized and ubiquitinated by β-TrCP (the E3 ubiquitin ligase β-transducing repeat-containing protein) leading to p-IκBα degradation by the proteasome. • A49 functions by binding to β-TrCP, so that even if IκBα is phosphorylated, it is not recognized and ubiquitinated by β-TrCP; thus, p-IκBα remains bound to NFκB in the cytoplasm.
K1L	NFκB inhibitor	Pseudogene, Affected by d2763 region.	• Inhibit NFκB signalling pathway by preventing the degradation of IκBα, but

		Truncated protein 98 aa compared to the 284 aa *wt* protein.	exact mechanism is **unknown**.
M2L	NFκB inhibitor	Deleted, part of d2763	• Reduces ERK2 phosphorylation and block p65 nuclear translocation, inhibiting NFκB.
A53R-A54L	TNFR (CrmC)	Deleted, part of DelIII region	- TNFR (TNF receptor)
E3L, C16, A46R, K7R, C6L*and *N2L	**IFN inhibitor(Anti-IRF3)**		
E3L		Conserved	See above
C16L		Mutated, partially (36bp) deleted by d3585 region, resulted in frame shift	• Disrupt the recognition of dsRNA by DNA-PK → inhibits IRF3 → inhibits IFN-β.
A46R		Conserved	It can block IRF3 pathway (See above)
K7R	Inhibitor to TLR-dependent and -independent IFN-β activation	Conserved	• Binds to DEAD-box RNA helicase 3 (DDX3), downstream of its previous target (which is TRAF6) → inhibit IRF3 → IFN-β.
C6L		Conserved	• Interacts with NAP, TANK, SINTBAD → prevents the activation of IRF3 and IRF7 by kinases like TBK1(TANK-binding kinase) and IKKϵ → leading to the inhibition of IFN-β.
N2L		Mutated, 15bp internal deletion and 1 aa substitution	• Inhibit IRF3 pathways (at later stages) after the IRF3 phosphorylation and nuclear translocation. • N2 enters nuclei.
B18R*and*B8R	**Soluble receptors**		
***B18R* (*B19R* in MVA)**	IFN-α/β binding proteins (receptors)	Fragmented. Protein is 234 aa in MVA while 353 aa in VACV CPN*. Called *B19R* in	• Inhibits IFN-α/β in solution or on cell surface, i.e. B18 can bind to the cell surface and neutralise IFN-α/β effect. • Binds to IFN-α, secreted by

		MVA, *B18R* in VACV and *B20R* in VARV)	leukocytes, and inhibits it more efficiently, whereas inhibition of IFN-β, secreted by fibroblast and epithelial, relies on VACV immunomodulatory proteins that act intracellularly.
B8R	IFN-γ soluble receptor	Fragmented. Protein is 272 aa in VACV, but 226 aa in MVA with 2 internal deletions and 5 individual substitutions.	• Binds to soluble IFN-γ. • It is similar to the cellular IFN- γ receptor, but lacks the transmembrane domain. - Both B18 and B8 bind to IFNs from many hosts, but do not bind efficiently to murine IFN.
H1L	VH1 dual phosphatase	Conserved/essential	• Dephosphorylates STAT1 and STAT2 →impairing the IFN-induced pathways.
E3L, K3L	**Inhibitors for IFN-induced antiviral proteins**		
E3L	Multifunctional • Inhibiting IFN-induced antiviral status • Anti-PKR activity • Anti-apoptosis	Conserved	• Inhibits IFN-induced antiviral protein by blocking PKR. - PKR (Protein kinase R) is activated by viral ds-RNA, which shuts down protein synthesis. - E3 binds to ds-RNA and prevents the sensing of ds-RNA, hence the activation of PKR, OAS, and other dsRNA sensors (see K3). • E3 binds and deactivate ISG15 too. • PKR inactivation by E3 is redundant by K3 protein (below)
K3L	• Anti- PKR activity • Anti-apoptosis	Conserved	• Shares some amino acid similarity with eIF2α, and become a pseudo-

			substrate for PKR and thus competitively inhibits the phosphorylation of eIF2α by PKR. • PKR could bind to K3 but does not phosphorylate it.
C7L*and *K1L		*C7L*: Conserved. *K1L*: Pseudogene.	• Host-range factors. • Speculation: C7 and K1 might support VACV replication by antagonism of currently undefined IFN-induced antiviral factor.
F1L, B13R*, and *B15R	**Cytokines inhibitors** All NFκB inhibitors can stop production of cytokines like IL-1 and TNF		
F1L	IL-1β inhibitor	Mutated, 12 bp deletion	• Binds to NLRP1 inflamosomes → stops the activation of caspase 1 → prevents the cleavage of pro-IL-1β.
B13R	IL-1β inhibitor IL-18 inhibitor	Split in VACV CPN*, more fragmented (Split) in MVA	• Binds to caspase 1 and prevents the cleavage of pro-IL-1β. Also, inhibit the caspase-1-induced cell death. • Because IL-18 precursor needs to be cleaved by caspase-1, B13 acts as IL-18 inhibitor as well.
***B15R* (*B16R* in MVA)**	Soluble IL-1Receptor (IL-1R)	Conserved	• Similar to cellular receptor of IL-1β. • Released from infected cells and binds to IL-1β, which is produced by uninfected cells, whereas infected cells production of IL-1β is inhibited by F1 and B13(above).
C12L* (*008L* in MVA) Not in CPN	IL-18 binding protein(inhibitor)	Slightly mutated, 18 bp deletion. Protein has internal deletion of 6 aa compared to not MVA VACV viruses)	• Different from cellular IL-18R, but similar to IL-18BP, which is a negative mediator for IL-18 action. • Binds to IL-18 → reduces IFN-γ→ reduces NK and $CD8^+$T cell responses. - IL-18 is called IFN-γinducing factor.

			• Inhibition of NF-κB or IRFby other VACV proteins, inhibits IL-18-induced transduction too.
***A53* (CrmC) and (CrmE in USSR strain)**	TNF Receptor (TNFR)	Deleted, part of DelIII. Fragmented in many VACV strain including VACV CPN and WR.	• Although TNF is affected by the blockage of NFκB activation, some strain of VACV, like Lister & USSR, has an active A53 which binds to TNFα - TNF (Tumour necrosis factor)
	Chemokine inhibitors Chemokines can also be inhibited by the inactivation of NF-κB or IRF, which are transcription factors for many pro-inflammatory cytokines and chemokines genes.		
A41L	CC-binding protein, soluble receptor	Conserved	• Binds to CCL21, CCL25, CCL26 and CCL28 but does not prevent them from binding to their receptors because of its low affinity. • Could disrupt the chemokine gradient in the site of infection, e.g. VACV WR lacking *A41L* was cleared faster due to quick recruitment of leukocytes. • A41 also binds heparin via a domain that overlaps with that used for CC binding.
B7R		Mutated, 15bp deletion	• Due to structural similarity and the CC binding domain, B7 is suggested CC-binding proteins.
***B23R* (*C14L-C13L* in MVA)**		Fragmented Deleted within d3585	• Due to structural similarity and the CC binding domain, B23 is suggested CC-binding proteins
***C23/B29R* (in ITR)**		Fragmented, truncated protein in VACV CPN, WR, and MVA strains	• CC-binding protein with high affinity in VACV Lister strain.
	Anti-Apoptosis VACV proteins		

Vaccinia anti-apoptotic genes, e.g. *F1L and N1* L	Anti- apoptosis (intrinsic pathways)		**Simplified general intrinsic apoptosis mechanism:** Intrinsic apoptosis stimuli→ bind to Bid or Bad (pro-apoptotic signals) → bind/activate to Bak and Bax (pro-apoptotic target) → oligomerise on the outer mitochondrial membrane→ Cytochrome C release → apoptosome → cascades → Apoptotic status. • F1 and N1 are two proteins with Bcl-2-like fold, which found in anti-apoptotic proteins. • Studies showed that virulence activity of F1 and N1 proteins are more important to VACV than their anti-apoptotic activities. **Extrinsic apoptosis** is triggered by the activation of TNFαreceptors and Fas receptors on cell surface.
F1L	Anti-Apoptosis (intrinsic pathway)	Mutated, 12 bp deletion	• F1 uses its Bcl-2-like fold to bind to Bak (a target of pro-apoptotic proteins like Bad or Bid). • Also, F1 is IL-1β inhibitor (see above).
N1L	Anti-Apoptosis (intrinsic pathway)	Mutated, partially deleted at the end of d4817 region	• N1 also has Bcl-2-like that binds to Bad or Bid → prevent their binding to Bak or Bax →anti-apoptosis. • N1 could also binds directly to Bax→ anti-apoptosis. • Also, N1 is NFκB inhibitor (see above).
B13R	Anti-Apoptosis (extrinsic pathway)	Split in VACV CPN*, more fragmented (Split) in MVA	• By inactivating caspase 1, 4, and 5 (group I caspases) and caspase 8, 9, and 10 (group III caspases). • Also, B13 can inhibit pro-

			inflammatory cytokines like IL-1β (see above)
E3L	Anti-Apoptosis (extrinsic pathway)		• E3 can prevent apoptosis by neutralising extrinsic apoptosis stimuli like ds-RNA. • Deletion of *E3L* induces apoptosis[92]
	Anti-NK cells VACV proteins		
A56R	Viral ligand to NK activating receptors	Mutated, DelIII affected the late element of *A56R*promoter.	• A56 can bind the activating receptors of NK: NKp46 and NKp30. • A56 binds and activates NKp46, which should lead to NK cytotoxicity, but A56 binding neutralisedNKp30 activity. • Many other VACV proteins, such as N1, F3, C12 and K7,could affect NK responses, but A56 acts in a direct way.

Table 1.2:Immunomodulatory genes and their functions in VACV with their status in MVA

[†]Gene nomenclature based on VACV CPN.* VACV CPN: Vaccinia virus Copenhagen strain. VACV WR: Vaccinia virus Western Reserve strain. Arrows (→) means "leads/leading to. "The six deletions in MVA genome are presented by their size: d4817, d2763,andd3585 see table 1.2. Adopted mainly from references: Smith *et al*[4] and Antoine *et al* [3], other references are mentioned in the table.

Chapter 2

Materials and methods

2.1. Materials

2.1.1 Commercial kits, reagents, and media

All commercial kits used in this DPhil thesis are listed below in table 1.1 that also shows the manufacturers, and the catalogue codes.

Kit	Manufacturer	Catalogue codes	Use purpose
Commercial Kits and reagents			
QIAprep Spin Miniprep Kit	QIAGEN	27104	Plasmid DNA extraction (mini)
QIAGEN Plasmid *Plus* Midi Kit	QIAGEN	12945	Plasmid DNA extraction (midi)
QIAGEN Plasmid *Plus* Maxi Kit	QIAGEN	12965	Plasmid DNA extraction (maxi)
QIAGEN Plasmid Maxi Kit	QIAGEN	12163	MVA-BAC extraction*(maxi)
Phaseprep™ BAC DNA	SIGMA-ALDRICH	NA0100	MVA-BAC extraction
EndoFree Plasmid Maxi Kit	QIAGEN	12362	Extracting plasmid DNA used for mice immunisation
QIAquick PCR/Gel Purification Kit	QIAGEN	28106	Purification of up to 10kb PCR products
MinElute PCR/Gel Purification Kit	QIAGEN	28006	Purification of up to 4kb PCR products
RNeasy Mini Kit	QIAGEN	74106	Purification of up to 100 µg total RNA from cells
GenElute™ Mammalian Total RNA Miniprep Kit	SIGMA-ALDRICH	RTN70	Purification of total RNA from cells
GenElute™ Mammalian Genomic DNA Miniprep Kits	SIGMA-ALDRICH	G1N70	Viral DNA extraction from infected cells.
DNAreleasy	Anachem Ltd.	LS02	Quick viral DNA extraction from infected cells.
Omniscript RT Kit	QIAGEN	205111	cDNA synthesis
QuantiTect SYBR Green PCR Kit	QIAGEN	204143	qPCR master mix
SensiFAST™ SYBR® Hi-ROX Kit	BIOLINE	BIO-92002	qPCR master mix
Zero Blunt® TOPO® PCR Cloning Kit	Invitrogen™	K2800-20	One Shot® TOP10 Chemically Competent E. coli
TOPO® TA Cloning® Kit	Invitrogen™	K4575-02	for Sequencing, with pCR™4-TOPO® Vector, One Shot® TOP10 Chemically Competent E. coli
In-Fusion® HD Cloning Plus	Clontech	638910	Cloning Kit, require 2 PCR products. No restriction digest is required.
LigaFast™	Promega	M8221	Rapid DNA ligation system
Restriction digest enzymes	New England BioLabs Inc	Various	DNA restriction digest
6X DNA Loading Dye	Thermo scientific	R0611	For gel electrophoresis
GeneRuler DNA Ladder Mix	Thermo scientific	SM0334	DNA marker
Tris-Acetate-EDTA (50X)	Fisher Scientific	BP1332-4	TAE
SYBR® Safe	Invitrogen™	S33102	DNA Gel Stain. Safe alternative to ethidium bromide.

Phusion® High-Fidelity PCR 2XMaster Mix with HF Buffer	New England BioLabs	M0531L	DNA Polymerase and PCR kit
Phire Hot Start II	Thermo scientific	F-122S	DNA Polymerase and PCR kit
Titanium® Taq DNA Polymerase	Clontech	639208	DNA Polymerase and PCR kit
10X Titanium® Taq PCR Buffer	Clontech	639141	DNA Polymerase and PCR kit
T4 DNA ligase	New England BioLabs	M0202T	Ligase
Halt™ Protease Inhibitor Cocktail (x100)	Fisher	PN78430	Supplement for lysis buffers
Protein A/G UltraLink Resin	Thermo Scientific	53132	Used in LIPS assay
Western Blot and ELISA			
ColorPlus™ Protein Molecular Weight Markers	New England BioLabs	P7711S	Protein marker
12% Precise Tris-Glycine Gels	Thermo Scientific	25262	Ready precast SDS-PAGE
Nitrocellulose Membrane, 0.2 µm, 1 roll.	BIO-RAD	162-0112	Nitrocellulose membrane, used in Western blot.
Nitrocellulose/Filter Paper Sandwiches, 0.45 µm, 7 x 8.5 cm, 50 pack	BIO-RAD	162-0215	Nitrocellulose membrane, used in Western blot.
Trans-Blot® Turbo™ Mini PVDF Transfer Packs	BIO-RAD	170-4156	PVDF membrane, used in the Semi-dry Blot transfer system for Western blot.
Anti-Renilla Luciferase Antibody, clone 5B11.2, Raised in mouse.	Millipore	MAB4400	Anti-rLuc primary antibody, (used in Western blot, ELISA, and Immunostaining)
Anti- MVA D10 polyclonal antibody, raised in rabbit against D10 peptide (see peptides table 1.4)	NeoBioLab	Customised	Anti-D10 antibody, used as primary Ab in Western blot and ELISA.
Mouse Anti-V5-TAG (Monoclonal Antibody) Clone: SV5-Pk5	AbD Serotec, UK	MCA2895GA	Anti-Pk monoclonal antibody, used as primary Ab in Western blot
Donkey anti-Rabbit IgG–alkaline phosphatase	Jackson labs	711-055-152	Anti-rabbit antibody (from goat) Used as **secondary** Ab in Western blot and ELISA.
Donkey anti-mouse IgG conjugated to alkaline phosphatase	Jackson labs	715-055-150	Anti-mouse antibody (from Donkey) Used as **secondary** Ab in Western blot
Goat anti-mouse total IgG conjugated to alkaline phosphatase	Sigma Aldrich	A3562	Anti-mouse antibody (from goat) Used as **secondary** Ab in ELISA.
SIGMAFAST BCIP/NBT tablet	Sigma Aldrich	B5655	Substrate (used in Western blot)

PNPP tablets (p-nitrophenylphosphate)	Sigma Aldrich	N-2765	Substrate (used in ELISA)
diethanolamine buffer	Thermo Scientific™ Pierce™	34064	Substrate buffer (used in ELISA)
Vaccinia virus Immunostaining			
Rabbit Anti-vaccinia virus Antibody	AbD SeroTec	9503-2057	Anti-VACV primary antibody, **(used in immunostaining for markerless virus titration)**
ECL™ Anti-Rabbit IgG-Horseradish peroxidase (from Donkey)	GE Healthcare, Life Sciences	NA934-100UL	Secondary antibody **(used in immunostaining for markerless virus titration)**
ImmPACT™ DAB	Vector Laboratories Ltd	SK-4105	Stained substrate **(used in immunostaining for markerless virus titration)**
ELISpot			
Anti-mouse IFN-γ mAb, AN18.	Mabtech, UK	3321-3-250	Anti-IFN-γ a monoclonal antibody, coating/capturing antibody **(used in ELISpot)**
Biotinylated anti-mouse-IFN-γ mAb, R4-6A2.	Mabtech, UK	3321-6-250	Anti-IFN-γ monoclonal antibody, detecting antibody **(used in ELISpot)**
Streptavidin alkaline phosphatase (AP) polymer	BIO-RAD	170-3554	Developing agent (**Used in ELISpot)**
AP Conjugate Substratre Kit,	BIO-RAD	170-6432	Alkaline phosphatase substrate **(used in ELISpot)**
Brefeldin A (Golgiplug)	BD Biosciences, UK		Golgiplug, used in ELISpot
Lipofectamine® 2000	Lifetechnologies™	11668019	Transfection Reagent
Fluorophore conjugated antibodies	eBioscience	Various	Flow cytometry
Renilla Luciferase Assay System	Promega	E2820	*In vitro* luciferase detection
ViviRen™ In Vivo Renilla Luciferase Substrate	Promega	P1231	*In vivo* luciferase imaging
BD Perm/Wash™	BD Biosciences	554723	ICS
Media and related reagents			
DMEM (Dulbecco's Modified Eagle Medium)	Sigma-Aldrich	D 5546	
OptiMEM® I Reduced Serum Media	Gibco (lifetechnologies™)	11058021	No phenol red
Minimum Essential Medium Eagle	Sigma	M3024	To make complete media to maintain splenocytes
TrypLE™ Express	Gibco (lifetechnologies™)	12605036	Trypsin reagent, with phenol red
Sodium Biocarbonate Solution 7.5%	Gibco (lifetechnologies™)	25080-094	Cell culture media additive
Formalin solution, neutral buffered, 10%	Sigma	HT501128	Histological tissue fixative, 4% Formaldehyde
LB tablet	Sigma-Aldrich		For LB broth

LB with agar	Sigma-Aldrich	L7025	For LB agar plates
Difco™ McConkey Agar	BD Biosciences		McConkey powder
Bacto™ Agar	BD Biosciences		Agar powder
M63 Medium	USBiological		Minimal agar plates for recombineering, used 31.2g in 400ml H_2O
Difco™ M9 Minimal Salts	BD	248510	
S.O.C. Medium	Invitrogen™	15544-034	Provided with the One Shot® DH5α™
Tryptic Soy Broth (TSB), a.k.a Soybean-Casein Digest Broth	Sigma-Aldrich	T8261	

Table 1.1: List of commercially kits, reagents and media

* This is based on flow-gravity and adding as double volume as recommended.

2.1.2 Chemicals

Chemical item	Provider
Agarose Ampicillin Kanamycin Chloramphenicol Dimethyl Sulfoxide Sodium Azide Terrific Broth Sodium Dodecyl Sulfate (SDS) Carbonate Biocarbonate Capsule Phosphate Buffer Saline solution PBS (Cat# D-8537) PBS tablets PBS powder (from Gibco not Sigma) PBS/0.05% Tween (Cat# P3563) PBS/Tween Sachets BSA (Bovine Serum Albumin) Ethanol (Molecular biology grade) Dulbecco PBS Ethylenediaminetetraacetic acid (EDTA) Foetal Calf Serum Dried Skimmed milk powder Hydochloric Acid (HCL) [1M] KHC03 [1mM] Na2EDTA NaSCN – Sodium Thiocyanate NH4Cl [0.15M] KH2PO4 K2HPO4 (NH4)2 SO4 FeSO4 Galactose 2-Deoxy-D-galactose (Cat#D4407) Sucrose Glycerol Leucine Biotin Pen/Strep [100U Penicillin 100µg Strep] L-Glutamine [4mm] Triton X-100	Sigma Aldrich, UK
Proteinase K	QIAGEN
Mowiol-DAPI mounting medium	Various provider
CASYton	Roche Diagnostics, Scotland
Methanol	Fischer Scientific, UK
Isopropanol	Fischer Scientific, UK
Difco™ M9 Minimal Salt 5x	BD Biosciences
M63 Medium	US Biologicals, USA
Isoflurane	From PPC

Table 1.2: List of chemicals

2.1.3 Primers

Primer code	Primer sequence	Use purpose
For B8-MVA		
Rec-B8-F	ttaaaatatattatcacttcagtgacagtagtcaaataacaaaca acaccATGGatgatAGCTACATCCCTAGC	Amplifying target DNA for recombineering
Rec-B8-R	gcatgctaagaaaagtggtaacatcatttgatgtgctaccgagaa atttaTCAGCACTGTCCTGCTCCTT	Amplifying target DNA for recombineering
ID-B8-F	Ttactgcagagggaaaatgc	ID-PCR for tPA-pb9-rluc at *B8R* locus
ID-B8-R	ACGCGAACTTTACGGTCATC	ID-PCR for tPA-pb9-rluc at *B8R* locus, a.k.a Screen.GalK.R
Seq-B8-F	Cgtcggatttgattccatag	Seq-PCR
Seq-B8-R	Tgaataatccgccagttacg	Seq-PCR
Pu-B8-F	Ccgcatttccaaagaatgat	Purity PCR
Pu-B8-R	gtgctgtcacgcacactttt	Purity PCR
For E3-MVA		
Rec-E3-F	atgataaaataaattagttttattgctggttgtgttagttctctc taaaaATGGATGCAATGAAGAGAGG	Amplifying target DNA for recombineering
Rec-E3-R	tgtatgattcaatgtataactaaactactaactgttattgataac tagaaTCAGCACTGTCCTGCTCCTT	Amplifying target DNA for recombineering
ID-E3-F	ctccagaaccagcatcacct	ID-PCR for tPA-pb9-rluc at *E3R* locus
ID-E3-R	ACGCGAACTTTACGGTCATC	ID-PCR for tPA-pb9-rluc at *E3R* locus, a.k.a Screen.GalK.R
Seq-E3-F	Tccaaacgcttttgtgatagt	Seq-PCR
Seq-E3-R	Ctccagaaccagcatcacct	Seq-PCR
Pu-E3-F	Acatcagcatccggcttatc	Purity PCR
Pu-E3-R	Tattgacgagcgttctgacg	Purity PCR
For F11-MVA		
Rec-F11-F	aattataaaaagtgaaaaacaatattatttttatcgttggttgtt TcactATGGatgatAGCTACATCCCTAGC	Amplifying target DNA for recombineering
Rec-F11-R	tctttgatggtgccagcatacgaattacaataatgcagaaactcg gttaaTCAGCACTGTCCTGCTCCTT	Amplifying target DNA for recombineering
ID-F11-F	atcattggcaacacccattt	ID-PCR for tPA-pb9-rluc at *F11R* locus
ID-F11-R	ACGCGAACTTTACGGTCATC	ID-PCR for tPA-pb9-rluc at *F11R* locus, a.k.a Screen.GalK.R
Seq-F11-F	Atcattggcaacacccattt	Seq-PCR
Seq-F11-R	Gccgagcattcatttactca	Seq-PCR
Pu-F11-F	Atagaatgcgagcagcgatt	Purity PCR
Pu-F11-R	gcaggatttacggcaacaat	Purity PCR
For mB8-MVA		
Rec-mB8.1-F	tggacatagtaatacatattattgaaaatatgatttttaaaaatt taaaatatattatcacttcagtgac	
Rec-mB8.2-F	tggacatagtaatacatattattcaaaatataatttttaaaaatt taaaatatattatcacttcagtgac	
Rec-mB8.3-F	tggacatagtaatacatattattgaaaatataatttttaaaaatt taaaatatattatcacttcagtgac	
Rec-IRES-B8-F	see recombineering database	

For MVA with inserts at TK		
TK-PhuUp-F	tcaatataaatgcgtgactataaaatattc	
TK-PhuUp-R	atacacacagcagttagttttaccacc	
TK-seq-F	ccatggatgacaactcaaaca	To sequence any insert within the TK locus
TK-seq-R	agcgtctgatttggtaactcg	
TK-ID-F	ccatggatgacaactcaaaca	To identify an insert within the TK locus
TK-ID-R	varies depends on antigens	
TK-Pu-F (J619)	ttttgaagcattggaagcaa	Purity PCR To confirm the absence of parental MVA, confirming a disturbed TK locus. Expected size is 278bp for parental MVA, and bigger for recombinant
TK-Pu-R (J622)	ctcggtttcctcacccaat	
For eB8-MVA		
Rec-eB8-F	ccgtgataggtatcgatgaaggacagttctttccagacat tgttgaattctcaacgatgatactctattgagaaaa	Amplifying target DNA for recombineering
Rec-eB8-R	tcgagtgcggctactataactatttttccttcgtt tgccatacgctcacatcagcactgtcctgctcctt	Amplifying target DNA for recombineering
ID-eB8-F	Use TK-ID-F	ID-PCR for tPA-pb9-rluc at TK locus
ID-eB8-R	gcagagccctctcttcattg	ID-PCR for tPA-pb9-rluc at TK locus, also called J989
Seq-eB8-F	UseTK-Seq-F	Seq-PCR
Seq-eB8-R	UseTK-Seq-R	Seq-PCR
Pu-eB8-F	UseTK-Pu-F	Purity PCR
Pu-eB8-R	UseTK-Pu-R	Purity PCR
For eE3-MVA		
Rec-eE3-F	ccgtgataggtatcgatgaaggacagttctttcca gacattgttgaattccctccagaatctccagaacc	Amplifying target DNA for recombineering
Rec-eE3-R	tcgagtgcggctactataactatttttccttcgtt tgccatacgctcacatcagcactgtcctgctcctt	Amplifying target DNA for recombineering - – Same as Rec-eB8-R and Rec-eB8-R
ID-eE3-F	UseTK-ID-F	ID-PCR for tPA-pb9-rluc at TK locus
ID-eE3-R	UseID-eB8-R	ID-PCR for tPA-pb9-rluc at TK locus
Seq-eE3-F	UseTK-Seq-F	Same as Seq-eB8-F
Seq-eE3-R	UseTK-Seq-R	Same as Seq-eB8-R
Pu-eE3-F	UseTK-Pu-F	Purity PCR
Pu-eE3-R	UseTK-pu-R	Purity PCR
For eF11-MVA		
Rec-eF11-F	ccgtgataggtatcgatgaaggacagttctttccagacattgtt gaattctgccgagcattcatttactc	Amplifying target DNA for recombineering
Rec-eF11-R	tcgagtgcggctactataactatttttccttcgtt tgccatacgctcacatcagcactgtcctgctcctt	Amplifying target DNA for recombineering - – Same as Rec-eB8-R and Rec-eB8-R
ID-eF11-F	UseTK-ID-F	ID-PCR for tPA-pb9-rluc at TK locus
ID-eF11-R	UseID-eB8-R	ID-PCR for tPA-pb9-rluc at TK locus
Seq-eF11-F	UseTK-Seq-F	Same as Seq-eB8-F
Seq-eF11-R	UseTK-Seq-R	Same as Seq-eB8-R

Pu-eF11-F	UseTK-Pu-F	Purity PCR
Pu-eF11-R	UseTK-pu-R	Purity PCR
For qPCR		
rLuc-F	ccctgatcaagagcgaagag	
rLuc-R	gtctaacctcgcccttctcc	
E9-F	gagtatagagcactatttctaaatccca	
E9-R	tcaactgaaaaggccatctatga	
Multiplex PCR		
ITR(002/192)F	acgggatcgcagtctttatg	5 primer pairs for MVA-BAC integrity used in Multiplex PCR. Expected sizes are discussed in the relevant section
ITR(002/192)R	ccggagacgtcatctgttct	
ATPase(143)F	ttcaccttcacaaaatacggagt	
ATPase(143)R	ttgctgtcgcacaaaatcat	
Kinase(039)F	tgccggataaaagtgggata	
Kinase(039)R	caaaattgggctccatcagt	
Rpoly(090)F	tacggttttggggtgacatt	
Rpoly(090)R	cgaccaccatatcctccatc	
RikkeF	atagaacttacgcaaatattagcaaaaat	
RikkeR	tggaaagcgggcagtga	
IF-AphAI-85A-F	gcggccgcatagatctcgatttattcaacaaagccacg	In-Fusion primers
IF-AphAI-85A-R	cagaattcccagatctgccagtgttacaaccaattaacc	
CMV-D10-F	cttgacacgaagcttggtaccatgaacttttacagatcta	To amplify D10 from MVA and has *KpnI* site plus 15 nt matching plasmid p1990 which has CMV promoter
CMV-D10-R	tgatggatatctgcagaattctcaatcatcctcagttaat	To amplify D10 from MVA and has *EcoRI* site plus 15 nt matching plasmid p1990 which has CMV promoter

Table 1.3: List of all primers used throughout the DPhil project

F: Forward primer. R: Reverse primer. ID-PCR: Identity PCR. Seq-PCR: flank-to-flank PCR that were then purified and sent for sequencing analysis.

2.1.4 Peptides and proteins

<table>
<tr><th>Peptide</th><th>Peptide sequence</th><th>Notes</th></tr>
<tr><td>E3</td><td>VGPSNSPTF</td><td>H2^{d} haplotype-restricted CD8^{+} T cell peptide, e.g. BALB/c mice</td></tr>
<tr><td>F2(G)</td><td>SPYAAGYDL</td><td>H2^{d} haplotype-restricted CD8^{+} T cell peptide, e.g. BALB/c mice</td></tr>
<tr><td>B8</td><td>TSYKFESV</td><td>H2b haplotype-restricted CD8^{+} T cell peptide, e.g. C57B1/6 mice</td></tr>
<tr><td>Pb9</td><td>SYIPSAEKI</td><td></td></tr>
<tr><td>P11</td><td>EWYDQSGLSVVMPVGGQSSF</td><td>CD8^{+} T cell peptide, used for BALB/c mice</td></tr>
<tr><td>P15</td><td>TFLTSELPGWLQANRHVKPT</td><td>CD4^{+} T cell peptide, used for BALB/c mice</td></tr>
<tr><td>T cell-specific peptide pool. 66 peptides covering the 85A Ag, synthesised by NeoBioLab.</td><td colspan="2">
<table>
<tr><td>1</td><td>MQLVDRVRGAVTGMS</td><td>34</td><td>VGLSMAASSALTLAI</td></tr>
<tr><td>2</td><td>RVRGAVTGMSRRLVV</td><td>35</td><td>AASSALTLAIYHPQQ</td></tr>
<tr><td>3</td><td>VTGMSRRLVVGAVGA</td><td>36</td><td>LTLAIYHPQQFVYAG</td></tr>
<tr><td>4</td><td>RRLVVGAVGAALVSG</td><td>37</td><td>YHPQQFVYAGAMSGL</td></tr>
<tr><td>5</td><td>GAVGAALVSGLVGAV</td><td>38</td><td>FVYAGAMSGLLDPSQ</td></tr>
<tr><td>6</td><td>ALVSGLVGAVGGTAT</td><td>39</td><td>AMSGLLDPSQAMGPT</td></tr>
<tr><td>7</td><td>LVGAVGGTATAGAFS</td><td>40</td><td>LDPSQAMGPTLIGLA</td></tr>
<tr><td>8</td><td>GGTATAGAFSRPGLP</td><td>41</td><td>AMGPTLIGLAMGDAG</td></tr>
<tr><td>9</td><td>AGAFSRPGLPVEYLQ</td><td>42</td><td>LIGLAMGDAGGYKAS</td></tr>
<tr><td>10</td><td>RPGLPVEYLQVPSPS</td><td>43</td><td>MGDAGGYKASDMWGP</td></tr>
<tr><td>11</td><td>VEYLQVPSPSMGRDI</td><td>44</td><td>GYKASDMWGPKEDPA</td></tr>
<tr><td>12</td><td>VPSPSMGRDIKVQFQ</td><td>45</td><td>DMWGPKEDPAWQRND</td></tr>
<tr><td>13</td><td>MGRDIKVQFQSGGAN</td><td>46</td><td>KEDPAWQRNDPLLNV</td></tr>
<tr><td>14</td><td>KVQFQSGGANSPALY</td><td>47</td><td>WQRNDPLLNVGKLIA</td></tr>
<tr><td>15</td><td>SGGANSPALYLLDGL</td><td>48</td><td>PLLNVGKLIANNTRV</td></tr>
<tr><td>16</td><td>SPALYLLDGLRAQDD</td><td>49</td><td>GKLIANNTRVWVYCG</td></tr>
<tr><td>17</td><td>LLDGLRAQDDFSGWD</td><td>50</td><td>NNTRVWVYCGNGKPS</td></tr>
<tr><td>18</td><td>RAQDDFSGWDINTPA</td><td>51</td><td>WVYCGNGKPSDLGGN</td></tr>
<tr><td>19</td><td>FSGWDINTPAFEWYD</td><td>52</td><td>NGKPSDLGGNNLPAK</td></tr>
<tr><td>20</td><td>INTPAFEWYDQSGLS</td><td>53</td><td>DLGGNNLPAKFLEGF</td></tr>
<tr><td>21</td><td>FEWYDQSGLSVVMPV</td><td>54</td><td>NLPAKFLEGFVRTSN</td></tr>
<tr><td>22</td><td>QSGLSVVMPVGGQSS</td><td>55</td><td>FLEGFVRTSNIKFQD</td></tr>
<tr><td>23</td><td>VVMPVGGQSSFYSDW</td><td>56</td><td>VRTSNIKFQDAYNAG</td></tr>
<tr><td>24</td><td>GGQSSFYSDWYQPAC</td><td>57</td><td>IKFQDAYNAGGGHNG</td></tr>
<tr><td>25</td><td>FYSDWYQPACGKAGC</td><td>58</td><td>AYNAGGGHNGVFDFP</td></tr>
<tr><td>26</td><td>YQPACGKAGCQTYKW</td><td>59</td><td>GGHNGVFDFPDSGTH</td></tr>
<tr><td>27</td><td>GKAGCQTYKWETFLT</td><td>60</td><td>VFDFPDSGTHSWEYW</td></tr>
<tr><td>28</td><td>QTYKWETFLTSELPG</td><td>61</td><td>DSGTHSWEYWGAQLN</td></tr>
<tr><td>29</td><td>ETFLTSELPGWLQAN</td><td>62</td><td>SWEYWGAQLNAMKPD</td></tr>
<tr><td>30</td><td>SELPGWLQANRHVKP</td><td>63</td><td>GAQLNAMKPDLQRAL</td></tr>
<tr><td>31</td><td>WLQANRHVKPTGSAV</td><td>64</td><td>AMKPDLQRALGATPN</td></tr>
<tr><td>32</td><td>RHVKPTGSAVVGLSM</td><td>65</td><td>LQRALGATPNAVPAP</td></tr>
<tr><td>33</td><td>TGSAVVGLSMAASSA</td><td>66</td><td>GATPNAVPAPQGA</td></tr>
</table>
</td></tr>
<tr><td>Peptide pool of 15-mer overlapping by 11 amino acids, covering the transgenic protein of pb9-rLuc. Peptides were</td><td colspan="2">
<table>
<tr><td>1;1;DAMKRGLCCVLLLCG;15;;;;</td><td>45;177;AIVHMESVVDVIESW;15</td></tr>
<tr><td>2;5;RGLCCVLLLCGAVFV;15;;;;</td><td>46;181;MESVVDVIESWDEWP;15</td></tr>
<tr><td>3;9;CVLLLCGAVFVSPSQ;15;;;;</td><td>47;185;VDVIESWDEWPDIEE;15</td></tr>
</table>
</td></tr>
</table>

synthesised by NeoBioLab.	4;13;LCGAVFVSPSQEIHA;15	48;189;ESWDEWPDIEEDIAL;15
	5;17;VFVSPSQEIHAMDDS;15	49;193;EWPDIEEDIALIKSE;15
	6;21;PSQEIHAMDDSYIPS;15	50;197;IEEDIALIKSEEGEK;15
	7;25;IHAMDDSYIPSAEKI;15	51;201;IALIKSEEGEKMVLE;15
	8;29;DDSYIPSAEKIGSKV;15	52;205;KSEEGEKMVLENNFF;15
	9;33;IPSAEKIGSKVYDPE;15	53;209;GEKMVLENNFFVETV;15
	10;37;EKIGSKVYDPEQRKR;15	54;213;VLENNFFVETVLPSK;15
	11;41;SKVYDPEQRKRMITG;15	55;217;NFFVETVLPSKIMRK;15
	12;45;DPEQRKRMITGPQWW;15	56;221;ETVLPSKIMRKLEPE;15
	13;49;RKRMITGPQWWARCK;15	57;225;PSKIMRKLEPEEFAA;15
	14;53;ITGPQWWARCKQMNV;15	58;229;MRKLEPEEFAAYLEP;15
	15;57;QWWARCKQMNVLDSF;15	59;233;EPEEFAAYLEPFKEK;15
	16;61;RCKQMNVLDSFINYY;15	60;237;FAAYLEPFKEKGEVR;15
	17;65;MNVLDSFINYYDSEK;15	61;241;LEPFKEKGEVRRPTL;15
	18;69;DSFINYYDSEKHAEN;15	62;245;KEKGEVRRPTLSWPR;15
	19;73;NYYDSEKHAENAVIF;15	63;249;EVRRPTLSWPREIPL;15
	20;77;SEKHAENAVIFLHGN;15	64;253;PTLSWPREIPLVKGG;15
	21;81;AENAVIFLHGNATSS;15	65;257;WPREIPLVKGGKPDV;15
	22;85;VIFLHGNATSSYLWR;15	66;261;IPLVKGGKPDVVQIV;15
	23;89;HGNATSSYLWRHVVP;15	67;265;KGGKPDVVQIVRNYN;15
	24;93;TSSYLWRHVVPHIEP;15	68;269;PDVVQIVRNYNAYLR;15
	25;97;LWRHVVPHIEPVARC;15	69;273;QIVRNYNAYLRASDD;15
	26;101;VVPHIEPVARCIIPD;15	70;277;NYNAYLRASDDLPKL;15
	27;105;IEPVARCIIPDLIGM;15	71;281;YLRASDDLPKLFIES;15
	28;109;ARCIIPDLIGMGKSG;15	72;285;SDDLPKLFIESDPGF;15
	29;113;IPDLIGMGKSGKSGN;15	73;289;PKLFIESDPGFFSNA;15
	30;117;IGMGKSGKSGNGSYR;15	74;293;IESDPGFFSNAIVEG;15
	31;121;KSGKSGNGSYRLLDH;15	75;297;PGFFSNAIVEGAKKF;15
	32;125;SGNGSYRLLDHYKYL;15	76;301;SNAIVEGAKKFPNTE;15
	33;129;SYRLLDHYKYLTAWF;15	77;305;VEGAKKFPNTEFVKV;15
	34;133;LDHYKYLTAWFELLN;15	78;309;KKFPNTEFVKVKGLH;15
	35;137;KYLTAWFELLNLPKK;15	79;313;NTEFVKVKGLHFLQE;15
	36;141;AWFELLNLPKKIIFV;15	80;317;VKVKGLHFLQEDAPD;15
	37;145;LLNLPKKIIFVGHDW;15	81;321;GLHFLQEDAPDEMGK;15
	38;149;PKKIIFVGHDWGAAL;15	82;325;LQEDAPDEMGKYIKS;15
	39;153;IFVGHDWGAALAFHY;15	83;329;APDEMGKYIKSFVER;15
	40;157;HDWGAALAFHYAYEH;15	84;333;MGKYIKSFVERVLKN;15;
	41;161;AALAFHYAYEHQDRI;15	85;337;IKSFVERVLKNEQ;13;
	42;165;FHYAYEHQDRIKAIV;15	86;341;VERVLKNEQ;9;incomplete
	43;169;YEHQDRIKAIVHMES;15	87;345;LKNEQ;5;incomplete
	44;173;DRIKAIVHMESVVDV;15	88;349;Q;1;incomplete
D10 peptide	CYYESLKKLTEDD	Used to generate rabbit Anti-D10 polyclonal antibody, customised for our experiment by NeoBioLab

Protein	Notes
rLuc recombinant	Recombinant *Renilla reniformis* Luciferase, from RayBiotech, Inc.

protein	Cat#: RB-15-0003P
85A recombinant protein	

Table 1.4: List of peptides and proteins

2.1.5 Cells

Cells	Source	Notes
CEF	AHVLA Scientific - DEFRA, UK	Primary chick embryonic fibroblast
BHK-21	ATCC (American Type Culture Collection)	Baby hamster Kidney fibroblast cell lines (Mesocricetus auratus, Syrian golden hamster)
DF-1 (a.k.a UMNSAH/DF-1)	ATCC	Embryonic fibroblast cell line (*Gallus gallus*, chicken) Used as an alternative of CEF.
VERO	ATCC	Kidney epithelial cell lines (*Cercopithecus aethiops*, Green African monkey)
HEK 293	ATCC	Embryonic kidney epithelial cell lines (*Homo sapiens*, human)
A549	ATCC	Lung adenocarcinoma epithelial cell lines (*Homo sapiens*, human)
SW102 *E.coli*	Søren Warming	See Warming *et al* paper [171]
One Shot® MAX Efficiency® DH5α™	Invitrogen™ Cat. No.12297-016	Competent Cells, used with In-Fusion cloning
Subcloning Efficiency™ DH5α™	Invitrogen™ Cat. No.18265-017	Competent Cells
One Shot® TOP10	Invitrogen™	One Shot® TOP10 Chemically Competent *E. coli*, used with Zero Blunt® TOPO® PCR Cloning Kit (see kits above)

Table 1.5: List of cells

2.1.6 Equipment and instruments

Equipment	Equipment trade name, Source
PCR machine	BIO-RAD
Agarose Gel and SDS-PAGE apparatuses	BIO-RAD
Heat block	Eppendorf
Water bath	Various
Bacteria plate incubator	Heraeus, Thermo scientific
Shaking incubator	Innova"44 New Brunswick Scientific
Cell culture incubator	Galaxy 170R, New Brunswick
Electroporation device	GenePulser Xcell, BIO-RAD
Vacuum pump	From KNF Laboratory Pumps
Vacuum pump	From Charles Austen Pumps
Plate vacuum pump, for LIPS	Millipore
Microfuge, Bench-top Centrifuges	Eppendorf
Centrifuges, Ultracentrifuge	Beckman Coulter
Vortex, tube shaker	Various
Plate shaker/rotator	The Belly Dancer, Stovall Life Sciences
Gel documentation	BioSpectrum AC imaging system
Fume hood	
DNA and protein quantitation system	NanoDrop, Thermo scientific
Spectrophotometer	DU®730, Beckman coulter
DNA Concentrator	DNA SpeedVac Concentrator, Thermo scientific
Microwave	Various
Pipettes	Gilson and RAININ
Pipette boy	Pipetboy, RAININ
Scale	Various
pH meters	Various
Class 2 biosafety cabinet	Mars, SCANLAF
Light Microscope	Leica
Epifluorescence Microscope	Leica
Sonication probe	Sonics & Materials, USA
Chemoluminescence reader	VARIOSKAN FLASH, Thermo scientific
ELISA plate reader	EL800, BioTek®
ELISpot plate reader	ELISpot reader system, AID
ELISA and ELISpot plate washing machine	Ultrawash Plus, DYNEX
Semi-Dry Western blotting	Trans-Blot® Turbo™ Transfer System, BIO-RAD
Flow Cytometers	1- CyAn™ ADP Analyzer, Beckman Coulter 2- BD LSR II, BD Biosciences
MoFlo flow cytometer	CyCLONE robotic module of a MoFlo flow cytometer, Dako Cytomation
Real time qPCR machine	Step One Plus, AB Applied Biosystem
Fluorometer	FluoSTAR OPTIMA F fluorimeter, BMG Labtech
Anaesthetic machine	
Liquid Nitrogen storage	Kseries Cryostorage System and CryoScience by Taylor-Wharton
Fridge, freezer, and -80 freezer	Various
Cell counter	CASY Cell Counter, Schärfe Systems, Germany
Aspirator	Vacusafe, IBS Integra BioSciences

Hood cabinet	Used for mice immunisation
18 MΩ.cm water distillation system	PureLab Ultra, ELGA
Living imaging system	IVIS system, Xenogen

Table 1.6: List of equipment and instruments

2.1.7 Consumable plastics and glassware

Item, source	Use purpose
Tubes	
0.2 mL PCR Tubes, Thermo Scientific.	PCR
0.5 mL Eppendorf Tubes, Treff Laboratories, Switzerland	
1.5 mL Eppendorf Tubes, Treff Laboratories, Switzerland	
2 ml screw-capped tubes,	Virus prep and bacteria glycerol stocks
Microvette tubes, SARSTEDT	For mice blood collection
14 ml Culture Tubes, BD Biosciences	For Flow Cytometry machine
15ml and 50 ml centrifuge tubes, Fisher, Corning Inc., and BD Falcon®	
7 ml Bijou, Sterilin - Thermo scientific	
50 ml universal tubes, Sterilin - Thermo scientific	
BD 5 ml Falcon® FACS tubes	Flow Cytometry
5 ml TruCool® Cryogenic Vials, Biocision	Liquid nitrogen cell vials
Ultracentrifuge 250 bottle	
Ultracentrifuge tube (Thinwall, Ultra-Clear™, 14 ml, 14 x 95 mm)	Beckman Coulter
Plates and flasks	
60mm Petri Dishes, Corning Inc.	
96-Well Tissue Culture Flat Bottom Plates, Corning Inc. (also 6-well and 24-well plates)	
96-Well U Bottom Plates, Corning Inc.	Splenocyte stimulation, in ICS
96-Well V Bottom Plates, Fischer	Spinning splenocytes, in ICS
96-Well flat bottom – Nunc Immuno Maxisorp Plates, Thermo Scientific	ELISA
96-Well flat bottom – Nunc Maxisorp White Plates, Thermo Scientific	Chemoluminescence
96-Well flat bottom – Black opaque plates	GFP detection by Fluorometer
96-well filter plate: MultiScreen HTS with hydrophilic Durapore PVDF membrane Barex/TiO_2, non sterile, opaque, 0.45µm pore size	LIPS Assay (Millipore Cat # MSHVN4B50)
96-Well filter plate: MultiScreen® Filter Plates with Durapore® Membrane	ELISpot, Millipore
T25/T75/T150/T175 Flask, Corning Inc.	25, 75, 150, and 175 cm2 surface
Black-walled, clear-bottom 96-well plate, (Grand Plates™, Germany)	Fluorometer
Miscellaneous	
Nalgene bottle top filter, 500ml 2.45mm, Fisher	Sucrose sterilisation
Cell Scrapper, Corning Inc.	Spleen mashing
70µm Cell Strainer, VWR, UK	
Tips	
Serological pipettes	
Pasteur pipettes	
2ml, 5ml, and 10ml Syringe, BD Plastipak	
26G, 0.45mm needle, BD Microlance	
0.2µm filter, Filter Sartorius.	
0.5ml U-100 Insulin syringe, BD Microlance	Mice immunization
Surgical blade, Swann-Moston	Mice tail-bleeding
Durham bottles	
Needles: 23G, 25G, and 26G, BD Microlance	

Syringe: 2, 5, 10, 20, 50 ml, BD Plastipak	
Filters: 0.2, 0.45, 0.8 µm, Minisart®, Sartorius Stedim	

Table 1.7: List of consumable plastics and glassware

2.1.8 In-house prepared buffers and solutions

Various solutions, buffers, and cell culture media were made in the Jenner institute's laboratories as described below. Such solutions are referred to accordingly in the methods section or in the thesis chapters. The list below also contains some information on the use of these solutions.

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

1.8.2 **Ammonium Chloride Potassium (ACK) Lysis Buffer:** 8.29g NH4Cl (0.15M), 1g KHCO3 (1mM), and 37.2mg Na2EDTA, made up to 0.8L in water. *Used in processing splenocytes and PBMCs.*

1.8.3 **Phosphate Buffer Saline (PBS) – Tablet – 0.01M:** NaCl 0.138M, KCl 0.0027M, pH 7.4: made by dissolving tablets in dH_2O. *Used in ELISA and Western blot; or autoclaved and used in ELISpot and ICS*.

1.8.4 **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. *Used in ELISA and Western blot*.

1.8.5 **Tris-acetate-EDTA (TAE) buffer:** Made up from 50x concentrate with dH_2O. *Used in Gel electrophoresis.*

1.8.6 **Loading Dye (for DNA):** This was used, sometimes, in replacement of to the 6X loading dye described in the commercial kits table, above. It is made of 0.4% orange G, 15% Ficoll® 400, 10mM Tris-HCl (pH 8.0), 50mM EDTA (pH 8.0) made up with dH_2O. *Used in Gel electrophoresis.*

1.8.7 **Reducing Laemmli 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. *Used in Western blot.*

1.8.8 **Buffer A (LIPS assay):**50mM Tris, 100mM NaCl, 5mM $MgCl_2$, 1% Triton X-100, made in 1L and stored at room temperature.

1.8.9 **Lysis Buffer (LIPS assay):**50mM Tris, 100mM NaCl, 1% Triton X-100, 50% glycerol, 1% EDTA (i.e. 1 ml for a 100 solution). Also, x100 Halt protease inhibitor cocktail was diluted 1:100 and added just before use.

1.8.10 **Lysis Buffer (**Western blot)**:** *Used to lyse attached cultured cells for Western blot.* Add 1ml of 2% Triton X-100, 100 ul of 100X protease inhibitors, and 1 mM EDTA (i.e. take 10 ul of 1M EDTA). Combine them in 10 ml PBS.

1.8.11 **Running Buffer** (Western blot)**:**To make (x10) buffer, add 121g Tris Base (MW = 121), 238 g HEPES (free acid MW = 238),and 10 g SDS (MW = 288) into 1 L ultrapure H_2O.

- Before use dilute 10-fold with water. The pH of the 1X buffer should be ~8.0; do not adjust pH.
- Final composition of the 1X buffer is 100 mM Tris, 100 mM HEPES, 1% (~3 mM) SDS, pH 8.0.

1.8.12 **Transfer Buffer** (Western blot)**:**Add 6 g Tris base,30 g Glycine, and 200 ml Methanol into 1 L H2O.

1.8.13 **Blocking Buffer (ELISA):** 10% skimmed milk powder (Tesco) in PBS/T (Sigma)

1.8.14 **Blocking Buffer (Western Blot):** 3% BSA in PBS

1.8.15 **10%sera DMEM:** Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, and 1% penicillin/streptomycin. *Used to maintain cell growth.*

1.8.16 **2%sera DMEM:** As 10% DMEM, except percentage of FCS lowered to 2%. *Used to maintain virus-infected cells.*

1.8.17 **Complete Medium:** Modified Eagle's Medium alpha-modification, supplemented with 10% FCS (heat-inactivated at 56°C, 30min), 2mM L-glutamine, 1% penicillin/streptomycin and 50 µM 2-mercaptoethanol. *Used to maintain splenocytes and PBMCs in ELISpot and ICS.*

1.8.18 **CMC Overlay:** 1:3 mix of 1 part CMC and 2 parts Dubelco Eagle Medium (with 2% FCS). *Used to in virus plaque assay.*

1.8.19 **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. This was made as a backup solution in case there is no CMC-overlay, and was never used.

1.8.20 **Sucrose Gradient:** 36% Sucrose (Sigma) in 18 Megohm ultra-pure H_2O, filter sterilized with a bottle top filter(500ml). *Used for virus purification.*

1.8.21 **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. *Used to reconstitute virus prep for in vivo immunisation.*

1.8.22 **M9 Medium (5x):** Solution prepared according to manufacturers' instructions (e.g., BD Difco) and autoclaved. Otherwise, prepared traditionally as: 6g Na2HPO4, 3g KH2PO4, 1g NH4Cl, 0.5g NaCl dissolved in 1L dH_2O and autoclaved. *Used in MVA-BAC recombineering*

1.8.23 **M63 Medium (5x):** 24 g KH2PO4 (anhydrous), 56 g K2HPO4 (anhydrous), 10 g (NH4)2SO4, 1 mL 2.5 mg/ml FeSO4 in 1 litre; check pH = 7 and autoclave. *Used in making minimal medium agar plates, for MVA-BAC recombineering*

1.8.24 **STET:** 8% sucrose; 5% Triton X-100; 50 mM EDTA; 50 mM Tris-HCl, pH 8.0.

1.8.25 **Minimal Medium Agar Plates:** Autoclave 7.5 g agar in 400 mL water and add 100 mL 5× M63 to molten agar. Equilibrate to 50°C. Add 0.5 mL of 1M MgSO4 (autoclaved); 2.5 mL of 0.2 mg/mL D-biotin (sterile filtered); 2.5mL of 9 mg/mL L-leucine (sterile-filtered); 500 µL of 12.5 mg/mL chloramphenicol in MeOH; and 5 mL of 20% D-galactose (autoclaved). Pour up to 20 Petri dishes. (All reagents can be obtained from Sigma). *Used to screen recombinant MVA-BAC clones*

1.8.26 **MacConkey Indicator Plates:** 16g agar in 400mL dH_2O and autoclaved. Add 20mL galactose and 400µl Chloramphenicol (final conc. 12.5µg/mL). *Used to screen $GalK^+$ or $GalK^-$ MVA-BAC recombinant clones.*

1.8.27 **FACS buffer:** PBS + 0.5% BSA + 0.05% Sodium Azide in PBS

2.2. Methods

2.2.1. Molecular biology methods

2.2.1.1. PCRs

Polymerase chain reaction (PCR) was used to amplify construct DNA that were used for cloning and recombineering, or to screen for the correct constructs after cloning or recombineering were perfumed. In this project there is a number of slightly different PCR that are described below and referred to throughout the thesis, with also the use of different primers that are listed in table 1.3.

2.2.1.1.1. Target DNA PCR

This PCR was used to amplify different inserts as target DNA for recombineering, using Phusion DNA polymerase due to its high fidelity rate; following this recipe and thermocycler program:

Recipe:
25 µl 2X master mix Phusion
2.5 µl Primer F (forward) (10µM). Final conc. 0.5µM
2.5 µl Primer R (reverse) (10µM). Final conc. 0.5µM
10 µl Betaine. Final conc. 1M

? µl (10 pg to 10 ng) template DNA
H_2O - volume up to 50µl

Total: 50µl

Program:
1. 98°C for 30 sec
2. 98°C for 5 sec
3. 60°C for 10 sec (primers-dependent*)
4. 72°C for 40 sec (amplicon-dependent)
5. 72°C for 5 min
6. Hold at 4°C

Repeat 2-4 steps 50 times

Box 1: Phusion 2X master mix PCR. * Almost all primers worked at Tm= 60°C when Betaine was added to the reaction.

However, when the 2X master mix Phusion PCR mix reaction was difficult to yield the desired product, especially when the amplicon is more than 4-5 kb, the following recipe was performed:

Recipe:
10 µl Phusion HF buffer
0.5 µl Phusion HF enzyme
1.25 µl of 8mM dNTPs
2.5 µl Primer F (of 10 µM solution)
2.5 µl Primer R (of 10 µM solution)
1.5 µl DMSO
? µl (10 pg to 10 ng) template DNA
H_2O - volume up to 50µl
Total: 50µl

Program:

1. 98°C for 30 sec
2. 98°C for 5 sec
3. 60°C for 10 sec (primers-dependent)
4. 72°C for 40 sec (amplicon-dependent)
5. 72°C for 5 min
6. Hold at 4°C

Repeat 2-4 steps 50 times

Box 2: Phusion Hot Start II PCR.

2.2.1.1.2. Identity PCR (ID-PCR)

This PCR was done to confirm the presence of inserts into either the BAC DNA or the viral DNA before proceeding further with the purification of the clones, either colonies (at the BAC stage) or virus plaques (at the viral infection stage). It also confirms the integration of inserts within the correct destination (insertion site). Therefore, the primers are designed to amplify the sequence that extends from the left region of the inserts and the right region of the adjacent vector backbones (either plasmid vector, the MVA-BAC backbone, or the MVA viral backbone), amplifying the junction between the vectors and the inserts. Here, I used Phire Hot Start II DNA Polymerase because of its cost-effectiveness with the following reaction:

Recipe:
4 µl Phire Hot Start buffer
0.4 µl Phire Hot Start enzyme
2µl of 8mM dNTPs
1µl Primer F (of 10 µM solution)
1µl Primer R (of 10 µM solution)
3.6 µl of 1:100 diluted BAC miniprep
8 µl H_2O
Total: 20µl

Program:
1. 98°C for 30 sec
2. 98°C for 5 sec
3. 60°C for 5 sec (primers-dependent)
4. 72°C for 30 sec (amplicon-dependent)
5. 72°C for 5 min
6. Hold at 4°C

Repeat 2-4 steps 35 times

Box 3: Phire Hot Start PCR.

2.2.1.1.3. Purity PCR

When ID-PCRs were correct, the purity of clones was then checked via Purity PCR, which screen for the complete absence of the wild type MVA-BAC DNA. This PCR is also used to confirm the absence of the parental MVA if recombinants were made using the conventional vaccinia recombination, and not using MVA-BAC recombineering, see

section 2.2.3.12. The primers for this PCR are designed to amplify the sequence of the insertion site, which was replaced by inserts. For example, if a construct is designed to be inserted at the *F11L* locus, deleting and replacing the *F11L* genes, the purity PCR should therefore amplify *F11*-specific sequence that should be positive only in the wild type, and not in the recombinant BAC or virus. The recipe of purity PCR is similar to the ID-PCR (Box 3).

2.2.1.1.4. Sequencing PCR (Seq-PCR)

This PCR was done following the Phusion polymerase recipes to amplify inserts from either the BAC DNA or from viral DNA; the primers are designed to cover beyond the insert sequences; this is often called flank-to-flank PCR. The products of this PCR are then purified (using QIAquick or MinElute purification Kits) and sent to a sequencing provider to confirm the sequence identity, the orientation of inserts, and the position of inserts within the neighbouring MVA loci. The Phusion DNA polymerase was used for its high fidelity to avoid introducing PCR-mediated mutation.

2.2.1.1.5. Multiplex PCR (BAC integrity PCR)

Following every BAC recombineering, the correct recombinant MVA-BACs were also checked for the integrity of the BAC DNA that should yield in intact genome and infectious viruses. This PCR must be done before proceeding to rescue MVA-BAC into infectious viruses. It is based on the Titanium Taq DNA polymerase and involves a mixture of five primer pairs targeting essential ORFs of MVA, distributed throughout the MVA genome, as described in the following box.

Recipe:
2 µl Titanium buffer
0.4 µl Titanium Taq enzyme
0.48 µl of 8mM dNTPs
4 µl Multiplex primer mix (5 primer pairs)
2 µl of 1:100 diluted BAC miniprep
11.12 µl H_2O
Total: 20µl

Program:
96°C for 15 sec
50°C for 10 sec
72°C for 1 min
Repeat for 35 times

Expected sizes:
232 bp
359 bp
444 bp
525 bp
592 bp

Box 4: Titanium Multiplex PCR.

2.2.1.1.6. Colony-PCR

To screen whether the bacterial colonies have the correct introduced inserts following DNA cloning, a PCR was performed on 10 to 15 individual bacterial colonies that were dissolved in 20 µl H_2O and lysed at 95ºC for 10 min. 10µl of lysed colonies were added to 10 µl of Titanium PCR master, below box. The primers used for colony screening varied according to the introduced inserts; the forward primers were always designed to bind to the right-hand side (towards 3') of inserts whereas the reverse primers were designed to bind to the left-hand side of plasmid vector backbones. This is to ensure that the colony-PCR amplifies over the junction between inserts and vectors. Titanium Taq was used as described in the box 5 below.

Recipe: 2 µl Titanium buffer 0.4 µl Titanium Taq enzyme 0.48 µl of 8mM dNTPs 0.4µ l Primer F 0.4 µl Primer R 6.32 µl H_2O 10 µl lysed colony Total: 20µl Program: 96°C for 15 sec 50°C for 10 sec 72°C for 1 min Repeat for 35 times

Box 5: Titanium Colony-PCR

2.2.1.2 DNA cloning

2.2.1.2.1. Restriction endonuclease digests

1 µl per restriction enzyme 2.5 µl 10x reaction buffer 25 µl 1:10 Bovine serum albumin (BSA) or as required 5 µl DNA template or as required depending on DNA concentration (maximum DNA is 1µg) 13 µl H_2O or as required Total: 20 µl

Box 6: Restriction digest mix.

2.2.1.2.2. Ligation reactions

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

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

Box 7: Ligation reaction mix.

2.2.1.2.3. In-Fusion cloning

Recipe: 2 µl 5X In-Fusion HD Enzyme Premix x µl *Purified PCR Fragment x µl ** Linearized Vector x µl dH_2O (as needed) 10 µl Total Volume Program: 50°C for 15 min Keep on ice

Box 8: In-Fusion reaction mix.
* Size-dependent, for <0.5 kb: 10–50 ng, 0.5 to 10 kb: 50–100 ng, >10 kb: 50–200 ng.
** <10 kb: 50–100 ng, >10 kb: 50–200 n.

2.2.1.3. Bacterial transformation

50 µl Maximum efficiency or Sub-cloning efficiency DH5α E. coli were thawed on ice, then 6 µl ligation reaction (or In-Fusion reaction) were added, and incubated on ice for 30 min. Following this, cells were heat-shock at 42°C for 45 sec and kept for 5 min on ice. Bacteria were grown for 1 hour in 250 µl S.O.C. medium at 37°C in a shaking incubator (220 rpm). Bacteria were then plated onto LB (Luria-Bertani) agar containing selection antibiotic(s) + screening indicators as required. Plates were incubated at 37°C over-night before storage at 4°C.

2.2.1.4. Gel electrophoresis

Digested plasmid DNA or PCR products were separated by 1% (or as required) agarose gel electrophoresis (100 V, 40 to 90 min) in 1x Tris-acetate-EDTA (TAE) buffer, and 1:10000 SYBR® Safe, and visualised on a UV-transilluminator (BiospectrumAC Imaging System, USA). The sizes of bands were estimated by comparison to the DNA marker (10K GeneRuler DNA Ladder, Thermo scientific).

2.2.1.5. Plasmid DNA extractions

The following kits were used to extract plasmid DNA in small (mini), medium (midi), or large (maxi) quantity as described in table 1.1 and following the manufacture protocols: QIAprep Spin Miniprep Kit, QIAGEN Plasmid Plus Midi Kit, and QIAGEN Plasmid Plus Maxi Kit. To extract PCR products from excised agarose gel or from PCR tubes, or to purify restriction-digested plasmids, excised from gels, QIAquick PCR/Gel Purification Kit or MinElute PCR/Gel Purification Kit were used (table 1.1). For DNA

vaccines, EndoFree Plasmid Maxi Kit was used to isolate a high concentration of DNA vaccine with endotoxin free yield, as recommended by the manufacturer protocol.

2.2.1.6. MVA-BAC DNA extraction

To screen MVA-BAC construct by ID-PCR, Purity PCR, or Seq-PCR, MVA-BAC DNA were extracted in small quantity (BAC mini prep) following a protocol that was based on one kindly supplied by Dr. Richard Wade-Martins, Department of Anatomy and Human Genetics, Oxford University, UK. First, obtained colonies were inoculated into cultures of 2 ml LB media, containing the appropriate antibiotic (i.e. 12.5µg/mL chloramphenicol) and incubated overnight with shaking at 32°C. Next, 1.5 ml of cultures was transferred into 1.5 ml microfuge tubes and cells were pelleted for 30s at ~13,200 rpm. The supernatants were removed and the bacterial pellets were resuspended in 70 µL STET by vortexing, added to 4-6 tubes at a time to avoid leaving the un-resuspended pellet in STET for a prolonged period. Pellets were lysed by adding 200 µl alkaline SDS while vortexing. Then solutions were immediately neutralised by adding 150 µl of 7.5 M ammonium acetate while maintaining vortexing. After cooling the solutions on ice for 5 min, they were centrifuged at ~13,200 rpm for 20 minutes at 4°C. The supernatants were then decanted into fresh 1.5 ml microfuge tubes and 250 µl room-temperature isopropanol was added, mixed by repeated inversion and then the DNA was pelleted by centrifuging at 10,000 rpm for 8 minutes. DNA pellets were washed with 200 µl room temperature 70% ethanol and spun again and left to air-dry for 5-10 minutes. DNA pellets (invisible) were eluted in 50 µl of TE containing 5 µg/ml RNAse A.

To isolate MVA-BAC constructs in large quantities with undisrupted integrity of the BAC (which is 200 kb in length) that is suitable for sufficient MVA-BAC rescue, the QIAGEN Plasmid Maxi Kit was used with doubling the volume of every buffer and using the gravity-flow columns. If this kit was not available, the more expensive Phaseprep™ BAC DNA kit was used with similar results, following the manufacturer protocols (table 1.1). The extracted MVA-BAC preps were kept at 4°C; and dealt with and pipetted via a cut off tip to avoid shearing the BAC DNA.

2.2.1.7. Viral DNA extraction

To screen recombinant viruses by ID-PCR, Purity PCR, or Seq-PCR, viral DNA was extracted from infected cells (BHK-21 in most cases), which have been frozen and thawed three times, following the protocol of either the GenElute™ Mammalian Genomic DNA Miniprep Kits or the DNAreleasy reagents (table 1.1).

For MVA-BAC genomic sequencing (chapter 7), MVA genome was extract from infected cells following the traditional phenol-chloroform DNA extraction protocol to obtain purer viral DNA. This protocol was performed as follow: First, 100 µl of purified virus was mixed with 40 µl of 10 % SDS, 10 µl Proteinase K, and 250 µl Tris-EDTA, and incubated at 56°C for 1 h. Second, 500 µl of phenol:chloroform:isoamylalcohol was added, mixed very well (until become cloudy), and centrifuged for 10 min. This step was repeated again and the upper aqueous layer was transferred to new tube. Third, equal volume of chloroform:isomylalcohol was added and the tube was spun, and the top aqueous layer was collected in a new tube. Fourth, the solution was then precipitated with 2.5 volumes of 100 % ethanol and 0.1 volume 3M sodium acetate. The mixture was incubated overnight at -20°C. Last, the tube was centrifuged 20 min at

4°C, and the pellet were washed with 70 % ethanol and left to air-dry. DNA was finally re-suspend in 10 mM Tris buffer.

In addition to three different quantifications by NanoDrop, DNA extracted with this protocol was run into gel electrophoresis to show the absence of smear DNA, which indicates DNA purity. The samples were sent for genomic sequencing that were kindly performed by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, Oxford University.

2.2.1.8. RNA Extraction

To extract total RNA samples from virus-infected cells, the RNeasy Mini Kit or the GenElute™ Mammalian Total RNA Miniprep Kit (table 1.1) were used according to the manufacturer protocol. RNA samples were then quantified using the NanoDrop and then 1µg RNA was used to synthesise cDNA for qPCR (see qPCR methods)

2.2.1.10. MVA-BAC recombineering

Background

Generation of the parent BAC carrying MVA (clone #26) was described by Cottingham *et al* [129]. Recently, Matthew G. Cottingham has also published the MVA-BAC recombineering protocol in the methods in molecular biology, volume 890 2012: vaccinia virus and poxvirology, editor: Stuart N. Isaacs [172], and this protocol was used to derive a range or rMVA as explained in details in the following sections.

Making MVA-BAC into SW102 *E.coli*

The MVA wild type genome was cloned into BAC DNA that includes CAT gene (Chloramphenicol *acetyltransferase*) as a selection marker. The *E.coli* strain SW102 is the *GalK* minus *E.coli* DY380 strain that also possess the Lambda red prophage, which encodes three modifying enzymes that facilitate the recombination between introduced inserts (target DNA) and the homologous region in the MVA-BAC. This SW102 strain was transformed with MVA-BAC and maintained at 32°C at all times, but when the recombination is desired bacteria are then grown at 42°C to activate the gene expression of Lambda red three enzymes. These genes are: *exo*, which has a 5'-3' exonuclease activity that produces single-stranded sticky ends at both ends of the double-stranded target DNA (insert); *beta,* which binds to the single-stranded DNA overhangs to protect them and facilitate the recombination; *gam,* which prevents the linear target DNA from being degraded by *E. coli Rec*BCD proteins [171].

Preparing electrocompetent SW102 cells

The modification of the BAC was done with a modified *E.coli* strain, SW102 that contains MVA-BAC (referred to as clone #26). To transform inserts to the clone #26, SW102 cells were prepared to become electrocompetent as follow: a mixture of this strain in 2 ml Luria-Bertani (LB) with Chloramphenicol (12.5 µg/ml) was inoculated in 12 ml culture tube (BD Biosciences) and incubated at 32°C overnight in a shaking water bath. Next day, the 2mL overnight culture was diluted in 50mL (LB), using a 250 ml baffled conical flask (Wheaton®, USA) with 50 µl chloramphenicol (12.5 µg/mL), and incubated at 32°C in a shaking water bath until OD600 of 0.6 was reached, measured using a spectrophotometer. The 50 ml culture was divided into two equal halves, i.e. 25 ml transferred into another 250 ml baffled conical flask and heat-shocked (induced) at 42°C for 15 min in a shaking water bath. The remaining 25 ml culture was left at 32°C as an uninduced control. After 15 min, both samples (induced and uninduced) were cooled for 5 min on ice/water bath slurry before transferring them to two pre-cooled 50 ml Falcon tubes (BD Biosciences). Samples were pelleted at 5000 RPM (Beckman) for 5 min at 0°C. Following this, the supernatant was poured off and pellet resuspended in 5 ml ice-cold distilled water (dH_20) by gently swirling the tubes in ice/water bath slurry. 25 ml dH_20 was added to the resuspended pellets to make up to 30 ml before centrifugation at 5000 RPM for 5 min. The washing and centrifugation step was repeated once more followed by removal of the supernatant and pellets were resuspended in 1000 µl dH_20. The samples were kept on ice ready to be transformed with the desired inserts (target DNA) by electroporation.

Electroporation of target DNA (inserts) into MVA-BAC

To electroporate the desired DNA inserts; in most cases, the insert was the model transgene (tPA-Pb9-rLuc) but with different homology sequences provided by long primers, 5 µl DNA of the model transgene PCR product was electroporated with 50 µl of induced or uninduced competent SW102 cells in a 0.1 cm cuvette (BIO-RAD) at 25 µF, 1.75 kV and 200 Ω using GenePulser Xcell (BIO-RAD); where µF (microfarad) indicates capacitance, kV (Kilovolts), and Ω (Ohm) is the unit of electrical resistance). Next, electroporated competent cell-DNA mixture was diluted in 1 ml LB broth followed by incubation in a shaking water bath at 32°C for 1 h.

Washing and plating of bacteria after recovery

Following the one-hour recovery, the bacteria were washed with M9 minimal salt to get rid of any rich LB media prior to selection on minimal media. The 1 ml culture was pelleted in 1.5 ml Eppendorf tube at 13,200 RPM at 4°C for 30 sec. Then, the supernatant was carefully removed with a pipette and pellets were resuspended in 1 ml M9 minimal salt. The washing and centrifugation step was performed three times in total, followed by resuspension of the pellets in 1 ml M9 minimal salt.

The next step was to plate on minimal medium agar plates. Three plates were used for every induced sample with 10 µl, 100 µl and the remaining sample, which was pelleted, resuspended in 100 µl before plating. The uninduced samples were also

plated once with 100 µl (as a negative control). Plates were then incubated for 3 to 5 days at 32°C in a cabinet type incubator (Heraeus, Thermo Scientific). After 5 days, the uninduced samples do not normally result in any colonies whereas the induced ones allow for bacterial growth. Three white colonies were then picked from the minimal medium plates (either from the 10 µl, 100 µl samples) and each colony was re-streaked on a GalK-containing McConkey agar plate and incubated for days at 32°C in a cabinet type incubator. Next, a pink and isolated colony was picked from each plate and re-streaked again on McConkey (GalK+) agar plates. This step was also repeated for a third time. Finally, isolated pink colonies were screened to confirm the recombinant MVA-BACs were obtained.

For some recombineering, the *KanR* gene was used as a selection marker instead of *GalK*. Thus, for KanR-based recombineering, the process was slightly different and quicker. There was no wash with M9 minimal salt or plating on minimal medium agar (and McConkey agar). Instead, induced and uninduced samples were spun for 1 min after the one-hour recovery, pellets were plated on Kanamycin-containing LB agar, in 10 µl, 100 µl, or the rest of the pellet. After 3-5 days incubation at 32°C, single colonies were screened to confirm the recombinant MVA-BACs were obtained.

Recombinant MVA-BAC screening

Obtained colonies were screened using colony-PCR to check the identity of insert. The integrity of the BAC was also checked using multiplex PCR (box 4). This initial screen was followed by MVA-BAC DNA extraction (BAC miniprep, described in point 2.2.1.6.),

the identity of the insert and purity of the MVA-BAC was carried using ID-PCR and purity PCR and the sequence identity of the insert was checked using Seq-PCR. The Seq-PCR products were sent for sequencing analysis (performed by the Source Bioscience, Oxford). All primers are listed in table 1.3 and more details on transgene construction are described below.

2.2.1.11. Large-scale extraction of the recombinant MVA-BAC

After the recombinant MVA-BAC constructs were confirmed by PCRs and sequencing analysis, a 500 µl of the bacterial miniprep culture of the correct clone was mixed with 500 µl 50% glycerol in 2 ml screw-capped tube and kept in -80°C freezer. A sample from the glycerol stock was then inoculated into 5 ml LB broth with chloramphenicol (12.5 µg/ml) in 12 ml culture tube (BD Biosciences, UK) and incubated at 32°C for 13 hours in a shaking incubator. The 5 ml culture were then poured into 500 ml LB media with chloramphenicol (12.5 µg/ml) in 1.5 L conical flask and incubated at 32°C overnight in a shaking incubator. The 500 ml culture was then pelleted at 15 RPM for 10 min and the MVA-BAC DNA was extracted following the manufacturer protocol of the QIAGEN Plasmid Maxi Kit or the Phaseprep™ BAC DNA kit. The extracted MVA-BAC DNA was eluted with TE (Tris-EDTA) buffer supplemented with 5 µg/µl RNase. This DNA was handled with a cut off tip (wide open 1000 tip) to avoid shearing the MVA-BAC and measured using NanoDrop, then kept at 4°C until MVA-BAC rescue.

2.2.2. Transgene constructions

2.2.2.1. Constructing IRES-tPA-pb9-rluc and tPA-pb9-rluc transgenes with MVA endogenous promoters (chapters 3 and 4)

The tPA-pb9-rluc construct was kindly provided in a plasmid vector (p1978) by Dr. Matt Cottingham, Oxford University; and it consists of a tPA (the leader sequence of the human tissue plasminogen activator) and pb9 (H2-Kd restricted murine CD8+ T cell epitope, SYIPSAEKI, from the *Plasmodium berghei* circumsporozoite protein (pb9) and rLuc8PV, a luciferase gene from *Renilla reniformis*. The rLuc8PV is an optimized rLuc by eight mutations, hence the "8", which allows measurement of rLuc activity in culture supernatant [173]. The rLuc8PV had a single T5NT, which was removed by PCR mutagenesis (ATT to ATC), to avoid transcription termination when inserted into poxviruses, since the "PV".

The tPA-pb9-rLuc-containing plasmid has a *GalK* gene as well, described by Warming *et al*[171]. Thus, both the tPA-pb9-rLuc construct and the *GalK* gene were amplified from the plasmid as one insert, to make the target DNA for MVA-BAC recombineering. The insert was amplified with 70 oligonucleotide (nt) recombineering primers, 20 nucleotides are insert-specific and 50 nucleotides match the destination insertion loci in MVA-BAC genome (i.e. homology sequences). These insertion loci were *B8R*, *E3L*, and *F11L*. Thus, three recombineering primer pairs were used to amplify the insert in three PCRs (as recombineering target DNA) using the following primers: (**For *B8R* locus**: Rec-B8-F and Rec-B8-R. **For *E3L* locus**: Rec-E3-F and Rec-E3-R. **For *F11L* locus**: Rec- F11-F and Rec-F11-R (See table 1.3 for primer sequences). The *GalK* was selection marker and was not removed from the recombinant MVA-BAC. The resultant

recombinant MVA-BAC clones were given codes: B8-MVA-BAC, E3-MVA-BAC, and F11-MVA-BAC; and their rescued respective viruses were named: B8-MVA, E3-MVA, and F11-MVA despite containing BAC DNAas well as *GalK.*

For constructing IRES-B8-MVA, the p2637 plasmid, containing IRES-tPA-pb9-rLuc was provided Dr. Matt Cottingham, Oxford University. Originally, Dr. Cottingham cloned the sequence of EMCV IRES, synthesized by GeneArt® (Life Technologies), into the p1978 plasmid (above). The IRES-tPA-pb9-rLuc insert was aimed for insertion at *B8R* locus in MVA-BAC genome. Thus, the insert, which also contains *GalK*, was amplified with a forward recombineering 70 nt primer (Rec-IRES-B8-F) and the Rec-B8-R primer (table 1.3).

2.2.2.2. Constructing tPA-pb9-rluc transgene with modified versions of pB8 promoter for (chapter 6)

The aim of chapter 6 was to mutate the core region of the pB8 promoter by nucleotide substitutions. To achieve this, the tPA-pb9-rLuc insert, amplified with Rec-B8-F and Rec-B8-R primers (described above), was used as a template for a second PCR. The second PCR utilises the Rec-B8-R as the reverse primer, but it has different forward long primers with sequences dependent on the desired nucleotide substation. Three second PCRs were performed to introduce three different substitutions. The substitutions were introduced by the primers Rec-mB8.1-F, Rec-mB8.2-F, and Rec-mB8.3 –F, each paired with the Rec-B8-R (table 1.3). For ID-PCR, purity PCR, and Seq-PCR, the three rMVAs (or MVA-BACs) with modified B8 promoters were performed using the same primers of B8-MVA.

2.2.2.3. Constructing tPA-pb9-rluc transgene with MVA conventional promoters for TK insertion locus (chapter 4)

Three conventional poxviral promoters (p7.5, mH5, and SSP) were used as comparative controls to the MVA endogenous promoters (chapter 4). The p7.5-MVA and SSP-MVA were kindly provided by Matt Cottingham and Toriste Orubu [156]. However, the mH5-MVA was derived as part of this thesis. First, the p2369 plasmid was kindly provided by Dr. Cottingham. This plasmid contains the construct tPA-pb9-rLuc flanked with homology sequences, matching the thymidine kinase (TK) locus, and *KanR* (Kanamycin resistance gene) selection marker to facilitate the recombineering, instead of *GalK*. The mH5 promoter sequence was digested from p1842 (synthesised by GeneArt and contain mH5 promoter with multiple cloning site) by KpnI and PstI restriction enzymes. The cut fragment was then subcloned into p2369 with the same enzymes, resulting in a new plasmid, named p3250. Next, a primer pair (TK-PhuUp-F and TK-PhuUp-R) that binds to the TK homology sequences in the p3250 was used to amplify the tPA-pb9-rLuc insert. The PCR product (recombineering target DNA) was purified and used in MVA-BAC recombineering to make the mH5-MVA. For sequinning the insert at the TK, TK-seq-F and TK-seq-R primers were used.

2.2.2.4. Constructing tPA-pb9-rluc transgene with MVA ectopic promoters for the TK insertion locus (chapter 4)

Three recombinant MVA-BACs have been made with endogenous promoter of *B8R*, *E3L*, and *F11L* loci as described above. All promoters drove the expression of the tPA-pb9-rLuc at their endogenous sites. So, to test the activity of these promoters in an ectopic state, i.e. inserted at the TK locus, each promoter was amplified along with the tPA-pb9-rLuc insert, which also contain *GalK* selection marker, from their respective MVA-BAC clones. For example, ectopic pF11 promoter was amplified from F11-MVA-BAC, along with the transgene to make eF11-MVA-BAC, which resulted eventually in eF11-MVA(ΔTK). The recombineering primer pairs have 70 oligonucleotides with 50 nt matching the TK insertion locus and 20 nt specific to the amplified insert, which depends on the promoter. For example, the F11-tPA-pb9-rLuc was amplified from F11-MVA-BAC using Rec-eF11-F and Rec-eF11-R primers (see table 1.3).

2.2.2.5. Constructing FP4b-mCherry insert to study the effect of TK inactivation (chapter 5)

Plasmid p3376 was kindly provided by the Vector Core Facility, the Jenner Institute, Oxford University. The plasmid contains mCherry gene driven by the p4B late promoter from Fowlpox virus (FP4b promoter) and flanked with TK homology sequences. The plasmid was purified and linearised with *AatII* restriction enzyme then used to derive rMVA in the conventional MVA construction method, see section 2.2.3.10.

2.2.2.6. Constructing p7.5-TIP transgene and MVA deletion mutants (chapter 7)

The TIP model antigen is a construct comprised the p7.5 early/late promoter driving an 82 amino acid epitope string (for Tuberculosis, Immunodeficiency virus and Plasmodium), which contains relevant epitopes from these various pathogens for vaccine-induced protection in mouse models, including the protective H-2K^{d}-restricted Pb9 epitope from Plasmodium berghei circumsporozoite protein (described previously [174]). The TIP construct was inserted into MVA-BAC by *GalK* recombineering at the TK insertion site by Cottingham *et al* [129]. The derived recombinant TIP/GalK-MVA-BAC clone was then used to remove *GalK* leaving the only TIP at the TK locus. The TIP-MVA-BAC was then used to insert the *GalK* into various insertion loci, replacing and deleting the native MVA ORFs as desired. This project resulted in a bank of MVA deletion mutants (ΔMVA-TIP viruses) that were either published in [129] or in (Alharbi *et al*, in prep). In this DPhil, two MVA mutants from the deletion bank were used in chapter 7 to study the effect of gene deletion on rMVA immunogenicity: the Δ15-MVA-TIP, which has 15 deleted genes, ΔA35-MVA-TIP that lacks *A35R*, in addition to non-mutated MVA-TIP as a control.

2.2.2.7. Constructing p7.5-85A transgene for MVA deletion mutants (chapter 7)

The plasmid p1228 was kindly provided by the Jenner institute. It contains TK homology sequences flanking the 85A antigen of *M. tuberculosis* that was used to generate MVA85A vaccine [175]. Since the p7.5 promoter driven 85A cassette was markerless, the *KanR* gene was required to facilitate MVA-BAC recombineering. Therefore, aminoglycoside phosphotransferase (AphA1) gene, which confer Kanamycin resistance (also named KanR) was amplified for In-Fusion cloning from a plasmid synthesised by GeneArt (p1841) using specific In-Fusion primers. The 85A containing plasmid (p1228) was digested with *BglII* restriction enzyme and the AphA1 PCR product had *BglII* recognition sites. Thus, In-Fusion enzyme was used, following manufacturer protocol to facilitate the ligation. The resultant plasmid, named p2796 was purified and screened by PCR and restriction map for confirmation. The 85A cassette was then sequenced and confirmed of having correct sequence. The 85A cassette was amplified with TK-PhuUp-F and TK-PhuUp-R recombineering primers and the PCR product was then used to derive the following. First, deriving Δ15-MVA-85A from the MVA-BAC of Δ15-MVA-TIP. Second, deriving ΔA35-MVA-85A from the MVA-BAC of ΔA35-MVA-TIP. Third, deriving MVA-BAC-85A from the parental unmodified MVA-BAC (a.k.a clone#26, see below). The MVA-BAC-85A serves as a control for 85A-containing MVA mutant as well as head-to-head comparison with the MVA85A vaccine (see chapter 7). Regarding PCRs, the 85A-containing viruses were treated similar to any virus with TK-inserted transgene. Therefore, for ID-PCR, TK-ID-F and TK-ID-R primers were used. For purity PCR, primers TK-Pu-F and TK-Pu-R were used. For Seq-PCR, TK-seq-F and TK-seq-R primers were used.

2.2.2.8. Constructing CMV-D10 and p7.5-mCHerry plasmids (chapter 3)

The plasmid p1990, which contains CMV promoter, was cut within the multiple cloning sites (MCS) with *KpnI* and *EcoRI* enzymes following restriction digest protocol and was then purified. MVA *D10R* ORF was amplified with CMV-D10-F and CMV-D10-R primer pair and the PCR product was purified. The purified open-cut vector and PCR product were ligated using In-Fusion cloning. The resultant plasmid was named p3286. In addition, the plasmid p1977 contains mCherry gene, driven by the p7.5 promoter was kindly provided by the Vector Core Facility, the Jenner Institute. Both p p3286 and p1977 were then amplified and extracted in large quantities, ready for transfection experiments.

2.2.3. Virology Methods

2.2.3.1. MVA-BAC rescue: Deriving Recombinant MVA-BAC Viruses

MVA-BAC constructs were rescued to infectious MVA using a Fowlpox virus of the strain 9 (FP9) that was used to provide poxviral transcriptional machinery. First, a nearly confluent T25 flask of BHK cells was infected with FP9-LacZ at multiplicity of infection (MOI) of 1.0 (i.e. 1 pfu per cell) in a volume of 2 ml OptiMEM. The flask was then incubated at 37°C (5% CO_2) for 2 hours. Second, during the incubation period, 20 µl of Lipofectamine was added gently to 600 µl OptiMEM per transfection followed by incubation at room temperature for 5 min. Third, 8 µg of BAC DNA was diluted in 600 µl OptiMem in a sterile 2 ml screw-capped tube, and 8µg of MVA-BAC (clone 26, i.e. wild type) was also diluted in 600 µl OptiMEM as control. After 5 min incubation, the Lipofectamine-OptiMEM mix was added to the BAC DNA-OptiMEM mix by gentle flicking, followed by incubation at room temperature for 20 min. Fourth, after infecting cells with FP9 (the 2-hour incubation), the media containing FP9 was aspirated and replaced with the transfection mixture (Lipofectamine and BAC DNA in OptiMem, which is 1.2 ml) using a cut off pipette tip (P1000 pipette), added gently (dropping) to avoid shredding the BAC DNA. Flasks were incubated at 37°C for 2 to 4 hours, then 1 ml fresh pre-warmed 2% sera DMEM was added and the flasks were incubated at 37°C (5% CO_2) overnight. Next day (day 2), cells were examined by epifluorescence microscope (Leica) to confirm the presence of green cells (normally green cells are very dim at this stage), cell were then further incubated for 4 days or until all cells were infected-dead.

To perform plaque assay, cell lysate was frozen-thawed three times and re-plated on fresh BHK (in 6-well plate) with neat, 1:10 and 1:100 dilutions in 2% sera DMEM for 2 hours, before replacing the media with 2 ml CMC overlay. The neat was 500 µl of the cell lysate and dilutions were made in 2% sera DMEM from the neat sample. Cells were then monitored for 2-3 days for the presence of green virus infected cells or plaques, because MVA encodes a GFP marker gene.

Although MVA-BAC constructs were undergone three rounds of re-streaking on GalK-containing agar plates, the recombinant viruses were also purified via plaque picking in addition to, in some cases, flow cytometry cell sorting using CyCLONE robotic module of a MoFlo flow cytometer (Dako Cytomation). The purified virus was confirmed by ID-PCR, purity-PCR, and Seq-PCR before recombinant viruses were bulked up in large quantity, all are explained below.

2.2.3.2. Plaque picking

This step was done to confirm that a rescued virus was not contaminated with FP9 or the parental MVA from the wild type MVA-BAC (clone 26). Thus, 2-3 days after incubation of 6-well plates (with CMC overlay), GFP-plaques were identified under a fluorescent microscope and an object marker attachment was used to label the identified plaques prior to purification. In the safety cabinet, a P10 pipette was set to 10 µl, then a 10 µl 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 2 ml screw-capped tubes containing 10 mM Tris (pH9). The tube were then frozen and thawed three times and stored at -80°C or used to make a serial dilution for first round of plaque-picking. The dilutions were prepared in 2% DMEM with a rigid vortexing between every pair of dilutions. The dilutions were then used to infect confluent monolayers of BHK cells in 6-well plate. The plate was incubated for 2 hours, and then the media were replaced with 2 ml/well CMC overlay (1:3 dilution) and the plate was incubated at 37°C (5% CO_2) for 2-4 days, monitoring the cells green virus infected cells. Once plaques were visible, a plaque was picked from the most diluted well for the second round of plaque purification. This process was repeated once more or until a pure virus was obtained. To obtain a pure virus, ID-PCR, purity-PCR, and Seq-PCRs were carried out on plaque picks viruses using primers as in table 1.3.

2.2.3.3. MoFlo sorting

In few cases, picked plaques were purified further using the sorting function of CyCLONE robotic module of a MoFlo flow cytometer (Dako Cytomation). For example, disrupting the TK gene with mCherry gene, resulted in rMVAs with green and red markers, suitable for MoFlo sorting. Thus, the confirmed picked plaques were used to infect T75 flask of confluent BHK cells, using 20 µl from the picks in 15 ml 2% sera DMEM. The flask were incubated at 37°C (5% CO_2) for 3 days. Next, the media were aspirated and the cells were covered with 5 ml TrypLE™ Express for 5 min at 37°C (5% CO_2) before neutralising the trypsin reaction with 10 ml 2% sera DMEM. The cells were spun for 5 min at 1500 RPM and resuspended in 25 ml 3% FCS PBS (PBS only should work just fine) and sent to the Jenner Institute Flow Cytometry Core Facility for MoFlo sorting. After the sorting, a 96-well flat bottom plate was received from the facility containing 50 µl sterile H_2O/well that should have a single infected cell, positive for GFP, and the red marker in this example. Next, fresh BHK cells (200 µl per well) were added to the 96-well plate to enable the virus in the sorted cells to replicate, and then the plate was incubated at 37°C (5% CO_2) for 2-3 days. The plate was checked under the fluorescence microscope, and the green fluorescing wells were harvested into 2 ml screw-capped tubes. The tubes were frozen-thawed three times and then the viral DNA was extracted using DNAreleasy (as described below) and used in screening PCRs.

2.2.3.4. PCR screening

A sample of 20 µl of picked plaques was mixed with 30 µl of DNAreleasy to extract the viral DNA using a thermocycler (PCR machine) with the program described in box 9 below. Next, 1 µl of the reaction was used as a template DNA for the ID-PCR, purity-PCR, and Seq-PCRs. The same primers were used as for MVA-BAC DNA at the bacterial stage, for primers see table 1.3.

65°C for 15 min (may be left longer if necessary) 96°C for 2 min 65°C for 4 min 96°C for 1 min 65°C for 1 min 96°C for 30 sec 20°C hold Or 75°C for 5 min 96°C for 2 min 20°C hold

Box 9: DNAreleasy thermocycling program.

When DNAreleasy was not available, the GenElute™ Mammalian Genomic DNA Miniprep Kit (see table 1.1) was used following the manufacturer protocol.

2.2.3.5. Virus Bulk-Up and Sucrose Cushion Purification

The confirmed and purified plaques were then grown in a T75 flask with a confluent monolayer of BHK cells in a total volume of 15 ml 2% sera DMEM, and incubated at 37°C (5% CO_2) for 3 days. The cells in T75 flask were then harvested, frozen-thawed three times, and used to infect confluent monolayers of BHK cells in 5 larger flasks, T175, using 3 ml cell lysate in 35 ml 2% sera DMEM for each T150 flask, followed by incubation at 37°C (5% CO_2) for 5 days.

Next, cells in the 5 flasks were harvested and underwent three round of freeze-thaw before they were spun 670 x g for 5 min. The supernatant was collected in a sterile 250 ml ultracentrifuge bottle, and the pellet was resuspended into 5 ml 10 mM Tris, mixed thoroughly by vortexing, sonicated at 57 Hz for 1 min (using sonicating probe, from Sonics & Materials), and then was spun at 670 x g for 5 min. The new obtained supernatant was then added to the previous supernatant in the 250 ml ultracentrifuge bottle. This bottle was then carefully balanced using a sensitive weighting scale and spun for 1 hour at 33 000 x g using the Optima L-80 XP ultracentrifuge. The 250 ml bottle was only opened in sterile conditions in a safety cabinet and the supernatant was discarded, leaving a large pellet that was resuspended in 5 ml 10 mM Tris, transferred to 15 ml tube, mixed by vortexing, and kept at -20°C ready for sucrose cushion purification.

Next day, the sample was thawed and loaded very gently on top of 7 ml 36% sucrose in ultracentrifuge thin tubes. The tubes were weighed very carefully compared to balance

tubes, and the weight difference between the tubes was less than 0.01 gram. Next, all tubes were inserted into the buckets of the SW40 swinging rotor of the Optima L-80 XP ultracentrifuge and closed tightly using the screw lids. The samples were spun at 30 000 x g for 2 hours at 4°C. The supernatant, which contains the sucrose and the impurities was removed leaving a small pellet that contains the viruses. The pellet was then resuspended in 800 µl 10 mM Tris and homogenised by vortexing and sonicating before making several aliquots.

Although purified rMVA came from a single plaque that was confirmed pure by PCRs, the PCR screening was repeated for the final virus preps. A sample of 20 µl aliquot was added to 30 µl of DNAreleasy (described earlier), and the extracted viral DNA were screened with ID-PCR, Seq-PCR, and purity PCR screening using exact primers that were used for their respective bacterial MVA-BAC DNA.

2.2.3.6. Sterility test

The purified recombinant viruses were then tested to determine their sterility from any contaminating aerobic (not anaerobic) bacteria and fungi. A Tryptic Soy Broth (TSB) 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. A sample of 10 µl from the purified virus was added to TSB media and incubated at 30-35□C for 3 days to test for bacterial contamination. A swap sample from skin surface was inoculated into TSB as a positive control. A negative control was included as well, which is a non-inoculated TSB. A sample from the 10 mM Tris buffer that was used to prepare the viruses was tested whenever there was suspicion of contamination.

2.2.3.7. MVA titration

The MVA-BAC construct encode GFP marker under the control of p4B late promoter from Fowlpox virus (FP4b promoter), which was inserted into the BAC DNA prior to constructing MVA-BAC. Thus, the titre of all recombinant MVA-BAC viruses was determined under the epifluorescence microscope by counting the green-fluorescent viral plaques. First, 10 µl of the recombinant purified virus was inoculated into 990 µl of 2% sera DMEM and mixed by thorough vortexing. This sample was the first dilution, which is 10^{-2}, and used to make serial dilutions up to the 10^{-8} with a thorough mixing between every pair of serial dilution. Duplicate serial dilutions were prepared for each purified virus. The last three dilutions, which are 10^{-6}, 10^{-7}, and 10^{-8} were then added to confluent monolayers of BHK cells in 6-well plate and incubated at 37°C (5% CO_2) for 2 hours. Next, the plates were retrieved and the media were replaced with CMC overlay

and were then incubated for 2-4 days at the same conditions. The plates were then examined under the epifluorescence microscope to detect green plaques. Only the wells that have a range of 20-100 plaques were counted; and the number of plaques in duplicate wells counted to determine the titre of each virus generated. After counting, the formula presented below was used to determine the titre in plaque forming unit (pfu), that is pfu/ml:

"Average number of plaques" x "serial dilution" x "dilution factor*" = "x" pfu/ml
e.g. (24+20) = 22 x ($1x10^7$) x 10* = 2.2 x 10^9 pfu/ml

* Note, as I added all of the serial dilution volume (1 ml, i.e. 10 ul virus + 990 media) to the wells, there was no dilution factor to adjust for, and the formula would be:

"Average number of spots" x "serial dilution" = "x" pfu/mL
e.g. (24+20) = 22 x ($1x10^7$) = 2.2 x 10^8 pfu/mL

In general, in case of colourless (no markers) MVAs, an immunostaining protocol would be used to titrate MVA (Jenner Laboratory Protocol Number: J111). Materials used for vaccinia (MVA) immunostaining are included in the material section although this protocol was never used for this DPhil project.

2.2.3.8. Growth rate of recombinant MVA

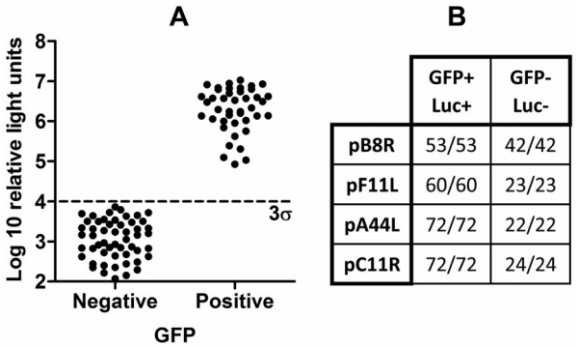
BHK cells ($5x10^4$ cells/well) in black-walled, clear-bottom 96-well plate (Grand Plates™) were inoculated in duplicate with rMVAs (suspended in 2%sera DMEM) at 0.5 MOI. The growth rates were determined by quantifying GFP fluorescence every 10 min for 48 h using a BMG FluoSTAR fluorometer equipped with 37°C + 5% CO_2 incubation.

2.2.3.9. Genetic Stability Assay

For genetic stability studies, rMVA had to be passaged at least 10 times before determining whether the tPA-pb9-rLuc transgene was still maintained and intact. **Passage 1:** BHK cells in T25 flasks were inoculated with 10µl of purified virus then incubated at 37°C (5% CO_2) for 2-3 days or until all cells were infected as determined by epifluorescence microscopy. Cells were then harvested, pelleted at 650 x g for 5 min, and then supernatant was discarded. The cell lysates were resuspended in 1 ml 2% sera DMEM and frozen-thawed three times. **Passage 2-10**: This process was repeated 9 times but this time cells were inoculated with 100 µl crude lysate from the previous passage. After passage 10, viruses were titrated on BHK cells 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-rLuc, i.e. the genetic stability of the transgene. Therefore, BHK cells in T150 flasks were infected at 0.001 pfu/cell and incubated at 37°C (5% CO_2) for two days. Next, cells were trypsinised and single GFP^+ cells were sorted into individual empty wells of 96-well plates (96-Well black opaque walled flat bottom plates) using the CyCLONE attachment of a MoFlo flow cytometer. Following this, the 96-well plate, which contains the GFP^+ individual cells were seeded with 5 x 10^4 BHK cells per well then incubated at 37°C (5% CO_2) for 2-3 days. Next, wells were scored positive or negative for GFP by epifluorescence microscopy. The GFP^+ and GFP^- wells were then harvested and the cell lysates were frozen-thawed three times and used to quantify rLuc activity (see luciferase assay method). The results were presented as $GFP^+/rLuc^+$ (stable), $GFP^+/rLuc^-$ (instable), or $GFP^-/rLuc^-$ (negative control) for passage number ten

of each virus. A figure from our previous publication is included below as an illustration of this method. Not all BHK wells were GFP^+, owing to unavoidable errors in the MoFlo droplet identification. 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^- wells (i.e., uninfected). Any genetic instability of the tPA-Pb9-rLuc at a given MVA insertion locus would result in absence of luciferase activity in a GFP^+ well.



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

A copy of figure 7 from Orubu *et al*[156]: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 (2) 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 labeled 3σ) was used to score GFP+and GFP^- wells luciferase positive (Luc^+) or negative (Luc^2). Wells in which cell monolayers were lost during processing were excluded. Raw data for the pB8R recombinant (A) and well scores for all viruses (B) are shown.

2.2.3.10. MVA recombination: Conventional method of recombinant MVA construction

The reporter gene mCherry, driven by the p7.5 promoter, in plasmid p1977 (kindly provided by Vector Core Facility, Oxford) was used to disrupt the TK gene to study the TK effect on rMVA immunogenicity (chapter 5). However, this was performed using a conventional method of recombinant vaccinia virus construction, and not using the MVA-BAC recombineering. The mCherry-containing plasmid was linearised using *AatII* restriction enzyme and was then purified using the MinElute PCR/Gel Purification Kit.

The FP4b-mCherry gene was used for TK gene inactivation in different viruses, such as E3-MVA, F11-MVA, and B8-MVA and that resulted in the new rMVAs named: E3-MVA(ΔTK), B8-MVA(ΔTK), and F11-MVA(ΔTK), see chapter 5. First, each of E3-MVA, F11-MVA, and B8-MVA was used to infect a confluent monolayer of BHK cells in 6-well plate at MOI = 1.0 in a volume of 1 ml OptiMEM. The plate was then placed in an incubator at 37°C (5% CO_2) for 2 hours. Second, during the incubation period, 4 µl of Lipofectamine was added gently to 300 µl OptiMEM per transfection followed by incubation at room temperature for 5 min. Third, 1.5 µg of the linearised plasmid was diluted in 300 µl OptiMEM in a sterile 2 ml screw-capped tube. After 5 min incubation, the Lipofectamine-OptiMEM mix was added to the plasmid-OptiMEM mix by gentle flicking, followed by incubation at room temperature for 20 min. Fourth, after infecting cells with the MVA-BAC viruses (the 2 hour incubation), media were aspirated and replaced with transfection mixtures (Lipofectamine and plasmid in OptiMEM, which is 0.6 ml total). Plates were incubated at 37°C for 2 to 4 hours, then 1 ml fresh pre-warmed 2% sera DMEM was added and the plates were incubated at 37°C (5% CO_2)

overnight. Next day (day 2), cells were examined by epifluorescence microscope (Leica) to confirm the presence of red cells (expression of mCherry gene), cell were then further incubated for 2 days or until all cells were infected-dead. Cells were frozen-thawed three times and re-plated on fresh BHK (in 6-well plate) with neat, 1:10 and 1:100 dilutions in 2% sera DMEM for 2 hours, before replacing media with 2 ml CMC overlay. The neat sample was 500 µl from cell lysates and dilutions were made in 2 % sera DMEM from the neat sample. 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.

The double fluorescent red and green plaques were picked and used to infect T75 flask of BHK cells. The infected cells were then trypsinised and sent for MoFlo sorting. The viruses were then treated exactly as explained in the MVA-BAC rescue. ID-PCR and purity PCR were performed on viral DNA to confirm the TK inactivation. As the PCRs were correct and the red fluorescent marker was detected throughout all purification steps, the FPp4-mCherry insert was not sequenced.

2.2.4. Non-immunological Read-out methods

2.2.4.1. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed to determine the level of rLuc transgene transcription in different rMVAs (chapter 3 and 6). First, such viruses were used to infect monolayers of BHK cells in 6-well plate at MOI of 1, and the plates were incubated 37°C (5% CO_2) for 24 h.p.i. or for the desired time-point. The supernatants were then removed and cells were harvested by scraping. The cell lysates were spun at 13 000 RPM for 1 min, then the pellets were used to extract the total RNA following the protocols of RNeasy Mini Kit or GenElute™ Mammalian Total RNA Miniprep Kit. The eluted RNA was kept on ice at all time, or stored at -80°C for later use, and quantified using NanoDrop. For each qPCR reaction, 1 µg total RNA was used to synthesise cDNA, using Omniscript RT Kit and following QIAGEN instructions.

The qPCR reaction was then set up to amplify rLuc gene (test) and *E9L* gene (endogenous control) using primer pairs described in table 1.3. Two wells in a 96-well qPCR plate were used for every sample (or dilution of a sample) to detect either rLuc or E9L; because I did not used specific labelled probes to detect those genes, besides that SYBR green master mix allows for one PCR reaction only, not duplex. Thus, 1 µl of cDNA was used with the SYBR green master mix (from QuantiTect SYBR Green PCR Kit or SensiFAST™ SYBR® Hi-ROX Kit) following the qPCR recipe, qPCR program, and qPCR plate layout, in the following boxes. The Step One Plus thermocycler was used for

qPCR amplification. Results were determined using the ΔΔCt method of qPCR, previously described by Livak and Schmittgen [176], with the following equation:

ΔCt value of a control virus (X)= Ct value of rLuc of sample X - Ct value of E9 of sample X
ΔCt value of a test virus (Y)= Ct value of rLuc of sample Y - Ct value of E9 of sample Y
ΔΔCt= ΔCt(Y) - ΔCt(X)
Relative fold change in rLuc gene expression is calculated as follow:
$2^{-\Delta\Delta Ct}$ =
This would be the relative fold change in rLuc gene expression by the virus Y, relative to that of rLuc by the virus x, where rLuc gene expression would always be given the value of "1".

Quantitect Master mix	10μl
Primer A (10pmol/μl) +Primer B (10pmol/μl) [This gives 0.5uM final concentration]	1μl
Water for PCR	7μl
Template cDNA	1μl
Total volume	20μl

Box 10: qPCR recipe. Based on Quantitect kit.

Hot-start activation	95oC for 10 minutes
Cycling -	95oC for 10 seconds [denature] 60oC (depends on Tm) for 40 seconds [annealing and extension]
Repeat 'Cycling' 40 times.	

Box 11: qPCR thermocycler program.

Neg	**Neg**	**Neg**	**S1L**	**S1L**	**S1L**	**S1L**		**S2L**	**S2L**	**S2L**	**S2L**
Neg+	**Neg+**	**Neg+**	**S1L+**	**S1L+**	**S1L+**	**S1L+**		**S2L+**	**S2L+**	**S2L+**	**S2L+**
H_2O	**H_2O**	**H_2O**	**S1R**	**S1R**	**S1R**	**S1R**		**S2R**	**S2R**	**S2R**	**S2R**
			S1R+	**S1R+**	**S1R+**	**S1R+**		**S2R+**	**S2R+**	**S2R+**	**S2R+**
10^{-1}	**10^{-1}**	**10^{-1}**	**10^{-2}**	**10^{-2}**	**10^{-2}**	**10^{-3}**	**10^{-3}**	**10^{-3}**	**10^{-4}**	**10^{-4}**	**10^{-4}**
10^{-5}	**10^{-5}**	**10^{-5}**	**10^{-6}**	**10^{-6}**	**10^{-6}**	**10^{-7}**	**10^{-7}**	**10^{-7}**	**10^{-8}**	**10^{-8}**	**10^{-8}**
10^{-9}	**10^{-9}**	**10^{-9}**	**10^{-10}**	**10^{-10}**	**10^{-10}**	**10^{-11}**	**10^{-11}**	**10^{-11}**	**10^{-12}**	**10^{-12}**	**10^{-12}**

Neg: cDNA from RNA of mock-infected cells.
H_2O: Water sample added, instead of cDNA.
S1: B8-MVA, added in quadruplicate.
S2: B8-IRES-MVA, added in quadruplicate.
L: rLuc primers.
R: Reference gene (*E9L*) primers
+: AraC treated samples
10^{-1} to 10^{-12}: Diluted cDNA sample, from RNA of cells infected with mH5-MVA

Box 12: qPCR 96-well plate layout.

2.2.4.1. *In vitro* Luciferase assay

Confluent monolayers of BHK cells (5 x 10^4 cells/well) in flat bottom 96-well plates were chilled on ice, and ice-chilled 2% sera DMEM was used to prepare duplicate rMVA inoculums at MOI of 1 or as desired. The inoculums were added to the cells, 200 µl per well and then spun at 600 x g for 1 hour at 1°C (called spinoculation). The spinoculation was performed to bind the viruses to the surface of cells, but with around 0°C to prevent the onset of viral infection, followed by the removal of the inoculums, which could have pre-made luciferase protein that could affect the luciferase read-out. Thus, after the spinoculation, the inoculums were removed and cells were gently washed twice with ice-chilled 150 µl PBS, and then 200 µl pre-warmed 2% sera DMEM were added and the plates were incubated at 37°C (5% CO_2) for 6 h.p.i., 24 h.p.i., or as desired.

For kinetic luciferase activity, a time course assay was performed by sampling 20 µl aliquot of supernatants immediately after infection and after the wash, and then at 2, 6, 12, 24 h.p.i. At 24 h.p.i., the remaining media were aspirated, followed by washing twice with 100 µl PBS. Cells were lysed with *Renilla* Luciferase Assay Lysis Buffer (Promega) and 20 µl of cell lysate were sampled. The *Renilla* substrate mixture was then prepared by adding 1 volume of *Renilla* luciferase assay substrate into 100 volumes of *Renilla* luciferase assay buffer (Promega). Samples of 10 µl from the 20 µl aliquots were added to 96-well white plate (Nunc maxisorp white plates), and then 50 µl of *Renilla* substrate mixture was added to each well and the plate was read immediately on the Varioskan Flash luminometer (Thermo Scientific). The luciferase results are presented as relative light units.

2.2.5. Immunology methods

2.2.5.1. Western Blot

Western blot was performed to detect the expression of rLuc protein (chapter 3), D10 protein (chapter 3), or Pk-tagged 85A protein (chapter 7) by rMVAs. In all cases, monolayers of BHK cells in 6-well plates were chilled on ice and then infected with rMVA at MOI of 1 using pre-chilled media to prepare the inoculums. The cells were spinoculated by spinning the plate at 600 x g for 1 hour at 1°C. The cells were then washed twice with 1 ml cool PBS, followed by addition of 1 ml pre-warmed 2% sera DMEM, and plates were incubated at 37°C for 24 hours. Next, the supernatants were collected in 1.5 ml tubes, and the cells were lysed with Western blot lysis buffer and transferred to 1.5 ml tubes. Cell lysate samples were then frozen-thawed for three times and spun at 13000 RPM for 3 min. The new supernatants of the cell lysates and the supernatant samples were then kept in -20°C until use.

For SDS-PAGE, aliquots of 20 µl of samples were mixed with the Laemmli sample buffer (reducing conditions), and boiled in a heat-block for 5 min. Samples were then cooled and loaded in 12% precast poly acrylamide gel (BIO-RAD) in addition to the ColorPlus™ protein marker, and run at 70 V until the samples have entered the gel, then at 100 V for 45 min. Next, the gel was transferred to a PVDF membrane, in ready-to-use packs using the Trans-Blot® Turbo™ Transfer System (BIO-RAD), which transferred proteins to the membrane in 7 min. Alternatively, the gel was placed in a 100 ml transfer buffer for 5 min and then placed in a nitrocellulose membrane/filter paper sandwich (BIO-RAD). This sandwich was then placed in the transfer apparatus (BIO-RAD), which was

assembled and kept in an ice-filled polystyrene box during the run. The transfer was then run for 1 hour at 12 V.

Membrane was removed and placed in bucket of 100 ml PBS and washed twice by changing the PBS and keeping the bucket on a plate shaker. When washed, membrane was then blocked with 50 ml 3% BSA/PBS (blocking buffer) and left on the plate shaker for 1 hour. Membrane was then washed twice by 10 min shaking in PBS/T, followed by two rounds in PBS. The membrane was incubated with a primary antibody, diluted 1:1000 in 20 ml blocking buffer, and left shaking for 1 hour at RT. The primary antibody varied according the tested protein. These primary antibodies against rLuc protein, D10 protein, or Pk tag are all described in table 1.1. Next, the membrane was washed twice with PBS-T followed by two washes with PBS as before. After the wash, the membrane was incubated with a secondary antibody of either the donkey anti-mouse-alkaline phosphatase or the donkey anti-Rabbit-alkaline phosphatase (dependent on the primary antibody used), diluted 1:3000 in 20 ml blocking buffer for 1 hour shaking at RT. This was followed 4 times of 10 min wash with PBS/T. The SIGMAFAST BCIP/NBT tablet was diluted in 10 ml water (vortex to dissolve). The nitrocellulose membrane was then laid on some Saran wrap and the BCIP/NBT solution was carefully pipetted on the top. The development was left for 5 – 10 min, and then the membrane was rinsed with water and left to dry for 5 min before scans and photographs were taken.

2.2.5.2. Immunofluorescence Assay (IFA)

A cover slip was placed in each well of 6-well plates, and the plate was seeded with A549 cells, non-permissive to MVA, in 10% DMEM media and incubated at 37°C until cells were 80% confluent. Cells were then infected with rMVA at MOI of 1 (similar to infection for Western blot). At 24 h.p.i, media were removed and plates were cooled down on water in a metal over ice, and wash with 500 µl ice-cold PBS once. Cells were then fixed by addition of 500 µl ice-cold 4% paraformaldehyde in PBS for 10 min on ice then 20 min at room temp. Cells were then washed twice with 500 µl PBS and quenched with 500 µl of 50 mM NH_4Cl in PBS for 5 min followed with two washes of 500 µl PBS. Cells were permeablised with 500 µl of 0.2 % Triton X-100 in PBS for 2 min and washed twice with PBS, then blocked with 0.5 % BSA in PBS (blocking buffer) for 30 min.

The primary antibody was the mouse anti-rLuc (Millipore), diluted 1:100 in blocking buffer. 100 µl of primary antibody was added on a parafilm, and then each cover slip was taken and inverted on the 100 µl drop. Cells (cover slips) were incubated in humidity (in a box with wet tissues at the bottom) for 1 h, followed by three washes with PBS, each for 10 min. Cover slips were then incubated for 1 h with Alexa488 conjugated anti-mouse secondary antibody, diluted 1:200 in blocking buffer, using drops in parafilm. Next, 40 µl of Mowiol-DAPI mounting solution (MD) was added to microscope slides. After 3-5 times of 15-20 min washes with PBS, cover slips were dipped into H_2O and then left to air-dry for 5 min. Cover slips were then inverted on the MD on slides, ready for epifluorescence microscope and confocal microscope.

This protocol was used to show the ability of B8-MVA and IRES-B8-MVA to express rLuc gene, and to determine if IRES insertion could affect the rLuc protein expression and localisation (Appendices, Figure S3.3)

2.2.5.3. Mice immunisation and sample collection

Immunisation experiments were conducted with female BALB/c (H-2K^d) mice that were purchased from Harlan Laboratories, Oxfordshire, UK. Mice were 6 – 8 weeks old at the immunization time. All procedures were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act (ASPA) [177] for the Project Licence (Prof. Adrian Hill's PPL 30/2889) and were approved by the University of Oxford Animal Care and Ethical Review Committee. All mice were housed at least 7 days for settlement prior to any procedure in the Wellcome Trust Centre for Human Genetics Animal Facility (Functional Genomics Facility (FGF)), under Specific Pathogen Free (SPF) conditions.

For immunisation, mice were kept in a clean glass box inside hood cabinet and anaesthetised with inhalation of a mixture of isofluorane (2 – 3.5%) and oxygen, using anaesthetic machine. Intramuscular (i.m.) vaccinations were into the tibialis muscle in a volume of 50 µl using 0.5 ml U-100 Insulin syringe (0.33 mm (29G) x 12.7 mm, from BD Microlance). Intraperitoneal (i.p.) vaccinations were given into the lower right or left quadrant of abdomen, avoiding hitting bladder, liver, or other internal organs. Viral vectored or DNA vaccines were prepared in sterile endotoxin free PBS, and kept in ice buckets after preparation until used for immunisation. Recombinant adenoviruses were dosed in infectious units (iu/ml), recombinant MVA were dosed in pfu/ml, and DNA vaccines were dosed in µg/mouse. All doses were calculated in a total volume of 50 µl (for i.m.) or 200 µl (for i.p.) per mouse.

Blood samples were collected via tail bleeding if the animal were not sacrificed or from cardiac puncture when sacrificing animals. For tail bleeding, mice were slightly warmed in a heating box inside a hood cabinet to allow the visibility of the tail vein and then were moved to a plastic restrainer. A surgical blade (Swann-Moston) was used to gently cut on a side of the tail, with pulling the tail back, and 8-10 blood drops were collected into 200 µl 10mM EDTA in PBS, in 1.5 ml tubes and used to prepare peripheral blood mononuclear cells (PBMC). For serum collection, 1.5 ml tubes had no solution to allow for blood clot and serum isolation.

Mice were sacrificed at the end of experiments by neck dislocation according to ASPA. The spleens were extracted in sterile conditions inside a hood cabinet. Upon sacrifice, blood for serum samples (not PBMC) was collected from the heart (cardiac puncture) immediately after the killing.

2.2.5.4. Animal sample processing

First, for preparing PBMC samples, 1 ml of ACK buffer was added to the 200µl blood samples and cells were centrifuged at 4000 RPM for 4 min. Supernatants were discarded and pellets were resuspended in another 1 ml ACK lysis buffer and centrifuged as before. Supernatants were removed and the cell pellet resuspended in approximately 1 ml 10% sera DMEM containing (the DMEM volume was dependent on subsequent protocols).

Second, for splenocytes preparation, spleens were crushed in PBS, passed through a sterile 70 µm cell strainer in a total volume of 15 ml PBS into 50 ml tubes and centrifuged at 1350 RPM for 5min. Supernatants were discarded and pellets were disrupted and resuspended in 5 ml ACK lysis buffer for maximum time of 5 min before addition of 25ml PBS. The tubes were inverted few times and centrifuged again as before, and then resuspended in 5 ml complete medium (see the buffers list). Splenocytes were counted using a CASY cell counter (Schärfe Systems, Germany) and resuspended at appropriate concentrations in complete medium.

2.2.5.5. Intracellular Cytokine Staining (ICS)

Cytokine producing $CD4^+$ and $CD8^+$ T lymphocytes were examined using intracellular cytokine staining (ICS). Cells were collected from spleens and blood as described above and placed into U-bottom 96-well plate (Corning Inc), $5x10^5$ cell/well. Each sample was re-stimulated with a given peptide or peptide pool (table 1.4) at a final concentration of 1µg/ml in the presence of Brefeldin A (GolgiPlug), and plates were incubated for 5 hours at 37°C. Some cells were not stimulated as controls. After re-stimulation, plates were stored at 4°C overnight. Next day, plates were centrifuged at 1350 RPM for 5 min at 4°C and pelleted cells were resuspended in FACS buffer(see buffers list) and transferred to V-bottom plates, and spun at same conditions. After removing the wash fluid, pelleted cells were washed with 200 µl FACS buffer and spun again as before. Cells were stained for surface markers with FITC conjugated mouse anti-CD8a, PacificBlue conjugated mouse anti-CD4 and mouse anti-CD16/CD32 (Fc block), all mixed in FACS buffer, diluted in 1:200, 1:200, and 1:50, respectively in 50 µl (per well) FACS buffer, and incubated for 30 minutes in the dark on ice. FACS buffer were added, 150 µl per well, and plates were spun as before. Cells were then fixed for no longer than 5 min on ice with Formalin solution (4% formaldehyde), 100 µl per well. After fixation, 100 µl (per well) of BD Perm/Wash™ (BD Biosciences) and plates were spun as before. Next, cells were washed twice with 200 µl BD Perm/Wash to wash and permeabilise cells in the same time. After permeabilisation, cells were stained intracellularly with anti-IFN-γ APC and/or anti-TNF-α FITC and anti-IL-2 PE, diluted 1:200 in Perm/Wash (50 µl per well) for 30 min in the dark, on ice. Samples were washed twice with 200 µl BD Perm/Wash™ followed by tow other washes with 200 µl FACS buffer and were finally resuspended in 200 µl FACS buffer and acquired on a LSRII or CyAn flow cytometer and data were analysed using FloJo software (TreeStar Inc.).

Background responses in unstimulated cells were subtracted from stimulated responses to each peptide prior to plotting the data on the GraphPad Prism (GraphPad Software).

2.2.5.6. Enzyme Linked Immune Spot (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 5 µg/ml in coating (carbonate-bicarbonate) buffer. Plates were blocked with complete medium at 37°C for at least 1 hour or at 4°C until use.

Day 2: First, PBMC ELISpot (Blood ELISpot), PBMCs were isolated as described previously and 50 µl of PBMCs were plated into duplicate wells ($5x10^5$ per well) and diluted two folds down the plate, to form $2.5x10^5$ and $1.25x10^5$ (each in duplicate rows); and then 50 µl of peptide mixture were added to each well. The peptide mixture was prepared at 1 µg/ml diluted in complete medium plus 0.25×10^6 (per well) naïve splenocytes. Negative control wells had PBMC and complete medium plus naïve splenocytes but no peptides.

Second, spleen ELISpot, spleens cells were re-suspended at $1x10^7$cells/ml and 50µl of cells were plated in duplicate wells ($5x10^5$ per well) and diluted two folds down the plate, to form $2.5x10^5$ and $1.25x10^5$(each in duplicate rows); and then 50 µl peptide diluted in complete medium was added to each well (sometimes, peptides were added before cells to improve the assay). For negative control, wells had splenocytes in complete medium but no peptides. Peptides were added at a final concentration of 1 µg/ml. In peptide pools, the final concentration was 1 µg/ml for each individual peptide. Plates were incubated at 37°C (5% CO_2) 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 1 µg/ml in PBS. After incubation, plates were washed 6 times as before followed by 1 h incubation with a streptavidin alkaline phosphatase polymer diluted to 1 µg/ml in PBS. Spots were developed by addition of colour development buffer (AP Conjugate Substratre Kit, Bio-Rad Laboratories, Inc.) and counted using the AID ELISpot reader system and AID software (AID: Autoimmun Diagnostika, Germany). Results are expressed as spot forming cells (SFC) per million cells. Regarding the analysis, background responses in media-only wells were subtracted from those measured in peptide-stimulated wells.

2.2.5.7. Mouse Whole IgG ELISA

Blood was collected from tail veins into microvette tubes, or by cardiac puncture upon animal sacrifice into 1.5 ml Eppendorf tubes. In both cases, blood was left to clot at 4°C overnight followed by centrifugation at 13,000 RPM for 4 min. Serum was removed and transferred into clean 1.5 ml tubes and stored at -20°C until use. Day 1: 96-well flat bottom plates (Nunc-Immuno Maxisorp Plates, Thermo Scientific) were coated at concentration of 2 µg/ml with capturing antigen/protein (e.g. rLuc or 85A purified protein, see table 1.4) in 50 µl PBS and left overnight at room temperature (RT). Day2: Plates were washed 6 times with 200 µl PBS/T and blocked with 100 µl 10% skimmed milk powder in PBS/T (blocking buffer). Sera were prepared in a 10-fold serial dilution in PBS/T and then 50 µl were plated in duplicate wells (no wash after the blocking step). Serum from a naïve BALB/c mouse was included as a negative control; positive control antibodies were also included in the plates, such as anti-rLuc antibody (Millipore, table 1.1) or a pool of anti-85A positive sera (from previously vaccinated mice, obtained after vaccinating mice 3 times with the MVA85A vaccine). After addition of sera, plates were incubated for 2 hours at RT and then washed as before. Goat anti-mouse total IgG conjugated to alkaline phosphatase (Sigma) was diluted 1:5000 in PBS/T, and then added (50 µl per well) and the plate was incubated for 1 hour at RT. Following a final wash, the substrate PNPP tablet (20 mg p-nitrophenylphosphate, SIGMA) was diluted in 4 ml diethanolamine buffer (Pierce™) plus 16 ml H_2O, and 100 µl per well was used to develop the reaction.

Optical density (OD) was read at 405 nm using the EL800 Microplate Reader (Bio-Tek®) and Gen5 software. Generally, serum antibody endpoint titres would be 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. Background responses in naïve mouse serum were subtracted from responses from test serum prior to analysis. However, raw data were plotted in chapter 4 and 7 instead of endpoint titre because the antibody responses were weak, and similar to the negative control, following one-shot MVA vaccinations.

2.2.5.8. Luciferase Immunoprecipitation System (LIPS)

The LIPS is an alternative method for the detection of antigen-specific antibody when antigen suitable for an ELISA (e.g. purified protein) is not available. The target antigen is first fused to *Renilla* luciferase, by cloning, to form recombinant proteins. LIPS is therefore based on the detection of luciferase light-emitting recombinant proteins for indirect quantitation of antibody titres to a target antigen. If the recombinant protein was not bound to antibodies, it would then be washed off and the there would not be light-emitting luciferase. However, in this project, LIPS was performed to directly measure antibody titres against luciferase, since the model antigen rLuc was fused only to the $CD8^+$ T cell-specific pb9 epitope, and not to any antigen. Also, rLuc, in itself, was the antigen and the reporting luciferase in our study. It was not sure whether using luciferase as a target antigen and a detection reporter would affect the efficiency of this assy.

Preparing luciferase samples: Generally, a plasmid that encodes the antigen of interest fused to rLuc gene would be first transfected into HEK 293 cells (in T25 flasks), but as the rLuc was, itself, the antigen of interest in our study, rLuc-containing plasmid (p????) was transfected into HEK 293 cells instead.

The cells were transfected using Lipofectamine and incubated 37°C (5% CO_2) for 24 hrs. Supernatant was removed and cells were then lysed with either lysis buffer or with *Renilla* Luciferase Assay Lysis Buffer; both buffers were supplemented with protease inhibitor cocktail (1:100). The luciferase activity in these crude lysates was determined

in relative light unit per ml (RLU/ml) using luciferase assay as described above. Working solution aliquots of $2x10^8$ LU/ml were then made, labelled, and kept in -80°C until required.

LIPS assay: 1 µl of mice sera was diluted 1:100 in buffer A in 1.5 ml tubes. The tubes were mixed by vortexing and used to make 2-fold serial dilutions in buffer A. Naïve mice serum was used as a negative control. Next, 50 ul from the lysate working solution ($2x10^8$ RLU/ml) was added to each well of a V-bottom 96-well plates, which made 10^7 RLU per well. 50 µl from each diluted serum sample was added to the appropriate wells of the V-bottom 96-well plate; some wells had lysate without serum to serve as a second negative control (or background). The plate was incubated for 1 h at room temperature on a rotary shaker. During the incubation, 30% suspension of protein A/G slurry was prepared in PBS, and 5 µl per well was added gently to wells in a new 96-well filter plate (Millipore Cat # MSHVN4B50). These 100 µl Ag-Ab samples were then transferred from the V-bottom plates to the appropriate wells in the filter plate (following the same layout) and the filter plate was incubated for 1 h at room temperature on a rotary shaker. Next, the plate was washed 8-times with 100 µl buffer A, using vacuum manifold (Millipore) followed by two washes with 100 µl PBS, using vacuum manifold. The wells were then filled with 50 ul PBS to prevent them from drying out. Next, 50 µl of *Renilla* luciferase substrate mixture was prepared, as described for in vitro luciferase assay, and added to wells and the plate was immediately read using Varioskan Flash luminometer (Thermo Scientific).

2.2.5.9. *In Vivo* Imaging

To assess the *Renilla* luciferase expression, *in vivo*, driven by different promoters in rMVAs, a bioluminescent imaging system (IVIS, by Xenogen Co.) was used. This uses a cooled, back thinned CCD camera and linked to a PC that runs live Image software. The imaging was performed using *Renilla* luciferase ViviRen™ substrate (Promega). It was purchased as 0.37 mg in 10 µl of DMSO from Promega (i.e. 60 mM) and was diluted in PBS with 10% sterile FCS to a final concentration of 0.150 mM, which is equal to 1mg/1kg (around 30 ug per female BALB/c mouse). The calculation and protocol were adopted from previously described protocol on testing *Renilla* luciferase *in vivo* [178,179].

Four female BALB/c mice per group, 6-8 weeks old, were immunised intramuscularly with $1x10^6$ pfu rMVA. At 2 or 4 hour post immunisation, mice were injected intravenously (tail vein) with 100 µl of prepared ViviRen substrate, and anaesthetised immediately and then put inside the IVIS machine within 120 sec. Images were taken after exposure time of 2 min. All mice were housed for 7 days and sacrificed, and then spleens were collected for ICS experiments.

Images were analysed using Living Image software (Caliper LifeSciences) and data were presented as total flux, which counts photons per second per square centimetre per steradian (photons/sec/cm2/sr) on a region of interest (ROI). ROI is an area drown around images of immunised mice legs. The colour scale was unified for all images. For example, in chapter 5, the scale was $5.6x10^3$ to $2.2x10^4$.

2.3. Statistics

Statistical analyses were performed only when there are differences in data to determine the significance of such differences. All statistical tests, such as *t*-test and one-way ANOVA, were applied using Prism software.

2.4. Analysis Software

2.4.1 DNAStar software package, from DNAStar®

2.4.2 GraphPad Prism, from GraphPad Software

2.4.3 FlowJo, from Tree Star Inc.

2.4.4 ImageJ, from National Institutes of Health, USA.

2.4.5 Microsoft Office®

2.4.6 AID EliSpot Software, from AID, Germany

2.4.7 Gen5™ Data Analysis Software, from Bio-Tek, USA.

2.4.8 Living image, version 4.3.1, from Caliper LifeSciences

2.5. Websites

2.5.1. Primer3: **http://primer3.ut.ee**, for primer selections.

2.5.2. Protein database:**www.uniprot.org**

2.5.3. Poxvirus Bioinformatics Resource Centre: **http://www.poxvirus.org**

2.5.4. GeneArt® (Life Technologies): **http://www.lifetechnologies.com**

Chapter 3

Internal Ribosome Entry Site Insertion into MVA Genome to Enhance Transgene expression and Immunogenicity

3. Internal Ribosome Entry Site Insertion into MVA Genome to Enhance Transgene expression and Immunogenicity

3.1. Introduction

This chapter presents the first approach of this DPhil project aiming to enhance transgene expression in rMVA. It was attempted by inserting an Internal Ribosome Entry Site (IRES) into MVA genome [180]. In general, the translation of cellular and viral messenger RNA (mRNA) would normally be initiated by a complex of eukaryotic initiation factors (eIFs) that bind to the 5'7-methylguanylate (m7G) cap structure at the 5' end of mRNA, the eIFs-cap complex would then recruit ribosomal units to begin translation (called canonical translation). Cells, and some large DNA viruses like VACV, express capping enzymes as part of their transcription machinery. However, the RNA genome of many RNA viruses and mRNA of some DNA viruses, as well as some cellular mRNA are translated in the complete absence of the cap structure by structural IRES elements that are able to recruit ribosomes [180]. An IRES element is an untranslated, structural RNA element that is found in many viruses and in some mammalian cells. It has been extensively used in recombinant DNA technology to express two proteins from one transcript. The IRES would be placed between two genes (downstream the stop codon of the first gene) and both genes would be transcribed as one mRNA, but the first gene transcript is translated by canonical translation whereas the second gene transcript is translated by the IRES (called bicistronic expression system).

IRESes can be classified into groups based on their virus family; their requirement for eIFs; or their structural complexity [180,181]. Highly structured IRESes require less eIFs. The encephalomyocarditis virus's internal ribosome entry site (EMCV IRES) is always found in the middle of the hierarchy of any of these classification systems [180,181].

This IRES is not very complex, but not very unstructured, in the structural ranking of IRESes. It is also in the middle of the ranking in terms of eIFs requirements as it does not require all eIFs, can function in the absence of eIF4E and eIF4GN, and does not involve ribosome scanning for the ATG start codon as it binds to the ribosome units at the ATG site [180,181]. EMCV IRES has been shown to be an optimal choice for recombinant protein expression amongst other IRESes [182,183]. In one report, EMCV IRES was used to drive the expression of uncapped mRNA that was produced by a T7/vaccinia *in vitro* expression system and the recombinant protein expression was enhanced by inserting EMCV IRES upstream of the transgene[184].

In the context of RNA capping, poxviruses can produce capped transcripts, but they also decap mRNA to regulate the temporal expression of their genes. Decapping transcripts in poxviruses is mainly carried out by D10 (expressed late), and to a lesser extent D9 (expressed early), decapping enzymes and they can decap both cellular and viral mRNA in vaccinia virus (VACV) infected cells. Both D9R and D10R genes are highly conserved across the *Poxviridae* family and share 25% sequence similarity [185]. The D10 decapping protein has been well-studied in VACV and shown to have a role in initiating late gene expression, and also has a role in down-regulating early genes by decapping early mRNA that is still present once the D10 enzyme is expressed. This is due to its specificity towards early mRNA as a substrate in general, as it does not appear to preferentially decap specific early transcripts [186]. Previous studies showed that deleting D10 yielded increased early mRNA and delayed late transcription. Not only this but it impaired VACV infectivity and slowed the viral growth [185]. Conversely, over-expression of D10 yielded a decrease in the late mRNA, decreased protein synthesis, and prevented the formation of infectious virions [185,186]

In the case of rMVA vectored vaccines, if the aim is to elicit $CD8^+$ T cells, an early promoter is "preferred" to drive transgene expression because most of the $CD8^+$ T cell immunodominant epitopes, from MVA proteins, in human and mouse are products of early genes. In addition, when human dendritic cells were infected with VACV (which is replication competent unlike MVA), early transcription persisted with no late protein expression [79,118]. Therefore, insertion of a vaccine transgene downstream of an early promoter would be ideal. However, since early gene transcripts are targeted by the D10 enzyme, we hypothesised that inserting an IRES upstream of a transgene driven by an early promoter, could potentially enable the transgene mRNA translation and prevent it from being decapped (mainly by the D10 protein). This could subsequently increase the expression of the transgene, eliciting improved $CD8^+$ T cell responses to the transgenic antigen in vaccinated animals. If decapping is not a prominent mechanism and does not severely affect the transgene transcript, then the IRES could still be useful in stabilising the transgene transcript by allowing it to persist (when MVA shifts the expression from early to intermediate and late genes by decapping and other regulatory proteins). Finally it would be informative to assess whether there is any transgene expression increase when two cis-regulatory elements are adjacent to each other; IRES at the RNA level, and promoter at the DNA level. IRES has been shown to drive gene expression in VACV despite the effect of an excess amount of D10 enzyme expressed *in trans*, suggesting that IRES could overcome decapping activity in poxviruses [186]. Thus, it could function in the same way when integrated into a rMVA genome and serve as an optional way to improve MVA-vectored vaccines.

In this chapter, I therefore studied the insertion of the EMCV IRES upstream of a reporter transgene (tPA-pb9-rLuc), which is made by the fusion of tPA (tissue plasminogen activator) leader sequence that directs the antigen to the secretory pathway of the cell to pb9 (an immunodominant malaria MHC class I H2-K^d-restricted epitope from *Plasmodium berghei* Circumsporozoite Protein) and then to the *Renilla* luciferase (rLuc) gene. This transgene is under the control of the early *B8R* promoter (pB8), at its natural *B8R* locus. This promoter was selected because the *B8R* gene is fragmented and not essential in MVA [3] and can be replaced with the transgene. Utilising the pB8 to express the tPA-pb9-rLuc transgene should result in early transcripts that are suitable substrates for the D10 decapping enzyme; allowing us to assess whether the IRES is then able to drive more expression in a cap-independent manner. So, two recombinant MVAs were made as described in the material and methods; one rMVA was named B8-IRES-MVA, which has 550 nucleotides of the EMCV IRES sequence upstream of the transgene, and the other MVA did not have the IRES (named B8-MVA) and used as a comparative control, illustrated in figure 3.1.1.

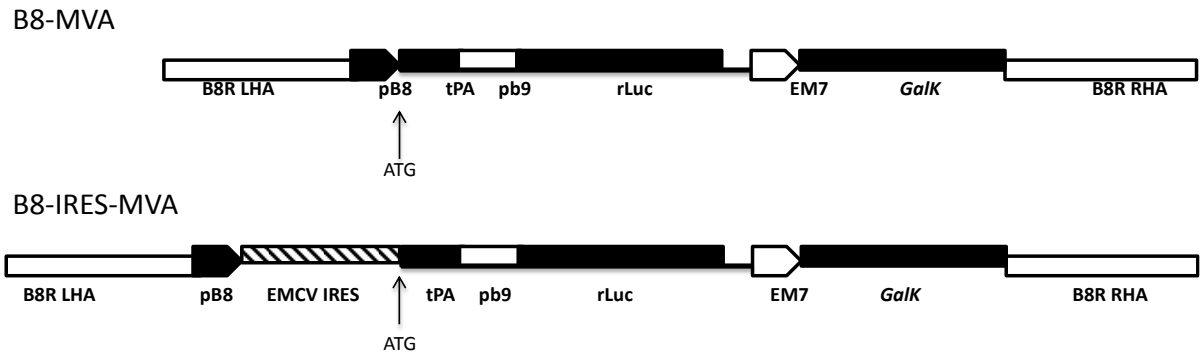


Figure 3.1.1: Schematic representation of the tPA-pb9-rLuc transgene.

The transgene consists of *Renilla* luciferase (rLuc), tPA leader sequence, and pb9 (the CTL epitope of *Plasmodium berghei* circumsporozoite protein) under the control of pB8R promoter. It also contains *GalK* bacterial selection gene controlled by a bacterial promoter (EM7). The transgene is inserted into B8-MVA virus (top), or fused to EMCV internal ribosome entry site (EMCV IRES) and inserted into B8-IRES-MVA (bottom). The ATG start codon of the transgene is indicated.

3.2. Results

3.2.5. Optimising the luciferase assay to detect the difference in protein expression of different rMVAs

The Renilla luciferase assay has been used before (Orubu, *et al*[156]) to detect the transgenic luciferase expressed by rMVAs. However, optimising the luciferase assay at the beginning of this DPhil project was important to be able to detect any small differences in the luciferase level. Using B8-MVA and B8-IRES-MVA (illustrated in figure 3.1.1), first, the impact of different multiplicity of infection (MOI) was determined. At either MOI of 0.3 or 3, the difference of luciferase level between the two rMVAs remained similar (Figure 3.1.2). Therefore, I decided to use a MOI of 1 for the remainder of the project, which is in the middle of the two MOIs tested above. MOI of 1 was used also because rMVA were tested in permissive cells with multiple cycles of (productive) infection. In addition, this MOI of 1 was previously used to assess transgene expression in similar studies [156]. This MOI however could have its limitations, as it might not be able to achieve a synchronized infection. Second, the pB8 is an early promoter and its activity might be stronger at early time points of the infection. Thus, a time course assay was performed to determine the best time point to detect the difference in the early expression (not only for this chapter, but because early promoters will be studied in the next chapters). BHK-21 cells were infected with B8-MVA or B8-IRES-MVA with or without AraC (cytosine arabinoside used to block DNA replication, hence allowing only early expression), and the supernatant was collected at different time points from 0 to 24 h.p.i (hours post infection). The difference in the luciferase level between the two viruses was similar in all time points, and therefore, the time point of 24 h.p.i. was selected to carry out other studies throughout the thesis

project (Figure 3.1.2). Third, measuring the luciferase in the supernatant or the lysate of infected cells was to be compared. Monolayers of BHK-21 cells were infected with the two viruses, and incubated for 24 h.p.i. The supernatants were collected and the cells were lysed with similar volume to the supernatant, in order to be consistent and avoid diluting the luciferase protein. Although the luciferase level in the lysate was a log higher than its level in the supernatant in any of the tested viruses, the differences between the two viruses remains similar regardless of the sample type. A combined supernatant and cell lysate of the same infected cells prior to the luciferase detection was therefore used to measure the total luciferase level. The difference in the total luciferase level between the two viruses was similar to that of either supernatant or cell lysate, indicating the possible use of the total luciferase (Figure 3.1.2). Fourth, to determine the effect of freezing condition on the level of luciferase, supernatant samples were kept in the fridge, or frozen down for two months, then were re-measured and compared to their previously fresh luciferase level. The result showed that although the luciferase level could slightly drop upon freezing, the difference between the two viruses remained the same (Figure 3.1.2). Therefore, fresh samples should be used in the project even though the frozen samples could still be used when necessary. Finally, these viruses were tested in different plate (e.g. 6 or 96 well plates) and did not show difference in the luciferase level although 6 well plates give the advantage of not scraping the cell monolayer when sampling, especially for time points assay.

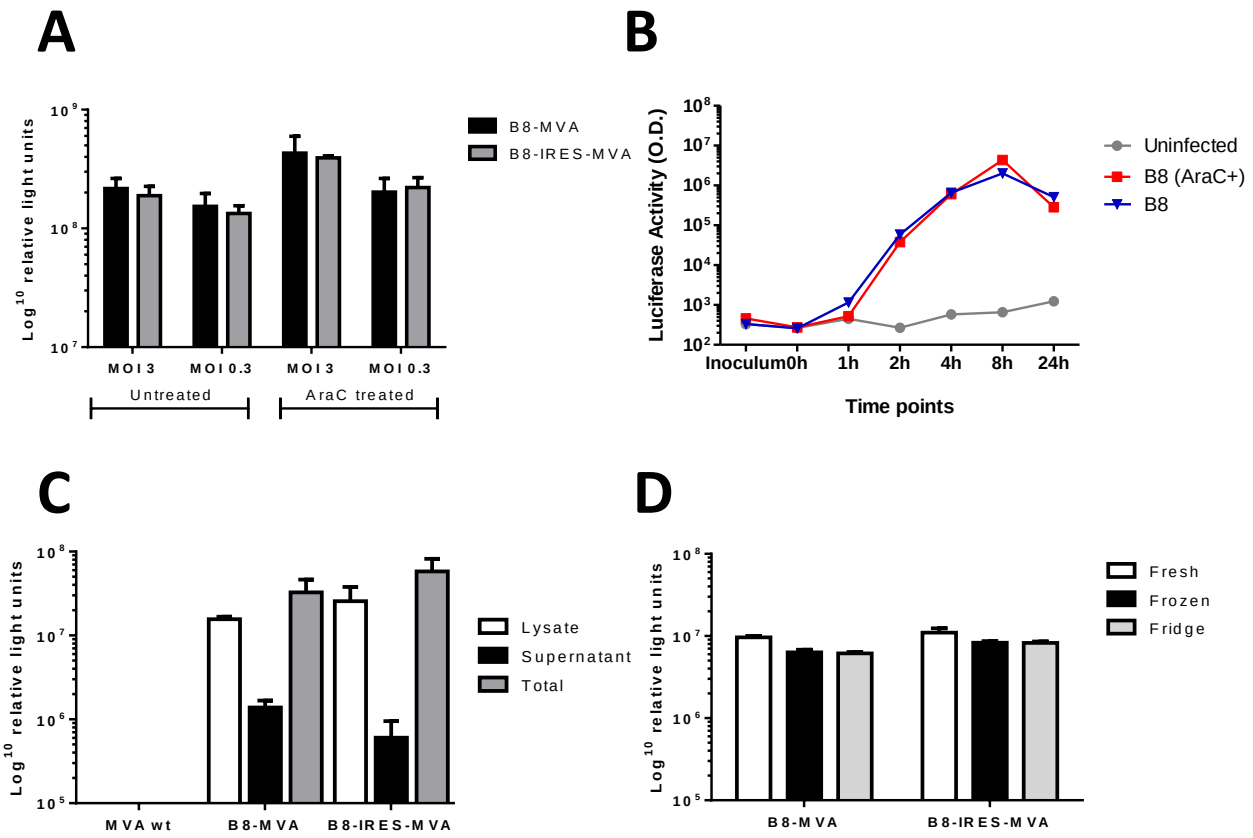


Figure 3.1.2: Optimization of rMVA luciferase expression detection.

BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of the pB8 (B8-MVA), or pB8 with EMCV IRES inserted upstream of the transgene (IRES-B8-MVA) at different MOIs **(A)**. Luciferase activity of supernatant samples collected from B8-MVA infected cells at different time points is plotted **(B).** At 24 h.p.i, the cells were lysed and cell lysate samples were compared to the supernatant taken at 24h.p.i. **(C).** Supernatant samples at 24 h.p.i. were kept in 4°C (fridge) or in -20°C (frozen) for 3 months and then measured again to show the effect of freezing on the recombinant luciferase **(D).** Luciferase expression was measured using *Renilla* luciferase system. MVA*wt was* included as a negative control. The data, shown in logarithmic scale, represent the mean of 4 wells with SD error bars. Data is representative of two independent experiments.

3.2.6. The effect of IRES Insertion on the Recombinant Luciferase Expression in vitro

The two rMVAs, B8-MVA and B8-IRES-MVA, were tested in the optimised luciferase assay described above. BHK-21 cells were infected with each virus at a multiplicity of infection of 1 (MOI = 1 plaque forming unit, pfu/cell) and incubated at 37°C overnight with or without AraC (cytosine arabinoside), which is used to block DNA replication and prevent late gene expression. 24 hours later, supernatant and cell lysate were collected and pooled from either AraC treated and untreated cells. Luciferase expression by B8-MVA or B8-IRES-MVA showed no significant difference (Figure 3.2). This experiment was repeated thrice independently and showed reproducible results at 24 h.p.i (hours post infection). Next, there was a concern that BHK-21 (cells permissive for MVA viral replication) might not be ideal for detecting any expression improvement. So, the HEK 293 cell line (non-permissive to MVA viral replication) was used and the expression profile was very similar to that achieved with BHK-21 cells (Figure 3.2). We had expected to see the effect of the IRES by driving more tPA-pb9-rLuc expression, especially when the cells were not treated with AraC and late gene expression occurred, which should allow for the expression of the D10 protein. However, our results showed that the IRES insertion did not appear to increase the transgene expression.

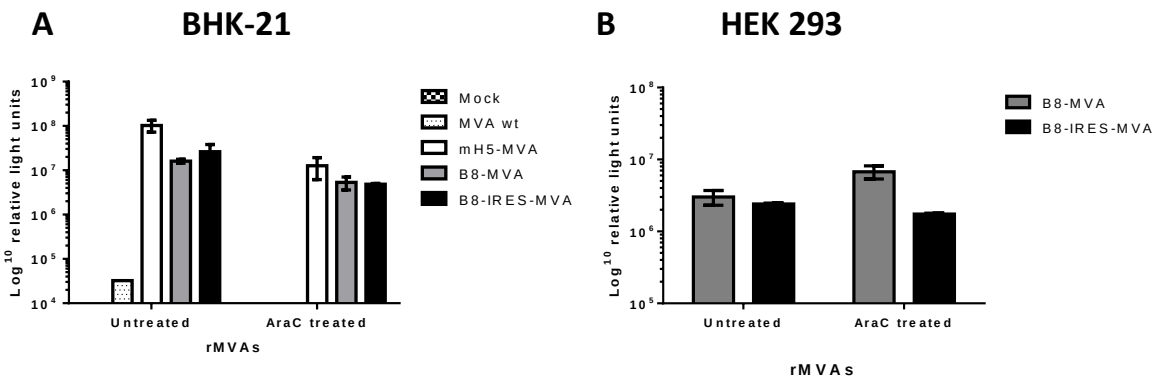


Figure 3.2: The effect of IRES insertion on luciferase expression of rMVA.

BHK-21 cells **(A)** and HEK 293 cells **(B)** were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of pB8 promoter, or pB8 promoter with EMCV IRES inserted upstream of the transgene at MOI of 1. Cells were either treated with AraC (to assess the early expression) or without AraC treatment (for the overall promoter activity). At 24 h.p.i, the cells were lysed, and added to the supernatant of the same wells, and the total level of luciferase expression was measured to determine the effect of IRES insertion on the transgene expression, using *Renilla* luciferase system. mH5-MVA as a positive control and MVA*wt* as a negative control were included (A). The data, shown in logarithmic scale, represent the mean of 4 wells with SD error bars. Data is representative of more than five independent experiments.

3.2.7. The effect of IRES Insertion on the Size of the Recombinant Luciferase Protein

IRES insertion did not yield more luciferase expression; however, I sought to determine whether the IRES sequence could be translated and become a part of the expressed luciferase protein, and then could have an effect on the size and the secretion of the recombinant luciferase. This could occur if the translation was initiated upstream of the native ATG start codon. Utilising the IRES's native ATG start codon was reported to be important for the IRES efficiency [182]; thus, the transgene was fused to the native ATG of EMCV IRES. However, IRESes in general have more than one ATG codon, any of which could act as a start codon, and EMCV IRES in particular has ten ATG codons located upstream of its native start codon [182,184]. One of these ten ATGs is in the same reading frame of the other annotated open reading frames (ORFs) in B8-IRES-MVA. If the initiation occurred at this upstream ATG, it would result in a larger transgenic protein in B8-IRES-MVA than in B8-MVA. This larger protein would be 58 kDa compared to the natural start codon-initiated protein, which is a 40.1 kDa protein if the tPA leader sequence is not cleaved or 36.9 kDa after the cleavage of the tPA sequence (as predicted *in silico*). Initiating the translation from an upstream ATG might also interfere with the tPA leader sequence and affect the secretion of rLuc transgenic protein, leading to accumulation and degradation of intracellular rLuc, and presumably to a lower than expected level of expressed luciferase.

A western blot was therefore performed to determine the size of the luciferase protein expressed by B8-MVA and B8-IRES-MVA recombinants. A rMVA with the modified H5 promoter (mH5) driving the expression of tPA-pb9-rLuc (mH5-MVA) was included as a positive control for its ability to drive strong luciferase expression [156], and

MVA*wt*(empty vector) was used as a negative control. The result showed a similar protein blot for all viruses, in either supernatant or cell lysate samples, with a size of around 50 kDa (Figure 3.3). Although this size is bigger than the predicted, which could be a result of post-translation modifications, it is similar to that of the control viruses, which indicates that the IRES does not impair the secretion of luciferase. Interestingly, the protein abundance seemed relatively similar in supernatant and in cell lysate samples, especially in mH5-MVA and B8-IRES-MVA (Figure 3.3.), which does not seem in a perfect agreement with the luciferase assay result. In the luciferase assay the level of luciferase expression was higher in cell lysate (around 10-fold) than in supernatant in any of the tested viruses (Figure 3.3). This could be because in cell lysate there is a population of immature or degraded luciferase protein that can still emit light when treated with substrate, but cannot bind to the monoclonal anti-rLuc antibody used in the Western blot. However, the Western blot is not quantitative and could also have a lower sensitivity limit compared to the luciferase chemo luminescence assay. Furthermore, the luciferase expression by B8-MVA and B8-IRES-MVA was also detected by immunofluorescence assay (images are presented in the appendices, Figure S3.3). Overall, although the IRES insertion did not increase the level of luciferase protein expression, it seemed to have no discernible effect on the size or the secretion of the transgenic protein.

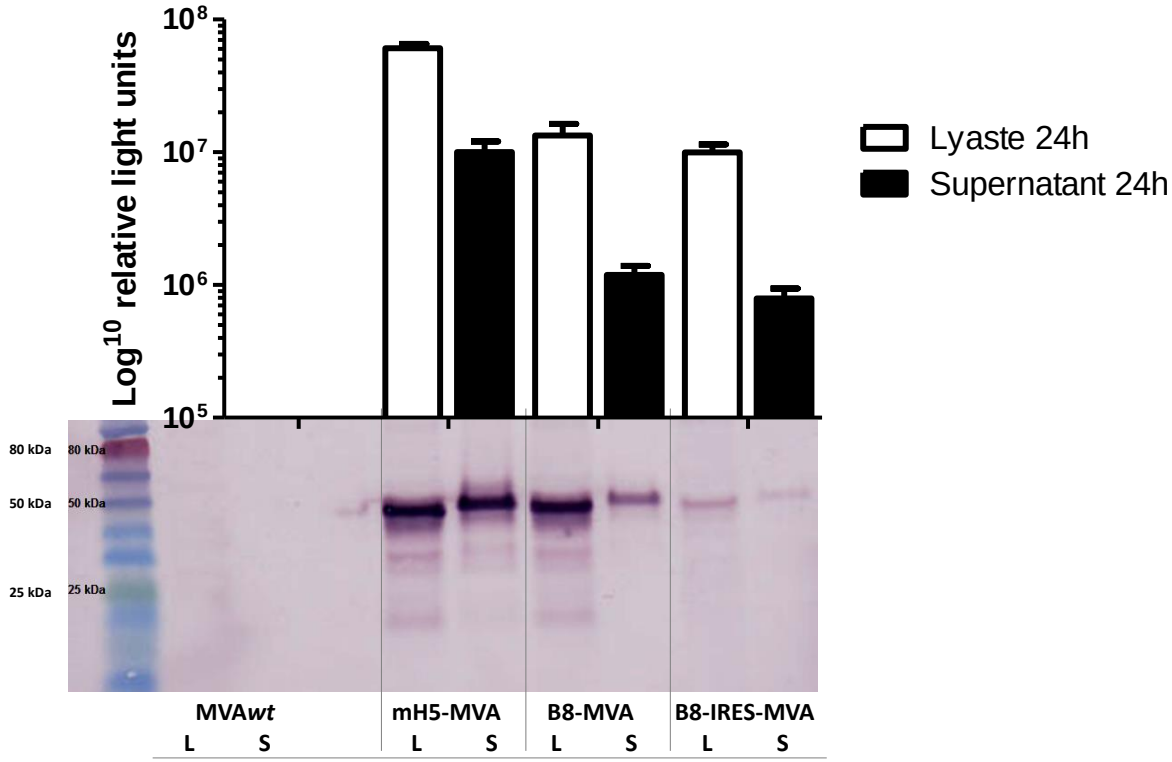


Figure 3.3: The effect of IRES insertion on secreted luciferase expression.

BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of pB8 promoter, or pB8 promoter with EMCV IRES inserted upstream the transgene at MOI of 1. At 24 h.p.i, the supernatants were collected and the cells were lysed, the level of luciferase expression in the supernatant or cell lysate was measured to determine the effect of IRES insertion on the secretion of expressed luciferase, using *Renilla* luciferase system, shown on a logarithmic scale **(Top)**, and western blot **(Bottom)**. mH5-MVA as a positive control and Non-recombinant wild type MVA (MVA*wt)* as a negative control were used. The samples were taken from same wells (cells) for both assays, shown in logarithmic scale for the luciferase expression (top), or the western blot (bottom). The data is representative of two independent experiments. L: Lysate from infected cells. S: Supernatant.

3.2.8. The effect of IRES Insertion on the Luciferase Transcript level

There was no observable higher expression of rLuc achieved by the IRES whose role is to increase the translation process; however, any decrease in the rLuc transcript level in B8-IRES-MVA would reduce the protein expression. Moreover, the IRES was inserted between the pB8 and the ATG start codon (see figure 3.1.1), resulting in prolonged length of the untranslated region. The untranslated spacer could be crucial for poxviral promoter efficiency according to Di Pilato *et al*[165]. Thus, a comparative ΔΔCt method of qPCR (a real-time relative quantitative PCR), described by Livak and Schmittgen [176], was used to determine whether the IRES insertion could interfere with the rLuc gene transcription. This method was performed using the MVA *E9L* gene as an endogenous control to determine the level of luciferase transcript (see Materials and Methods). In ΔΔCt method of qPCR, it is critical to have the difference in the Ct values of the tested gene (rLuc) and the endogenous control gene (*E9L*) similar over a serial dilution of a RNA template. These values are then subtracted and the slope of the ΔCt is plotted. As long as the slope ≤0.1 (Figure 3.4A and 3.4B), the amplification efficiency of both *E9L* and rLuc is reliable and the ΔΔCt can be calculated to show the relative change in gene expression. Therefore, monolayers of BHK-21 cells were infected with the rMVAs, in duplicates, at a MOI of 1, and incubated at 37°C for 24 hours. Next, the supernatants were removed and cells were washed with PBS before extracting the total RNA and synthesising cDNA. The cDNA samples from cells infected with either B8-MVA or B8-IRES-MVA were run as templates in qPCR and the ΔΔCt values were then calculated and presented as fold change in transgene expression. The result of two replicates of this experiment showed no difference in the relative quantification of luciferase transcript in both viruses, presented as relative fold change in expression

(Figure 3.4C). Inserting IRES thus did not appear to interfere with the rLuc gene transcription, and interestingly the prolonged untranslated spacer sequence, by around 550 nucleotides, did not seem to have an effect on the pB8 activity.

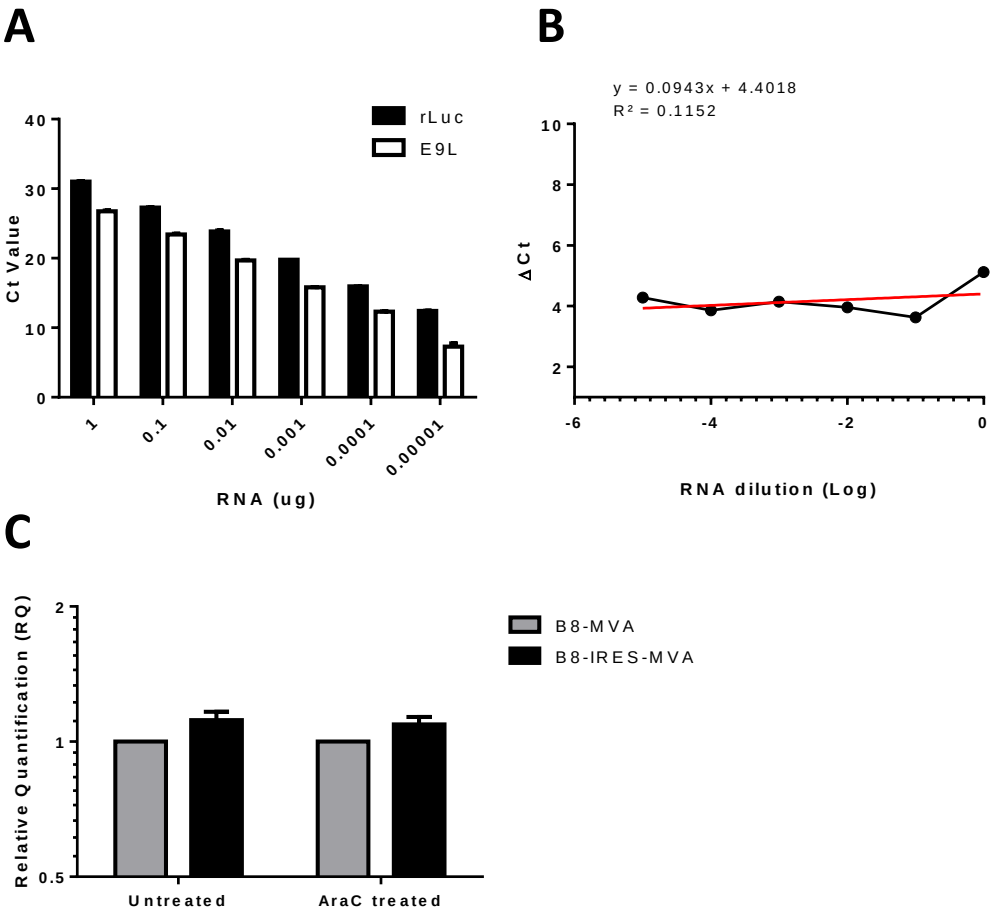


Figure 3.4: The effect of IRES insertion on luciferase transcripts level in rMVA

BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of [B8 promoter, or pB8 promoter with EMCV IRES inserted upstream of the transgene at MOI of 1. Cells were either treated with AraC (to assess the early transcription) or without AraC treatment (for the overall transcription). At 24 h.p.i, the cells were lysed, and the total RNA was extracted and used to synthesise cDNA for the qPCR using the ΔΔ Ct method to determine the relative change fold in the rLuc gene expression. The validation of ΔΔ Ct method is shown at the top panel. The Ct values of *E9L* and rLuc are presented against RNA template concentration in µg **(A)** to show the amplification efficiency of these two genes. The ΔCt of every RNA dilution is plotted **(B)** with a slope of 0.094 (the acceptable range should be at least 1, according to Livak and Schmittgen [176]). Based on this method, the relative change fold in rLuc gene expression of IRES-B8-MVA (relative to that of B8-MVA) is presented **(C).** The mean is shown with SD error bars. The Data is representative of two independent experiments.

3.2.9. *In vivo* immunogenicity of pb9-rluc transgenic antigen

The ultimate aim of the IRES insertion study was to improve the MVA vaccine vector by obtaining stronger transgene expression, which could contribute to higher immunogenicity. Although stronger expression was not observed using IRES *in vitro*, I continued the study by performing an *in vivo* immunogenicity experiment in mice; to see whether the use of the IRES *in vivo* would mirror the findings in cell lines *in vitro*. Two groups of four female BALB/c mice were immunized with B8-MVA or B8-IRES-MVA as described in the materials and methods. The percentage of antigen-specific IFN-γ producing $CD8^+$ splenic T cells was determined, seven days after a single shot of MVA, by intracellular cytokine staining and flow cytometry. There were no significant differences between the groups in response to the transgenic peptide (pb9) or to the MVA vector-specific F2(G) and E3 peptides (Figure 3.5). This result showed that IRES insertion into the rMVA genome did not yield higher cellular immune responses, presumably because the IRES did not increase the transgene expression either *in vivo*.

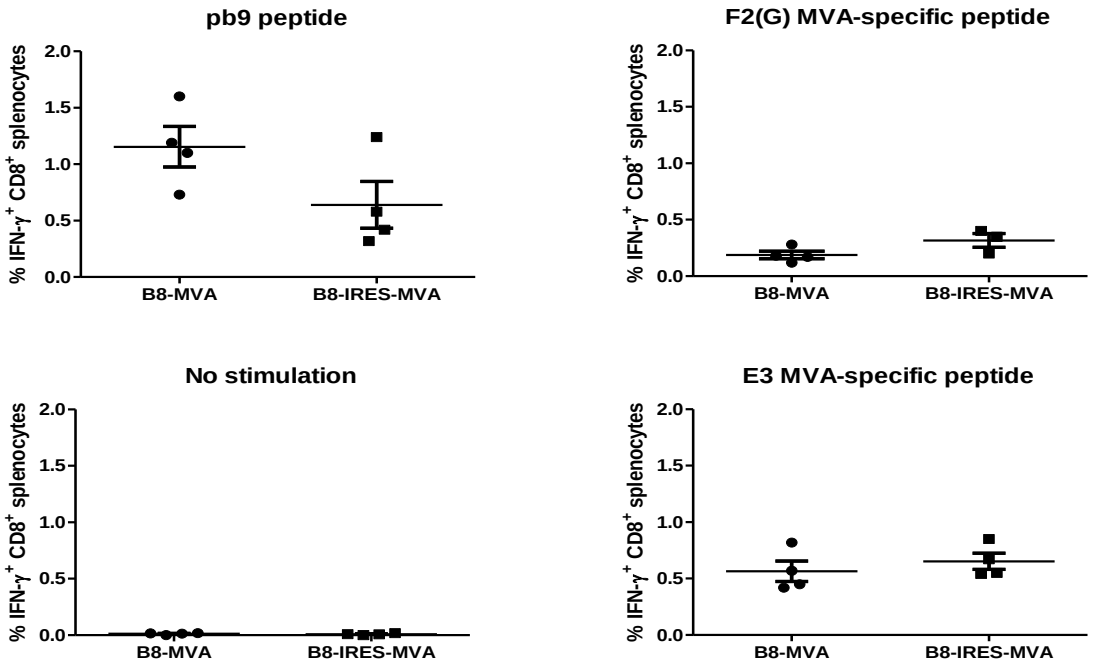


Figure 3.5: In vivo cellular immunogenicity of rMVAs with IRES inserted upstream of the transgene (tPA-pb9-rLuc).

Two groups of female BALB/c mice (n=4) were immunised with the respective rMVA and seven days post immunisation, the intracellular cytokine staining (ICS) and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in response to *in vitro* re-stimulation with pb9 peptide, or MVA-specific peptide. These values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with SEM error bars are shown. Data is representative of two independent experiments.

3.2.10. IRES functionality in the presence of the D10 decapping protein

The EMCV IRES has not (to our knowledge) been reported as non-functional, in particular when used in bicistronic expression vectors, which allow the simultaneous expression of two proteins separately but from the same RNA transcript [182]. Previously, the EMCV IRES functionality in a bicistronic expression system was assessed by performing a Western blot on HEK 293 cell lysate transfected with a bicistronic vector expressing Influenza virus haemagglutinin 1 antigen (H1HA) under the control of a CMV promoter[187], and a fused antigen of Influenza nuclear protein and matrix 1 protein (NP-M1) under the control of the EMCV IRES (identical to the one used in B8-IRES-MVA). The EMCV IRES was clearly able to express the Influenza NP-M1 fused antigen, with the correct expected size (Alharbi *et al*, in prep; data from Senthil Chinnakannan). This confirmed the functionality of the EMCV IRES sequence used here in driving cap-independent expression. However, it remained possible that the IRES would not function when integrated into the recombinant B8-IRES-MVA. Therefore, I sought to test its functionality in the context of decapping activity. Thus, the main decapping enzyme (D10 protein), amplified from the MVA genome, was cloned downstream of a CMV promoter (previously described [188,189]) in a mammalian cell expression plasmid, named CMV-D10. This was used to overexpress the D10 protein to determine whether the IRES would function in the tested cell line. This experiment should also reveal whether the decapping actually occurs as a result of the MVA D10 protein. I noticed that the MVA D10 enzyme has 3 amino acid substitutions (due to point mutations), which are absent in VACV (Figure S3.1, in appendices). Notably, the VACV D10 activity was shown to be inhibited by induced point mutations [190]. However, the reported point mutations in the VACV D10 protein were in the active

motif whereas those I found in the MVA D10 protein were not, and may not have a functional effect on the MVA D10 (Figure S3.1). Therefore, to test the functionality of EMCV IRES in the presence of an excess amount of MVA D10 protein, monolayers of BHK-21 cells in 6-well plates were transfected with 10 μg of CMV-D10, or irrelevant plasmid expressing mCherry gene (red fluorescent marker [191]) driven by the poxviral p7.5 promoter, or left with no transfection. Twelve hours later, cells were infected with either B8-MVA or B8-IRES-MVA at a MOI of 1, or left uninfected. The untransfected cells were also infected with either of the viruses to serve as controls. Pools of lysate and supernatant were collected 24 h.p.i. The luciferase level was reduced by CMV-D10 treatment in the cells infected with either B8-MVA or B8-IRES-MVA as compared to the control (untransfected, but infected cells, figure 3.6). In contrast, the level of luciferase in cells treated with mCherry plasmid did not drop in both viruses, which suggested that the reduction in the luciferase level is specifically due to the D10 treatment. However, the presence of the IRES did not improve the rLuc expression in a cap-independent manner as hypothesized. Therefore, we concluded that the D10 decapping protein is active in MVA, but the EMCV IRES is not functional in B8-IRES-MVA.

3.2.11. D10 decapping activity reduces MVA viral growth

The D10 protein, which was shown to be active in rMVA in the previous experiment, has been reported to act on the majority of capped cellular and poxviral mRNA (regardless of what these mRNA molecules encode), and is efficient in decapping RNA of 24-309 nucleotides in length [192]. Although the D10 enzyme does not have a clear specificity, there is more chance of decapping cellular and early poxviral mRNA as opposed to intermediate and late poxviral transcripts [192]. Therefore, to determine if the D10 overexpression had a deleterious effect on MVA growth by decapping, I imaged the cells that had been transfected with CMV-D10 and infected with either B8-MVA or B8-IRES-MVA (used for the IRES functionality experiment above). It was clear from the fluorescent microscopy at 24 h.p.i. that the D10 treatment reduced the green fluorescent protein (GFP). The GFP gene is inserted into rMVAs, part of MVA-BAC, and therefore its expression is linked to the viral growth (Figure 3.7). This suggested that the overexpression of MVA D10 protein did reduce the viral growth despite the three amino acid differences in comparison to VACV. This reduction was unlikely due to the transfection process, as the irrelevant transfection with mCherry-expressing vector did not reduce the viral growth, whilst the mCherry red fluorescence was also detected confirming successful transfection in the control. Because the mCherry gene is driven by a poxviral promoter, its expression would only occur in cells that are both infected and transfected. Overall these data suggest the D10 decapping enzyme is functional in MVA, but the EMCV IRES upstream of a transgene at the *B8R* locus does not prevent its activity.

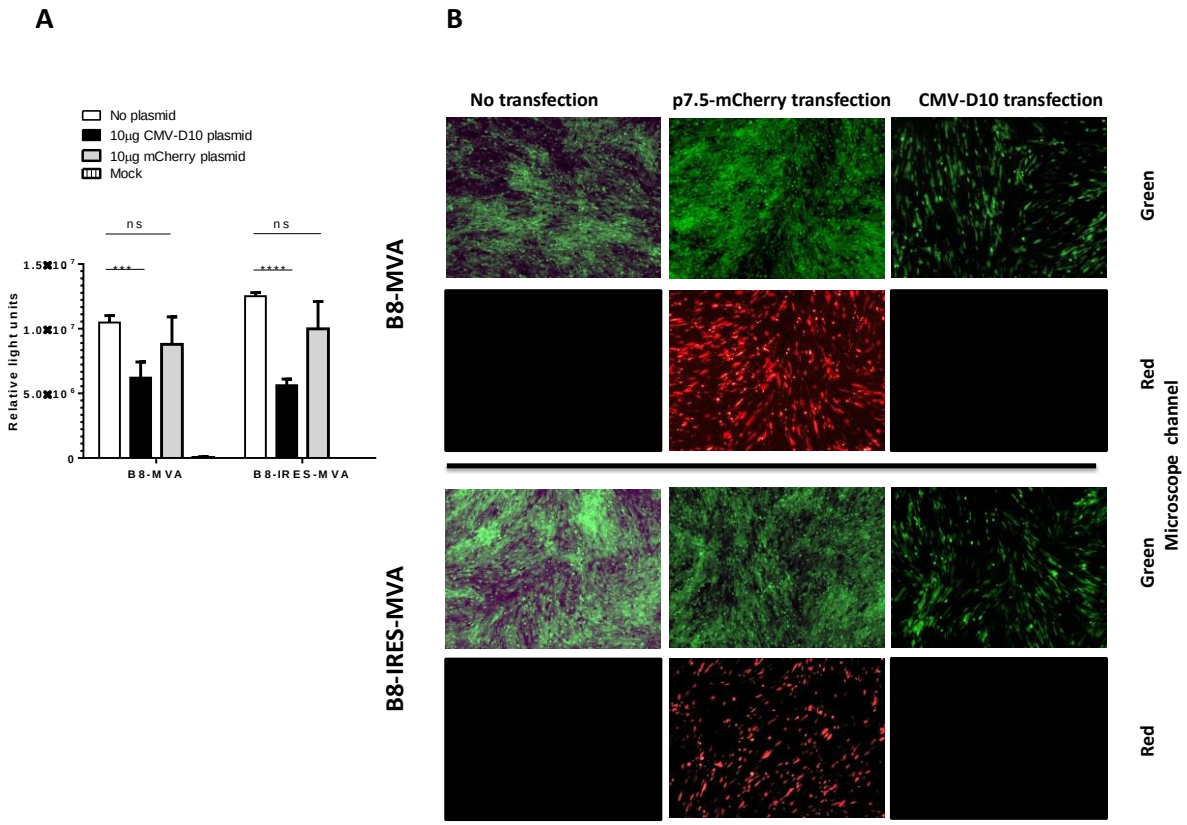


Figure 3.6: The effect of IRES insertion on luciferase expression of rMVA in the presence of overexpressed D10 decapping protein.

A: BHK-21 cells were transfected with CMV-D10 plasmid, p7.5-mCherry (irrelevant transfection control), or left untransfected (no plasmid). 12 h later cells were infected with either B8-MVA or B8-IRES-MVA at MOI of 1. At 24 h.p.i., the cells were lysed, and added to the supernatant, and the total level of luciferase expression was measured using the *Renilla* luciferase system to determine the effect of IRES insertion on the transgene expression in the presence of an excess amount of D10 decapping protein. **B:** The cells were imaged to show the effect of transfection with D10-expressing vector or mCherry expressing vector (irrelevant plasmid) on GFP expression, which reflects the viral growth. The data represent the mean of 4 wells with SD error bars and are representative of two independent experiments. ***, $p = 0.001$. ****, $p < 0.0001$. ns, not significant by two-way ANOVA with Bonferroni post-test

3.3. Discussion

An IRES insertion approach was attempted to enhance rMVA immunogenicity by increasing transgene expression, aiming to overcome de-capping activity, or if not, at least by stabilizing the transgene transcript. The EMCV IRES has been used in a bicistronic expression system and proved functional in expressing influenza virus proteins in a cap-independent manner. Thus, the same EMCV IRES sequence was used to try and increase the expression of luciferase in rMVA. Initially two rMVA vectors encoding the tPA-pb9-rLuc transgene at the *B8R* locus were generated. In one vector the EMCV IRES was inserted upstream of the transgene and controlled by the endogenous B8 promoter, which is an early promoter leading to an early transcript that should be a suitable substrate for the D10 decapping enzyme. These viruses were tested *in vitro*, where the D10 protein should be expressed late in the MVA life cycle, i.e. when cells were not treated with AraC (an agent used to block DNA replication and study the early gene expression in MVA); and the IRES could also potentially help by increasing the luciferase expression, at least, in the untreated cells. However, IRES insertion did not seem to protect the tPA-pb9-rLuc transcript from decapping by measuring luciferase expression in BHK-21 (permissive to MVA) or HEK 293 cells (non-permissive) in the presence or absence of AraC treatment. Western blotting showed that the protein expressed by either B8-MVA or B8-IRES-MVA virus is similar and around 50 kDa, suggesting that the IRES insertion did not affect the translational ATG start codon. It also showed that the IRES did not appear to interfere with the luciferase secretion, suggesting that the tPA leader sequence was not affected by the upstream inserted IRES sequence. Relative qPCR also showed that the IRES inserted between the promoter and the ATG start codon (thus elongating the untranslated spacer), did not

affect the pB8 activity. This contradicts a previous report emphasising the importance of the spacer region for poxviral promoter activity [165]. Finally, the IRES did not appear to improve the cellular *in vivo* immunogenicity when the rMVAs were tested in mice, presumably because it could not drive more transgene expression in the mouse model. Taken all together, these data led us to conclude that either the IRES is somehow impaired when inserted into the rMVA genome or that decapping is not a very prominent mechanism in MVA; at least in this experimental setting.

The optimal sequence of the EMCV IRES should have a number of characterizations to ensure efficient expression, according to Bochkov and Palmenberg [182]. First, the EMCV IRES should start at the preferred 5' boundaries of EMCV IRES and end at the 3' minimum boundaries of EMCV IRES (highlighted in figure S3.2, appendices). Second, it should utilize the defined ATG start codon at the 3' end of the IRES, and not any of the other upstream ATG codons within the IRES sequence. Third, it should contain the A6 loop (6 consecutive residues of adenine), which is the native and optimal sequence, and not the A7 loop (7 consecutive residues of adenine), which was introduced in some EMCV IRES versions to slightly reduce the IRES-controlled expression for specific applications (see [182]). Therefore despite the fact that the EMCV IRES functioned efficiently in our bicistronic expression system, we also ensured that the EMCV IRES sequence used here is the optimal sequence (Figure S3.2) according to these characterizations.

Bochkov and Palmenberg had also warned that placing the IRES immediately next to the 5' cap, i.e. between the expressed gene and its promoter, in monocistronic expression vectors might cause the IRES to fail or interfere with the translation process. However, although the IRES in our B8-IRES-MVA is slightly mimicking the

monocistronic systems, I did not detect any obvious interference with the luciferase translation (tested by Western blot). Yet, it is likely that the IRES RNA did not fold into a functional structure when it was placed too close to the B8 promoter, and a spacer sequence was required between the IRES and the promoter. A similar situation was previously noted when the EMCV IRES was inserted into VACV genome downstream of the T7 bacteriophage promoter. The IRES did not efficiently translate the uncapped transcript provided by the T7/vaccinia system until the T7 structured loop was inserted between the T7 promoter and the EMCV IRES, which obviously acted as a spacer [184]. In addition, it is likely that the transgene transcript is capped despite the presence of the IRES RNA so the ribosomes could be attracted to its cap structure rather than the IRES.

Nevertheless, I continued to assess the IRES functionality in the context of decapping. An over-expression system was set up by transfecting BHK-21 cells with a D10-mammalian expressing plasmid prior to infecting with the rMVAs. This system aimed to show the role of the EMCV IRES in expressing the early transcripts when the D10 decapping protein (acting on these early transcripts) is over-expressed. However, the IRES in B8-IRES-MVA did not increase the level of luciferase as compared to the control (B8-MVA). In D10-transfected cells, both B8-MVA and B8-IRES-MVA showed a lower level of luciferase than the controls (B8-MVA and B8-IRES-MVA without D10 transfection). This suggested that the D10 decapping enzyme is indeed active and could affect the transgene transcription, but that the rLuc transcript was not protected from this effect by inclusion of the IRES. Decapping enzymes in poxviruses are well studied in VACV and reported to have very conserved genes across the *Poxviridae* family. Here I studied decapping enzymes in MVA rather than in VACV. Interestingly,

an excess amount of the D10 protein reduced the viral growth as compared to the irrelative transfection of mCherry marker. This supports the previous studies [186], carried out on VACV, that the over-expressed D10 decapping enzyme targets early mRNA transcripts and impairs the viral growth. Although other groups [190] have reported that individual point mutations in the active motif of D10 could inhibit its activity in VACV, I report here that the MVA D10 protein appeared to function in spite of having three amino acid substitutions, not in the active motif, as compared to VACV D10 protein.

Overall, our data suggest that inserting the EMCV IRES between a poxviral promoter and the ATG of a transgene, integrated into the MVA genome, does not increase the transgene expression or the *in vivo* cellular immunogenicity.

3.4. Recommendations for future research

For future studies, it should be noted that our study concerned only the EMCV IRES; and there are various IRESes shown in the literature to require fewer eukaryotic initiation factors (eIFs). One IRES sequence was reported to require no eIFs and even no ATG start codon to initiate the translation because the ternary initiation structure (eIF2-GTP-Met-tRNA) is unnecessary. This IRES is found in the intergenic region (IGR) of dicistrovirus (an insect virus) [193]. It might be beneficial to have an IRES that recruits the ribosome units directly without any eIFs involved to overcome any concern over the suitable host cells [193].

Chapter 4

Utilizing Endogenous Poxviral Promoters to Enhance the Transgene Expression and *in vivo* Immunogenicity in Recombinant MVA

4. Utilizing Endogenous Poxviral Promoters to Enhance the Transgene Expression and in vivo Immunogenicity in Recombinant MVA

4.1 Introduction

As discussed in the thesis introduction, the use of a strong promoter has been linked to the enhancement of protein expression in many different expression vectors [142]. In poxviral vectors, it is essential to use a poxviral promoter [20] for transgene expression as the virus replicates in the cytoplasm and requires its own expressed transcription factors, not the cellular transcription machinery. When a reporter gene driven by the VACV p7.5 promoter was inserted into the cellular genomic DNA, into the nucleus, it was not transcribed by cellular enzymes, indicating that vaccinia transcription factors could not enter the nucleus, or if they did enter they could not interact with the cellular genomic DNA [146]. This is also because of the specificity of poxviral promoters to their respective poxviral transcription factors [145]. Poxviral genes, promoters, and transcription factors are divided into early, intermediate, and late classes, depending on their expression and involvement stages in poxvirus infections.

The use of optimal promoters to drive transgene expression is a well-known approach to enhance the cellular immunogenicity of T cell-inducing vaccine vectors such as MVA [61]. Conventionally, VACV vectors and vaccines were designed to utilise promoters like the p11, which is active at the late stage of the virus infection (late promoter) [20,147] or the p7.5 promoter to control transgene expression. The interest in the p7.5

promoter came from its ability to express protein at both early and late stages of vaccinia virus replication [150]. The vaccinia virus p7.5 promoter was used in fowlpox virus (FPV) to drive transgene expression. The transcription in FPV was initiated, at early and late element, and terminated at termination sequence similar to that of p7.5 in VACV, driving the same transgene [194]. This showed a genetic conservation as well as a universal approach of using poxvirus cross-strain promoters like p7.5. Similarly, the 4b late promoter from FPV (FP4b) shares genetic similarity to its VACV counterpart, utilises similar late element to that of VACV late core region, and is often used cross-strain in recombinant VACV and MVA [195]. For example, in this DPhil project, all rMVAs encode a GFP marker, inserted at the deletion III region, and driven by the FP4b. However, if the level of expression is very critical, a comparison should be performed to assess the activity of cross-stain exogenous promoter as a comparative study on the late p11 promoter driving the same transgene in either FPV or VACV revealed that VACV was able to express higher level of late transgenic protein [194].

The active core sequence of the p7.5 was dissected in a very comprehensive study, by Davison and Moss [162], in which they generated 99 VACV mutants by substituting the 33-nucleotides in the core sequence. Then, each mutant with a different mutated promoter was used to control the expression of *Beta-galactosidase* (β-gal), as a recombinant antigen, with or without AraC treatment. This study showed some absolutely crucial residues that prevent expression of the β-gal gene when substituted. This study was then used to establish the consensus core sequence of vaccinia early promoters. The same virologists dissected the late 28kDa promoter in the same strategy and described the consensus core region of vaccinia late promoters [163]. As

some VACV mutant increased the β-gal expression, this strategy was used later to optimise other early or late promoters. Therefore, a short synthetic promoter (SSP) was then produced by combining the optimal nucleotide of each residues of both early and late core sequences [160], which was also described by B. Moss' group [159]. Next, Wyatt *et al*, optimised the promoter of H5R ORF by two nucleotide substitutions, and introduced the modified H5 (mH5) promoter [61]. Although the mH5 was not compared to the original H5 promoter, its activity was superior to that of p7.5 and SSP, in driving β-gal expression. The mH5 showed stronger early activity whereas the SSP showed strong late activity. Several studies have reported the superiority of mH5 over the other two promoters in driving the expression of different antigens as well as enhancing cellular immunogenicity, which is summarised in table 4.1. In one of these studies, Hopkins *et al*[141] reported an extensive study on using three different promoters in MVA, testing the p7.5, mH5, SSP. They found that there was not always a correlation between *in vitro* expression and *in vivo* cellular immunogenicity, especially with the SSP. The SSP had the strongest *in vitro* activity, but the use of the mH5 promoter to drive transgene expression induced the highest magnitude of transgenic epitope-specific $CD8^+$ T cells.

There is a preference to use early poxviral promoter to enhance $CD8^+$ T cell responses that came mainly from promoter studies such as the above by Hopkins *et al* [141] or Wang *et al* [142]. An early report by Coupar *et al* (1986) showed the importance of using an early promoter in VACV-based vaccine for priming and re-stimulating T cells [143]. Moreover, the majority of $CD8^+$ T cell-specific epitopes in VACV are products of early genes, controlled by early promoters, in both humans [196] and mice [113]. In

addition, based on a study that used MVA to infect human monocyte derived dendritic cells (DCs), which are professional antigen-presenting cells (APC), MVA continues to express its early genes with no late protein expression. In an early report, priming cytotoxic T cells by dendritic cells were only reported when a transgene expression in DCs was controlled by an early promoter [144]. The early transcription was also reported to occur very late in the infection at the viral assembly stage, which support the use of early promoters [157].

In the light of this, it could be explained why the SSP was the optimal promoter *in vitro* but was second to mH5 in terms of *in vivo* cellular immunogenicity, in Hopkins *et al* study. The SSP late activity was much stronger than its early activity, a finding that had also been shown before by other reports [67,160]. This would also explain the high cellular immunogenicity achieved with the mH5, but not with the SSP, because of the mH5 strong early activity. Therefore, designing MVA-based T cell-inducing vaccines should take into consideration the use of early or early/late promoters.

In this chapter, first, I attempted to study conventional promoters (the p7.5, mH5, and SSP) to conclude which one of these is the optimal in terms of cellular immunogenicity. Several studies, summarised in table 4.1, have compared two or all of these three promoters with different antigens either *in vitro*, *in vivo*, or both. Hopkins *et al*[141] have reported the most extensive study on comparing these promoters; however, examining the early activity of promoters was missing. Hence, I ensured to assess the early activity of promoters by treating cells with AraC. Moreover, the mH5 sequence was different and shorter in Hopkins *et al* study, which also encouraged repeating the comparison study with the long mH5 sequence, reported originally by Wyatt *et al*[61]. In addition, as the

optimal promoter would be used as the benchmark comparator control to search for a stronger MVA promoter, I preferred to replicate the comparison study with our tPA-pb9-rLuc reporter transgene before proceeding further and testing endogenous promoters with the same reporter transgene.

Study	Technique	Insert	Promoter	Insert expression(1)		% Early activity (2)	Fold in early activity (3) (mH5 vs. others)	Immunogenicity (in BALB/c)
				AraC+ Early	AraC- Total			
Wang et al. [142]	Semi-quantitative W.B.	pp65 (CMV)	p7.5	Not done				
			mH5	0.90	2.25	40%		NA
			SSP	0.13	2.23	6%	7-fold	
Wyatt et al. [67]	β-Gal activity	*LacZ*	p7.5	0.21	0.535	39%	5-fold	NA
			mH5	1.1	2.6	42%	NA	
			SSP	0.32	8.24	4%	3-fold	
Hopkins et al. [141]	Immuno-fluorescence	Pk tag	p7.5	Not done	~28		NA	mH5 is stronger **(4)**
			mH5	Not done	~258		NA	
			SSP	Not done	~270		NA	
This DPhil	Luciferase activity	pb9-rLuc	p7.5	22827225	73882325	30%	3.5-fold.	Significant 1.7-fold
			mH5	78702975	183750000	42%	NA	NA
			SSP	39475725	1160000000	03%	2-fold	Not significant 1.3-fold

The mH5 promoter sequence used by research groups		
Study	The mH5 sequence	Notes
Original H5 promoter	AAAAAATGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAATT-38	-38 to AUG of H5R
Wyatt et al. [67]	AAAAAtTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAATA-38	-38 to the AUG
Our study	AAAAAtTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAATAATCATAAATA-56	-56 to the AUG of pb9-rLuc
Hopkins et al. [141]	Aaaaattgaaattttatttttttttttttggaatataaata-17	-17 to the AUG of HIVA

Table 4.1: *In vitro* and *in vivo* comparison of the p7.5, mH5, and SSP promoters by different studies

Top table: Different groups assessed poxviral promoter activity, using different transgenes (inserts) and techniques. **(1)** Early (AraC+ treated samples) and overall (untreated samples) expression of different inserts is presented. **(2)** Percentage of promoter early activity is presented by dividing values in AraC+ over AraC-. **(3)**A more reliable and frequently used calculation is the fold difference in promoter early activity. This shows the fold of early activity differences between two viruses, without normalising to the total amount of expression (used by Wang et al. [142] and Wyatt et al. [67]). **(4)** Immunogenicity was not done in most studies, here, only Hopkins et al. [141] showed that compared to either p7.5 or SSP, the mH5 promoter enhanced statistically significant difference in terms of cellular responses to H epitope in HIVA vaccine.

Bottom table: The sequence of mH5 promoter used in different studies. The mH5 sequence was different and shorter in a study by Hopkins *et al*[141].

Promoter	Sequence	Length (bp)
pB8	tcaacgatgatactctattgagaaaacatactcttaaaat tggatctacttatatatttgatcgtcatggacatagtaat acatattattcaaaatatgattttaaaaatttaaaatat attatcacttcagtgacagtagtcaaataacaaacaacac cATG	162
pE3*	cctccagaatctccagaaccagcatcacctatagatgacg ggttaatcagatccacattcgatagaaacgacgaaccacc agaggatgatgaataaaaaaatgataaaataaattagttt tattgctggttgtgttagttctctctaaaaATG	150
pF11*	tgccgagcattcatttactcaattatggaaaccatagatc cggtgtatatatctcagttcagttataaagaattatacgt tagtagctcttataaagatattaatgaatccatgagtcag atggtaaaattataaaaagtgaaaaacaatattattttta tcgttggttgttacactATG	180

Table 4.2: Sequences of ectopic promoters

Nucleotides upstream of the ATG start codon of the transgene in the rMVA with endogenous promoters were amplified along with the transgene (tPA-pb9-rLuc), and inserted into the TK locus of a wild type MVA-BAC construct. The core promoter regions are underlined. ***** These two ectopic promoters were inserted at the TK locus in the opposite direction of their endogenous counterpart.

The search for the optimal vaccinia virus promoter has not ended, and it focuses on either finding novel strong promoters or optimising the existing ones. In terms of the latter, chapter 6 will focus on optimising vaccinia promoter whereas this chapter presents a search for novel and strong MVA endogenous promoters. The approach of searching for a stronger endogenous promoter involves the replacement of MVA non-essential or fragmented ORFs, with a transgene exactly at the ATG start codon, without changing the upstream sequence. It provides the advantage of studying the promoter at its natural context without any concern over homologous recombination, which although very rare, it might occur between two homologous copies of promoters such the p7.5. However, this is not suggesting that the recombination between the exogenous p7.5 at the TK (thymidine kinase gene) and the two copies of native p7.5, driving *C23L* and *B29R* ORFs, is a major issue. Hundreds of MVA-based vaccines that utilised p7.5 have been produced and tested in preclinical and clinical trials without any reported issues on genetic stability (observation by scientists at the Jenner Institute). A more important objective of studying endogenous promoters is to create new insertion loci at the sites of the endogenous promoters in MVA. This would be of great value for developing a multivalent MVA vaccine for complex pathogens such as *Plasmodium falciparum* or human influenza where a single antigen might not be sufficient to induce protective immunity and a multi-antigen vaccine will be soon in much more demand. The use of poxviral endogenous promoters was first described by Panicali and Paoletti in the early 1980s [49,52]. It was then tested in a recombinant vaccinia virus encoding multiple vaccines against multiple pathogens [56]

However, the technology of the time was not able to precisely utilise the endogenous promoters without containing additional nucleotides, which result in the upstream sequence being transcribed and fused to the recombinant protein. Recently, in a published paper, we improved the search for endogenous promoters by using MVA-BAC recombineering technology to precisely insert transgenes, and replace MVA ORFs, at the ATG start codon and use endogenous promoters to drive transgene expression without any changes to the transgenic proteins [156]. We described the use of pB8, pF11 promoters as being similar to, or better than, the p7.5 promoter and SSP in terms of protein expression and *in vivo* immunogenicity.

Here, I extend our work on the endogenous promoters with the following aspects. First, examining the optimal conventional promoter and use it as a benchmark comparative control for the rest of the thesis work on promoters. Second, examining the use of pE3 promoter in addition to retesting of pB8 and pF11 promoters. Third, determining the transgene expression and immunogenicity achieved with the pB8, pE3, and pF11 ectopic promoters in contrast to their endogenous counterparts.

In this chapter, rMVAs that drive the tPA-pb9-rLuc were made in four panels, explained with a schematic representation (Figure 4.1). First, rMVAs with the p7.5, mH5, or SSP conventional promoters, driving the transgene at the TK locus.The mH5-MVA was made for this DPhil project and was compared to p7.5-MVA and SSP-MVA that were previously made by Orubu *et al* [156].Second, rMVAs with endogenous promoters, pB8, pE3, or pF11, driving the transgene at their loci.The B8-MVA and E3-MVA were

made for this DPhil project and the F11-MVA was previously made by Orubu *et al* [156].Third, rMVAs with ectopic promoters, pB8, pE3, or pF11, driving the transgene at the TK locus. These were named; eB8-MVA(ΔTK), eE3-MVA(ΔTK), and eF11-MVA(ΔTK), and were all made for this DPhil project. Fourth, rMVAs with endogenous promoters, pB8, pE3, and pF11, driving the transgene at their loci, with the TK locus disrupted by insertion of the fluorescent marker gene, mCherry. These were named; B8-MVA(ΔTK), E3-MVA(ΔTK), and F11-MVA(ΔTK), and were all made for this DPhil project. Briefly, the endogenous promoters were utilised by replacing their native ORFs with the transgene exactly at the ATG start codon in a wild type MVA-BAC construct. These were then used as parental viruses to insert the mCherry gene into the TK locus. The ectopic promoters were made by amplifying 150 to 180 nucleotides upstream of the ATG of the *B8R*, *E3L*, and *F11L* ORFs along with the transgene and inserted into the TK locus of a wild type MVA-BAC construct (table 4.2), explained in depth in the materials and methods.

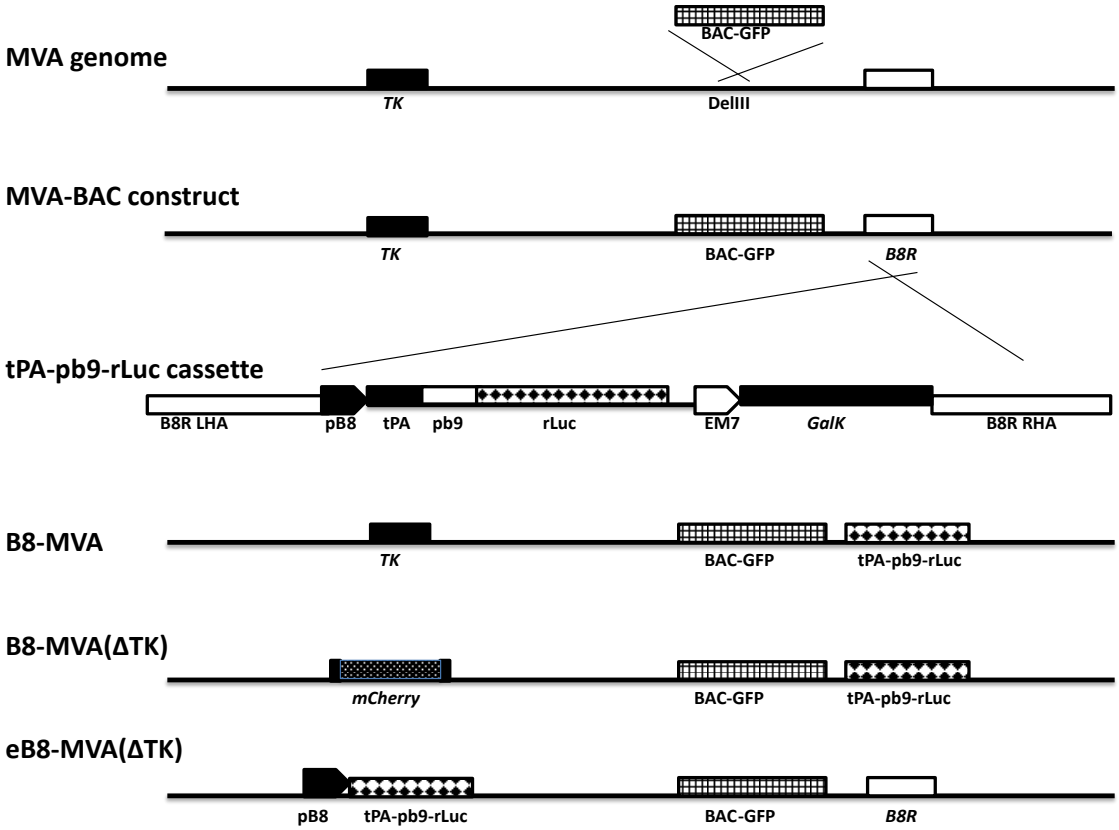


Figure 4.1: Schematic representation of different rMVAs used in this project, with *B8R* locus and pB8 promoter as examples.

Top: **MVA genome,** a gene of green fluorescent protein (GFP), driven by FP4b poxviral promoter, and a bacterial artificial chromosome (BAC) are inserted into the deletion 3 (DelIII) region of MVA genome, resulting in **MVA-BAC construct.** The **tPA-pb9-rLuc cassette** (transgene) is flanked with the *B8R* left (LHA) and right (RHA) homology sequences. Recombineering was performed to insert the transgene into MVA-BAC at the *B8R* locus, using its endogenous pB8 promoter, resulting in **B8-MVA.** A red fluorescent protein (mCherry) was inserted into the he thymidine kinase (TK) locus of B8-MVA to inactivate the TK activity, resulting in **B8-MVA(ΔTK)**. The transgene were then amplified from the B8-MVA along with the pB8 promoter and flanked with TK homology sequences, and inserted at the TK, resulting in **eB8-MVA(ΔTK)**. The "e" indicates ectopic promoter. The same approach was used with pE3 and pF11L. The tPA-pb9-rLuc transgene, used in all rMVAs to test various promoters, consists of *Renilla* luciferase (rLuc), tPA leader sequence, and pb9 (an immunodominant H2-K^d-restricted $CD8^+$ T cell epitope from *Plasmodium berghei* circumsporozoite protein). It also contains *GalK* selection marker controlled by EM7 bacterial promoter.

4.2 Results

4.2.1. Conventional MVA promoter as a comparative control

It was important to determine the best MVA conventional promoter to use as a comparative control in our experimental setting, before constructing a range of different rMVAs with different endogenous promoters to enhance the transgene expression. The p7.5, mH5, and the synthetic early late (SSP) promoters, which have been extensively used in recombinant vaccinia viral vectors, were selected from the literature for being strong promoters. Each one of these promoters was utilised to drive the reporter transgene (tPA-pb9-rLuc) inserted at the thymidine kinase (TK) locus as explained in the materials and methods (viruses were named: p7.5-MVA, mH5-MVA, and SSP-MVA). BHK-21 cells were infected with these rMVAs and either treated with AraC or left untreated. The total luciferase was measured (as explained and performed in chapter 3) to determine early promoter activity, in AraC treated cells, or the overall promoter activity, in untreated cells. The SSP promoter drove the highest *in vitro* luciferase expression in the untreated cells, which indicates its strong activity, followed by the mH5 promoter. In contrast, SSP-driven luciferase dropped below the level of the mH5-expressed luciferase when AraC was used to block the DNA replication (Figure 4.2 A). This indicates the strong late activity of the SSP promoter, showing also the strong early activity of mH5 promoter. The p7.5 promoter; however, drove lower expression compared to mH5 or SSP with or without the AraC treatment.

Next, these rMVAs were tested for cellular immunogenicity in BALB/c mice. Seven days after a single injection of $1x10^6$ pfu rMVA, IFN-γ secreting splenic $CD8^+$ T cells was determined using intracellular staining (ICS) and flow cytometry and using the pb9

transgenic peptide. The *in vivo* mouse immunogenicity of pb9 was enhanced when the mH5 promoter was used to a level higher than with p7.5 and SSP (Figure 4.2 B). The immune responses were statistically higher in the mH5-MVA group as compared to the p7.5-MVA, which replicates results from various groups in the literature (table 4.1). Although this difference was statistically significant, it is smaller than what have been previously reported (see table 4.1). This could be due to immune "break-point" where high doses such as 10^6 pfu might be very strong to allow for the real differences to be detected. Therefore, bigger differences might be detected by the use of smaller doses such as 10^{3-5} pfu that can best detect the real differences driven by used promoters. Immune responses elicited by the SSP-MVA were lower than mH5-MVA, even though not statistically significant, these responses did not correlate with the strong SSP activity *in vitro.* This result supports the notion that early vaccinia expression enhances cellular immunogenicity, particularly that the mH5 promoter is stronger at early expression.

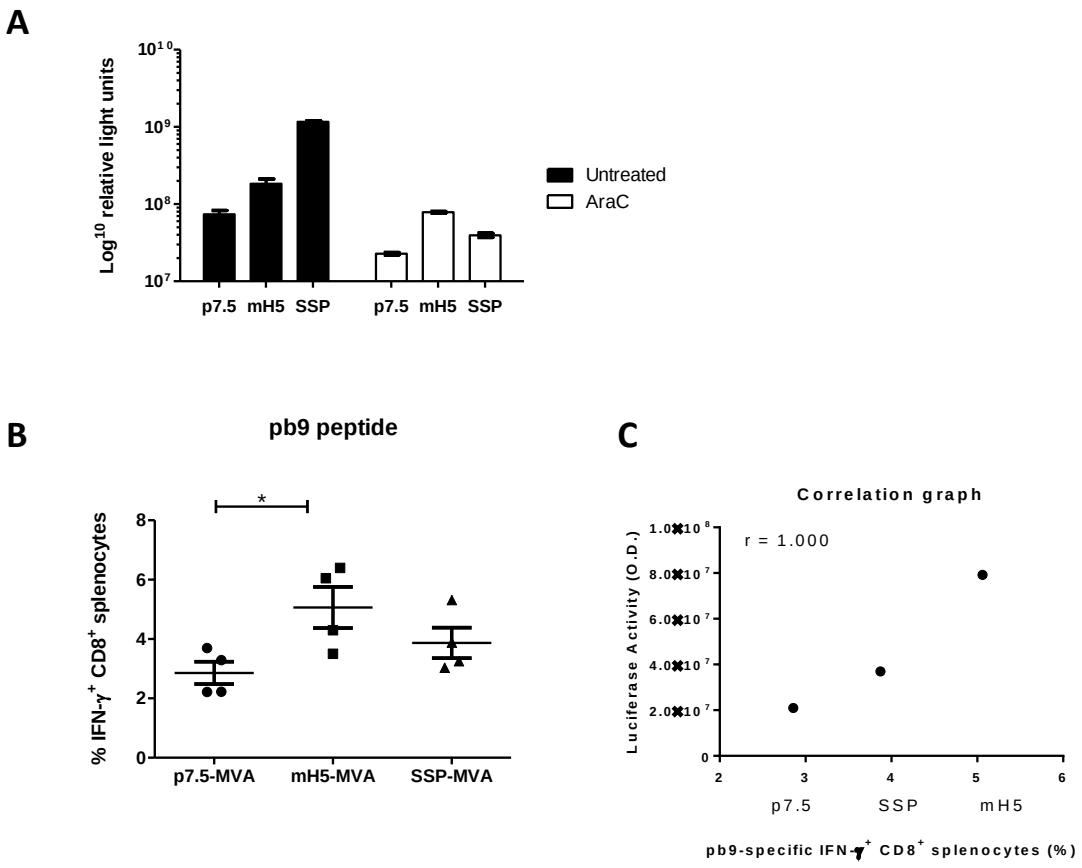


Figure 4.2: Transgene expression and immunogenicity of rMVAs with different conventional promoters.

(A): BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene controlled by the p7.5, mH5, or SSP promoters, at MOI of 1. Cells were either treated with AraC (closed bars) to assess the early expression or without AraC treatment (open bars) for promoter overall activity. 24 h.p.i, cells were lysed, and added to supernatants and the total level of luciferase expression was measured using *Renilla* luciferase system to determine promoter activity. Data, shown in logarithmic scale as relative light unit, represent two independent experiments. **(B):** Same viruses were used to immunise three groups of female BALB/c mice (n=4), i.m. at 10^6 pfu. Seven days post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in response to in vitro re-stimulation with pb9 peptide. These values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with the SEM error bars are shown. Data is representative of two independent experiments. *****$p< 0.05$, significant by One-way ANOVA with Dunnett's post-test. **(C):** Mean of values from graphs A (AraC treated) and B are plotted against each other to show the correlation between early promoter activity *in vitro* and *in vivo* cellular immunogenicity. The correlation coefficient (r) is 1.000, which indicates perfect correlation using nonparametric Spearman correlation test.

4.2.2. The cellular immunogenicity of rMVA with p7.5 or mH5 promoter in outbred mice

The comparison of p7.5 and mH5 promoters has been presented in a number of research articles. This comparison is often reported in favour of mH5 being stronger in terms of protein expression as well as the elicited cellular immunogenicity (Summarised in table 4.1); some of these studies have also included the early late short synthetic promoter (SSP). Our data (Figure 4.2) also agree with the conclusion that mH5 is the stronger promoter, which contributes to higher immunogenicity. However, vaccines, in general, should ultimately be tested in, and designed for, humans or animals that are very heterogeneous and outbred. We, therefor, wanted to compare those two most studied promoters (p7.5 and mH5) in the outbred experimental animal. The SSP promoter was not included in the outbred animal study as it is often disregarded in favour of mH5 when it comes to using strong early expression and *in vivo* cellular immunogenicity (see table 4.1). CD-1 outbred mice were selected for immunisation with either p7.5-MVA or mH5-rMVA driving the same tPA-pb9-rLuc transgene. CD-1 mice are the most widely used outbred mice, purchased from Harlen Laboratories [197]. Two groups of 12 mice were immunised at a dose of 10^6 pfu rMVA and were sacrificed one week later and spleens were collected. A pool of 85 overlapping peptides with 11 amino acid length, offset by 4 amino acids, covering the transgenic antigen (pb9-rLuc) was synthesised (by Mimotopes) and used in ICS to assess the cellular immune responses elicited by these rMVAs. There was no difference between the groups as tested in either ICS (Intracellular cytokines staining) or ELISpot (Figure 4.3). Although the peptide length was 11 amino acids, designed to increase the chance of MHC class II presentation, no $CD4^+$ T cells recognised the peptide pool in the ICS experiment. This could indicate the absence of genuine MHC-II restricted peptides

in the peptide pool, which could induce $CD4^+$ response. Interestingly, the ELISpot result was in a similar pattern to that of ICS. This experiment was performed twice, and did not detect differences between the promoters in the outbred mice population. However, based on the immunogenicity data (in BALB/c mice) as well as its strong expression profile, mH5-MVA was selected to be a comparative promoter control in the rest of this DPhil promoter studies.

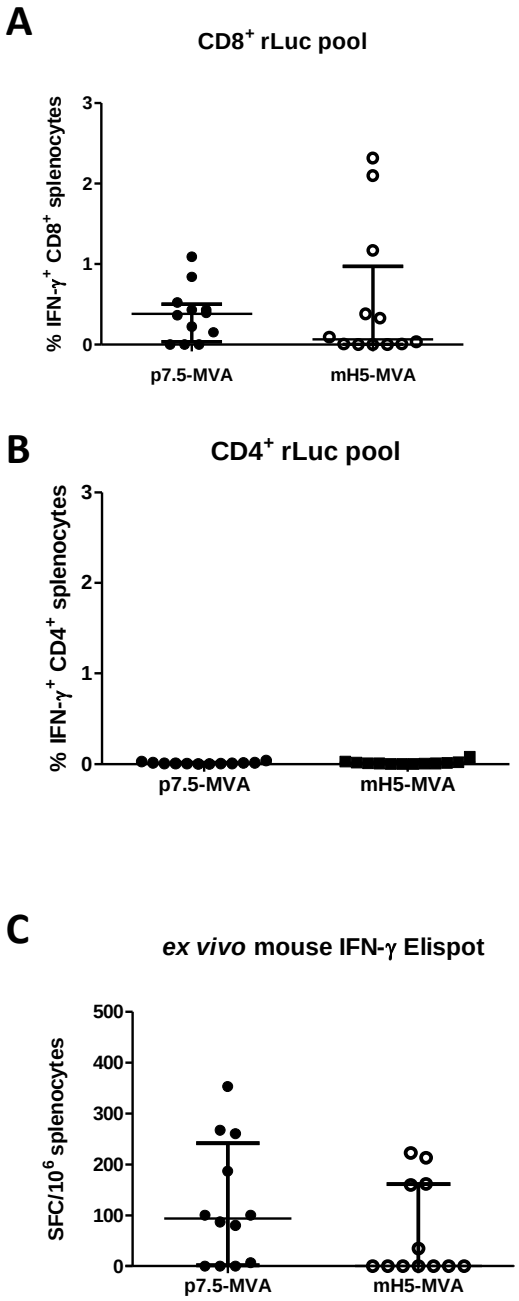


Figure 4.3: *In vivo* cellular immunogenicity of rMVAs with different conventional promoters in CD-1 outbred mice.

Two groups of female CD-1 mice (n=12) were immunised with rMVAs with either p7.5 or mH5 promoter driving the tPA-pb9-rLuc transgene expression. Seven days post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T **(A)** or $CD4^+$ T **(B)** splenocytes in response to *in vitro* re-stimulation with a peptide pool covering the transgenic antigen. These values are presented, with the median of each group with interquartile ranges, after subtracting the values of unstimulated cells for every mouse (sample). **(C):** The same peptide pool was used to determine IFN-γ-secreting cells in *ex vivo* ELISpot. The median of each group with interquartile range is shown. Data is representative of two independent experiments.

4.2.3. The luciferase expression profile controlled by different endogenous promoters

Two rMVA viruses were derived with the endogenous pE3 and pB8 promoters utilised at their natural sites to drive the transgene (tPA-pb9-rLuc) by replacing the original ORF of *E3L* or *B8R* as explained in the materials and methods (chapter 2) and as presented in the schematic representation (Figure 4.1). These two rMVAs were tested beside the F11-MVA, using the pF11 endogenous promoter, made previously in the Jenner institute by Orubu *et al*[156]. The mH5-MVA and p7.5-MVA were also included as comparative promoter controls. The luciferase assay was performed as described in chapter 3. The expression controlled by p7.5 and mH5 was similar to what we observed before confirming mH5 superiority. All of the three rMVA with endogenous promoters (pB8, pE3, and pF11) showed similar or lower expression of the transgene compared to mH5-MVA in early expression condition controlled by AraC treatment. In addition, without AraC treatment, the expression profile did not significantly change although the p7.5 and mH5-MVA exhibited slight increases presumably due to their late activity. The activity of the pE3 and pF11 was very similar to their activity in the early expression condition, which indicates that they are almost exclusively early promoters (Figure 4.4).

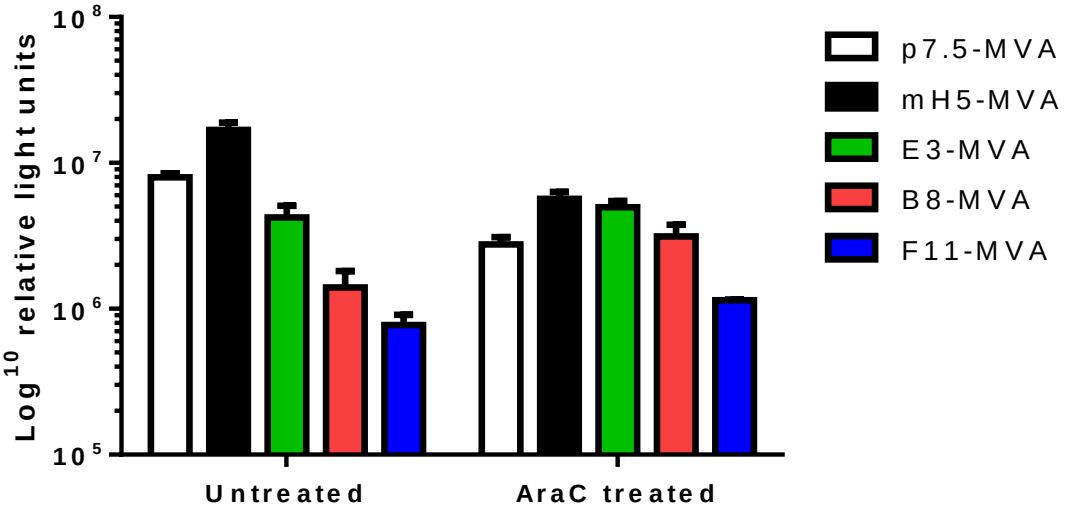


Figure 4.4: Luciferase expression of rMVAs with different endogenous promoters.

BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of different endogenous promoters at MOI of 1. Cells were either treated with AraC (to assess the early expression) or left untreated (for promoter overall activity). 24 h.p.i, cells were lysed, and added to supernatants and the total level of luciferase expression was measured to determine promoter activity, using *Renilla* luciferase system. P7.5-MVA and mH5-MVA were included as positive controls. The data, shown in logarithmic scale, represent the mean of 4 wells with SD error bars. Data is representative of more than five independent experiments.

4.2.4. *In vivo* cellular and humoral immunogenicity of rMVAs with different endogenous promoters

The *in vivo* immunogenicity elicited by the rMVAs with different endogenous promoters was tested by vaccinating four BALB/c mice per group, with 10^6 pfu rMVA/mouse. Mice were sacrificed after seven days, and spleens were collected. The cellular immune responses were measured by intracellular cytokines staining using pb9, a CD8-restricted transgenic epitope and vector-specific F2(G) and E3 epitopes (Figure 4.5). Interestingly, the pb9 immunogenicity confirmed the observed correlation between the *in vitro* expression and the *in vivo* immunogenicity shown for p7.5-MVA and mH5-MVA in figure 4.2 (as compared to figure 4.5 E). Strikingly, I did not observe that correlation with any of the endogenous promoters (Figure 4.5 E). In fact, F11-MVA, which showed the lowest expression profile *in vitro* (see figure 4.4), resulted in the highest *in vivo* immunogenicity (Figure 4.5). This immunogenicity was significantly higher than the "bench mark" mH5-MVA control. Therefore, I presented the ratio of the $CD8^+$ T cell responses to the transgenic peptide over the responses to the vector-specific peptide, an approach often used to assess the effectiveness of a promoter in improving the immunogenicity (Figure 4.5 D). This approach was used by Baur *et al* for their improved tandem promoter pHyb [155]. F11-MVA showed a significantly higher ratio than both of the controls, mH5-MVA and p7.5-MVA.

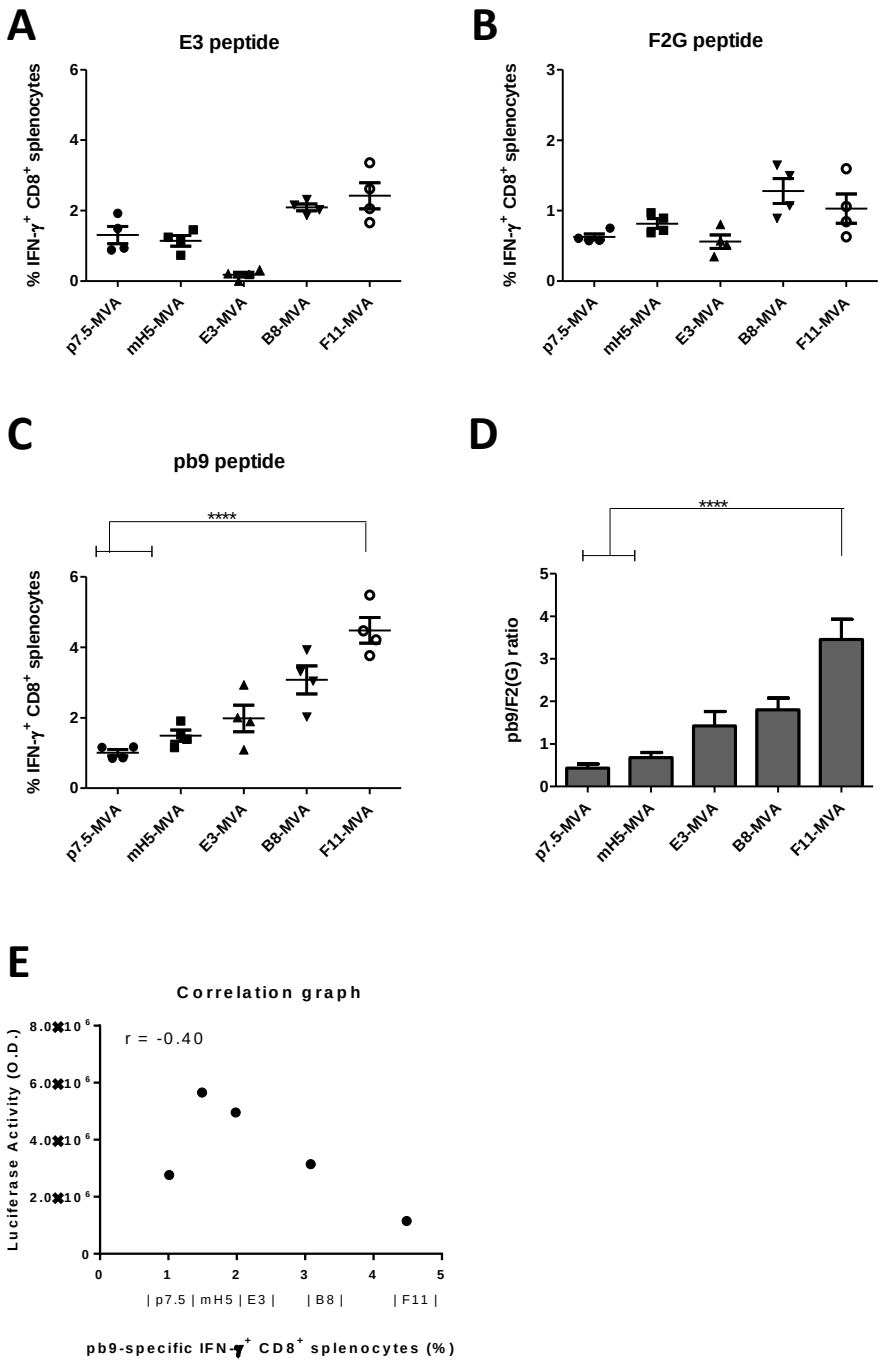


Figure 4.5: *In vivo* cellular immunogenicity of rMVAs with different endogenous promoters.

Five groups of female BALB/c mice (n=4) were immunised with the respective rMVAs and seven days post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in response to *in vitro* re-stimulation with MVA-specific peptide (A and B), or with pb9 peptide (C). The ratio of the transgenic peptide (pb9) response over the F2(G) vector-specific peptide response was calculated and presented (D). All values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with SEM error bars are shown. Data is representative of two independent experiments. **** $p = 0.0001$, significant by One-way ANOVA with Bonferroni's Multiple Comparison Test. **(E):** Mean of values from graphs C are plotted against mean of values from figure 4.4 (AraC treated) to test the correlation between early promoter activity *in vitro* and *in vivo* cellular immunogenicity. The correlation coefficient (r) is – 0.40, which indicates lack of correlation using nonparametric Spearman correlation test.

On the other hand, although the aim of this project is to look at different ways of improving cellular immunogenicity of rMVA, it was also of interest to determine the antibody responses when different promoters are used. The Luciferase immunoprecipitation system (LIPS) was performed using a lysate that expressed the luciferase protein upon transfection with the tPA-pb9-rLuc. This assay did not detect any mice anti-Renilla luciferase serum antibodies from any vaccinated group (Figure 4.6). LIPS is designed to indirectly report the binding activity of antibodies by measuring the substrate binding to the luciferase that is fused to an antigen (see materials and methods); however, here the luciferase was used as an antigen as well as the reporting agent at the same time. The serum antibodies as well as the substrate should bind to the same luciferase. So, this might inhibit each other binding in the LIPS or might affect the sensitivity of the assay (see LIPS assay diagram, Figure S7.2, Appendices).

An ELISA assay was therefore set up to accurately measure the antibody responses in mice sera, using a recombinant *Renilla* luciferase. The result of the ELISA confirmed the LIPS assay and none of these rMVAs was able to induce detectable serum antibodies after one vaccination dose of rMVA (Figure 4.6). The anti-rLuc commercial antibody detects the recombinant *Renilla* protein, and it was also able to detect rLuc expressed by viruses like mH5-MVA and B8-MVA, in Western blot (Figure 3.3, chapter 3). Thus, the lack of detectable level of anti-rLuc antibody in mice sera could be due to lack of humoral immunogenicity of a single shot of 10^6 pfu MVA. The induction of antibodies by MVA vector is often reported to be dependent on the target antigen, doses, and vaccination regimens.

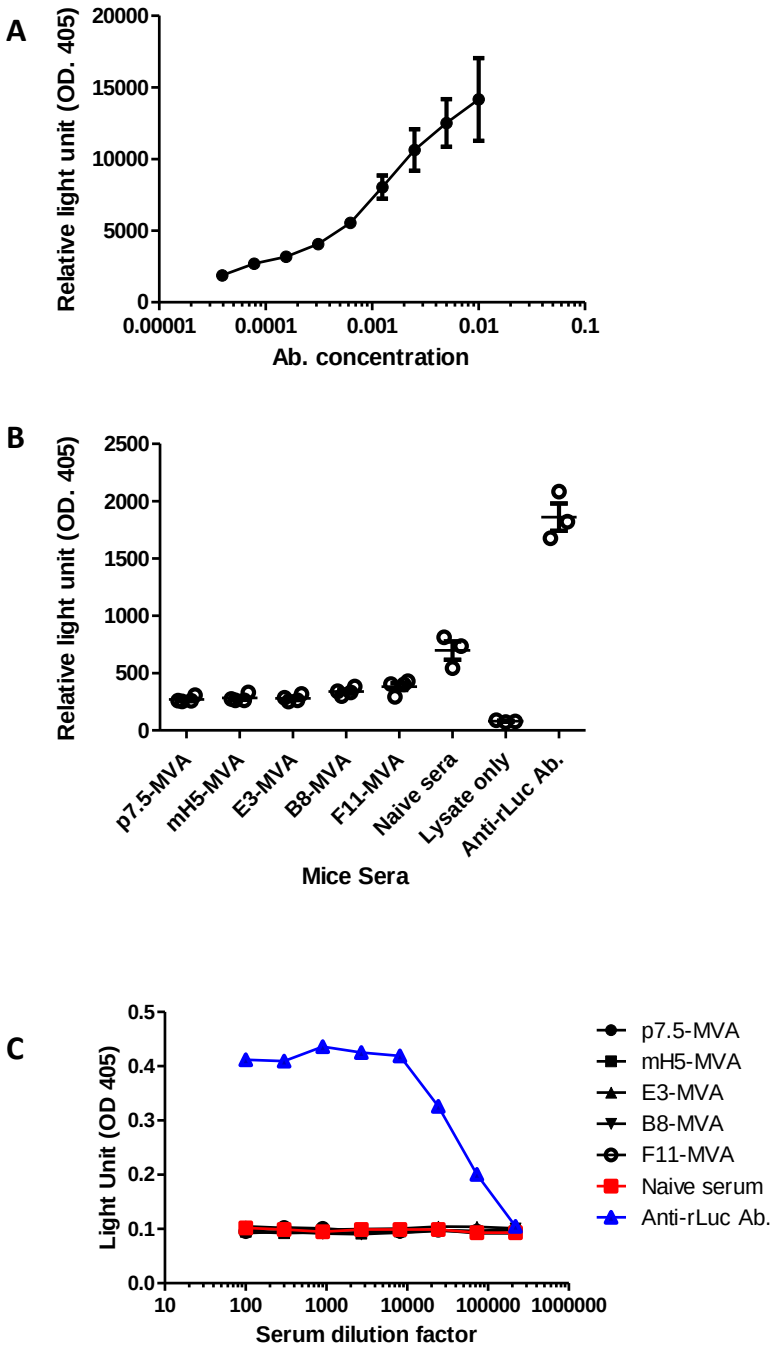


Figure 4.6: *In vivo* humoral immunogenicity of rMVAs with different endogenous promoters.

Five groups of female BALB/c mice (n=4) were immunised with the respective rMVAs and seven days post immunisation, the sera were collected and used to assay mice sera anti-rLuc antibodies. **(A):** LIPS assay was performed to show the commercial monoclonal Anti-rLuc antibody binding activity in this assay over serial dilution. **(B):** The commercial Anti-rLuc antibody was used as a positive control (blotting only the lowest concentration) to test mice sera from the respective mice groups in LIPS assay. The mean and SEM error bars are shown. **(C):** ELISA was performed to accurately measure antibody responses in mice sera from the respective groups. Values, in ELISA, are the mean of triplicates for every dilution of every sample; the average of 4 mice's mean values is presented here. Anti-rLuc antibody was used as a positive antibody control and sera from unvaccinated mice were pooled and used as a negative antibody control. Data is representative of two independent experiments.

4.2.5. Utilising endogenous promoters at the TK as an exogenous insertion site

The endogenous promoters proved efficient in driving early expression and eliciting stronger *in vivo* immunogenicity compared to the mH5 promoter. So, it was interesting to determine whether the activity of these promoters would be different when used out of their natural existence at a different locus such as the TK gene, i.e. used as ectopic promoters.

Three rMVAs were derived with the tPA-pb9-rLuc transgene inserted at the TK locus along with the sequences of pB8, pE3, or pF11 promoters. This was made by amplifying the transgene along with 150 to 180 nucleotide sequences upstream of the transgene's ATG start codons, which are originally the start codon for *B8R*, *E3L*, or *F11L* loci, in the B8-MVA, E3-MVA, and F11-MVA. These sequences contain the early promoter core region of each promoter, according to Yang *et al*[198]. The amplified sequences were then inserted into the TK locus of a wild type MVA-BAC construct, using MVA-BAC recombineering as explained in the materials and methods. This resulted in rMVAs with the ectopic pB8, pE3, and pF11 (named eB8-MVA(ΔTK), eE3-MVA(ΔTK), and eF11-MVA(ΔTK)), inserted into the TK locus. It also resulted in an inactivated TK gene. I therefore controlled the experiment by disturbing the TK locus in rMVAs with endogenous promoters (i.e. B8-MVA, E3-MVA, and F11-MVA) by insertion of mCherry marker gene into the TK locus. This resulted in three new rMVAs, B8-MVA(ΔTK), E3-MVA(ΔTK), and F11-MVA(ΔTK), which express mCherry fluorescent marker at the TK locus.

After the TK was inactivated, the rMVAs with ectopic versus endogenous promoters were, then, tested for their expression profile in the luciferase assay. None of the three tested ectopic promoters showed significant different expression compared to their endogenous counterparts (Figure 4.7). This suggested that the promoters have similar activity and drive the transgene expression regardless of the insertion site.

Although the minimum promoter sequence was not established for any of the tested promoters, the long sequences of 150 to 180 nucleotides upstream the ATG start codon, which was extended beyond early promoter element (table 4.2), did not seem to have any impact on promoter activity.

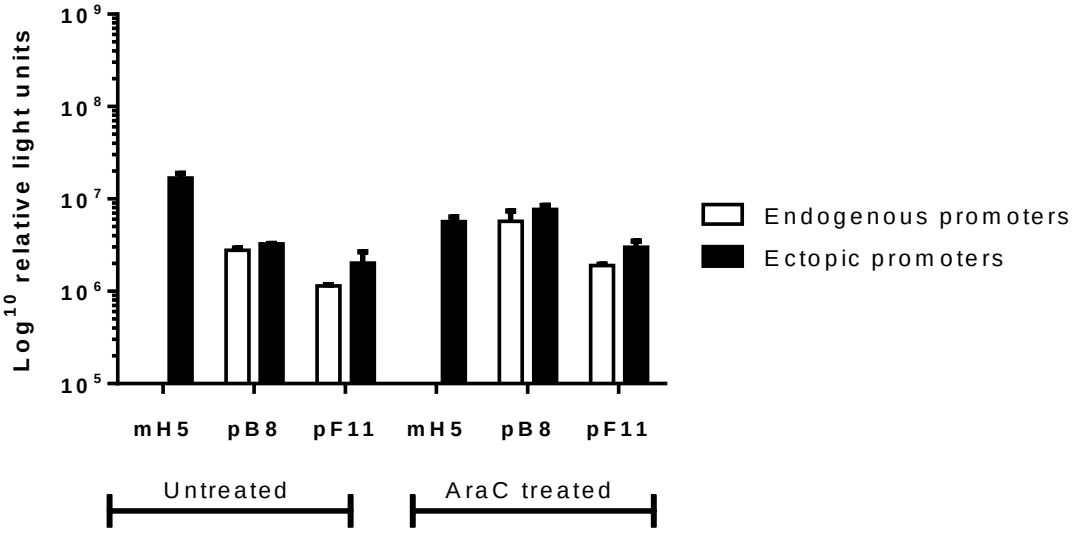


Figure 4.7: Activity of different endogenous and ectopic MVA promoters.

BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of endogenous promoters at their natural loci (open bars), or these promoters at the TK locus, i.e. ectopic promoters (closed bars) at MOI of 1. Cells were either treated with AraC or left untreated as indicated. 24 h.p.i, cells were lysed, and added to supernatants and the total level of luciferase expression was measured to determine the promoter activity, using *Renilla* luciferase system. The data, shown in logarithmic scale, represent the mean of 4 wells with SD error bars. Data is representative of three independent experiments.

Next, the *in vivo* immunogenicity of these rMVAs with ectopic or endogenous pB8, pE3, and pF11 promoters was performed as explained in the material and methods. Every rMVA pair, with the same promoter, showed similar $CD8^+$ T cell responses as measured by the responses to the pb9 peptide (Figure 4.8). This experiment was performed twice and the mH5-MVA was included as a comparative control. The rMVA with either ectopic or endogenous promoters, especially pE3 and pF11, showed significant increase in the $CD8^+$ T cell responses as compared to mH5-MVA (by one way ANOVA). The ectopic promoters resulted in similar cellular immunogenicity to their endogenous counterparts. This suggests that the promoters themselves are the driving mechanism behind the improved immune responses rather than their location in the MVA genome.

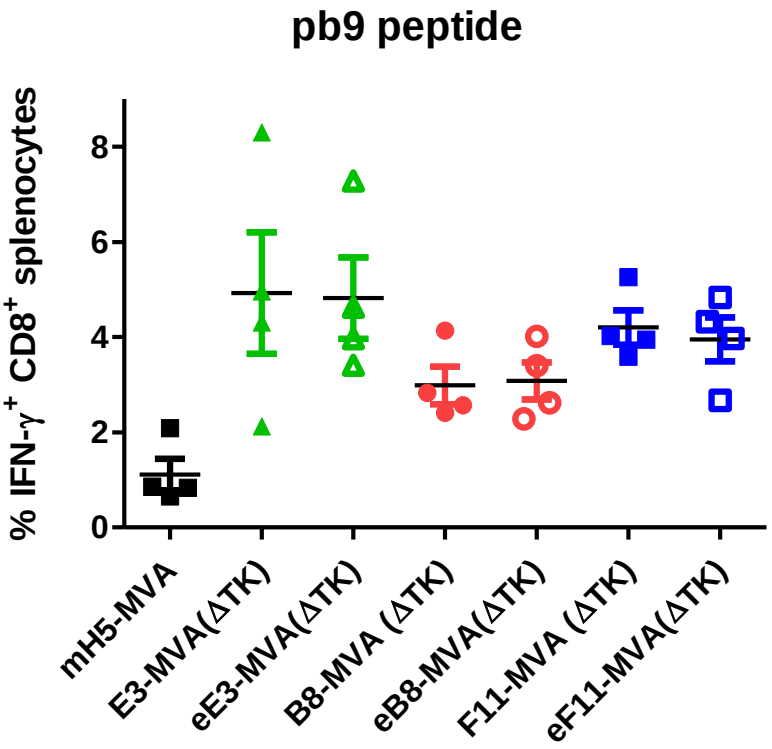


Figure 4.8: *In vivo* cellular immunogenicity of rMVAs with different endogenous and ectopic promoters.

Six groups of female BALB/c mice (n=4) were immunised with the respective rMVA with different endogenous promoters (with disrupted TK gene) or with ectopic promoters at the TK locus (resulted also in disrupting TK gene). Seven days post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in response to *in vitro* re-stimulation with pb9 peptide. All values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with SEM error bars are shown. Data is representative of two independent experiments. All group are statistically significant as compared to the mH5-MVA, except B8-MVA(ΔTK) and eB8-MVA(ΔTK), as tested by One-way ANOVA (with Bonferroni's post-test).

4.3 Discussion

Promoters in poxviral vectors could be important for designing the optimal vector for a given vaccine. Here, I attempted a study to define the best conventional promoter that can be used as a benchmark comparative control for searching for a stronger MVA promoter. The most studied conventional promoters are the p7.5, modified H5 (mH5), and the short synthetic promoter (SSP). The three promoters have early and late core sequences, and were able to drive high level of tPA-pb9-rLuc transgene expression. The SSP promoter was the strongest of the three, especially in the overall expression where its late element should be active. It has been reported that the SSP has a very strong late activity [67,142,159]. It can also be seen from table 4.1 that using different antigen, the SSP showed its superior late, and overall, activity. However, treating cells with AraC should block vaccinia late gene expression, under which conditions, the mH5 promoter was the strongest of the three, which is also consistent with some other studies showing that mH5 has stronger early activity [67,142] than the p7.5, summarized in table 4.1. The cellular immunogenicity study also showed that using mH5-MVA elicited statistically significantly more immune responses as compared to using p7.5-MVA or SSP-MVA. So, mH5 enhancement of *in vivo* immunogenicity can be assumed to result from its strong early activity because the early activity hierarchy *in vitro* is consistent with *in vivo* immunogenicity. This supports other studies in the relevance of early promoters for enhancing cellular immune responses [143]. Yet, the reason behind the enhanced immunogenicity of early-expressed antigen is not understood. Some found that antigen presentation on MHC-I molecules is blocked in the late stage of VACV infection although this can be antigen-specific effect [199].

Therefore, the mH5 promoter was selected to be the benchmark comparator control for the rest of promoter studies in this thesis. However, all vaccines produced by the Jenner institute, Oxford University, utilised the p7.5. Therefore, any improvement over the mH5 should also be an improvement over the p7.5. Yet, the comparison of p7.5 versus mH5 promoters in heterologous outbred mice population did not reveal any significant differences in terms of cellular immunogenicity. The outbred mice were used as the best available model to mimic the clinical trial where MVA-based vaccines are tested in humans. So, promoter comparison should ideally be conducted in clinical trials, should any promoter enhance vaccine immunogenicity.

Next, different MVA promoters were studied at their natural context, a.k.a. endogenous promoter. The *E3L* and *B8R* ORFs were replaced with the tPA-pb9-rLuc transgene, controlled by the endogenous promoters of those ORFs. The two promoters were then compared to the mH5, but also to the previously made pF11, of the *F11L* ORF, driving the same transgene. None of these endogenous promoters (pE3, pB8, and pF11) was able to improve the *in vitro* luciferase expression, and the mH5 remained the strongest promoter. However, testing these rMVAs in mice revealed that all endogenous promoters contribute to similar or enhanced $CD8^+$ T cell responses compared to that of mH5-MVA. In fact, F11-MVA enhanced statistically significant immunogenicity. Again, as I did not observe a correlation between *in vitro* expression and *in vivo* immunogenicity of mH5-MVA and SSP-MVA, here I did not see a correlation between *in vitro* expression and *in vivo* immunogenicity of mH5-MVA and F11-MVA. The reason behind the lack of correlation in both cases is likely to be the strong early activity of mH5 and F11 promoters, respectively. The $CD8^+$ T cell responses is often

associated with strong early expression of MVA, and the majority of $CD8^+$ T cell-specific epitopes are products of early genes of vaccinia virus, both in humans[196] and mice [113]. The lack of correlation could also be a result of experimental differences. It is likely that testing MVA promoter activity in BHK-21 cell lines, even with AraC treatment, did not mimic the early expression *in vivo*. Therefore, in the next chapter I tested the expression driven by mH5 and pF11 promoters in permissive cells as well as *in vivo*. A previous report concluded that immune responses to a recombinant antigen delivered by MVA were not found to correlate with the expression level, but rather with the clearance of the antigen [139].

Regardless of correlation with *in vitro* expression level, the pF11 endogenous promoter is superior to mH5 in inducing cellular immunogenicity. This was confirmed by making different rMVAs with pF11 to drive the ME-TRAP, a malaria antigen or NP-M1, a Flu fused antigens (Alharbi *et al* in prep, data from colleagues). Both of those antigens are in clinical trials, controlled by the p7.5 promoter.

Regarding humoral responses, none of rMVAs induced detectable level of rLuc-specific antibodies after a single shot of 10^6 pfu as tested by LIPS and ELISA. This might be a low dose that was not sufficient to induce detectable antibody responses. In fact, previous preclinical studies in mice and monkeys showed that antibody induction by MVA vector is dependent on the target antigen and the dose [91,98]. These studies also reported the importance of applying the best possible regimen to induce strong antibody titres. For instance, vaccinating mice with MSP1 antigen (a malaria merozoite

surface protein 1) delivered via MVA, DNA-prime MVA-boost, or FPV-prime MVA-boost vaccination regimens did not result in detectable antibody responses. In contrast, priming with the human adenovirus type 5 vector (AdHu5) and boosting with MVA, both encoding MSP1, resulted in higher titres of neutralising antibodies that protected mice from murine malaria challenge [98]. Therefore, it is not surprising that one shot of rMVA, assessed 7 days post immunisiation failed to induce detectable levels of antibodies. Our promoter comparison study should benefit from a more efficient regimen to determine whether a promoter could enhance antibody responses.

Next, it was sought to determine whether the pB8, pE3, and pF11 strong activity, and the improved immunogenicity could still be achieved when those promoters are inserted as ectopic promoters at a different locus, such as the TK. This would also reveal whether the insertion site could affect the transgene expression and immunogenicity. Utilising ectopic promoters resulted in homologous sequences of 150 and 180 nucleotides; and although genetic stability assays were not performed, homologous nucleotides did not affect the genetic stability of many MVA-based vaccines that utilise 240 of the p7.5 promoter, which has two natural copies in addition to the third copy that drives vaccines. This comes from observation by the Jenner institute scientists who developed tens of p7.5 utilising MVA-based vaccines, many of which have been manufactured by IDT, a German biotech company, and tested in clinical trials. One of the latest examples is testing MVA85A in around 3000 infants in Africa [175]. Recombinant MVAs with endogenous promoters; however, were tested and proved genetically stable as we published previously [156].

Utilising ectopic pB8, pE3, and pF11 promoters at the TK locus did not show any difference in terms of transgene expression or immunogenicity despite the fact that the minimum sequence of these promoters has not yet been established. This similar immunogenicity was observed regardless of the presence of the ORFs of these promoters. For example, the *E3L* ORF is intact in E3-MVA. Thus, it clearly demonstrates that the immune responses, enhanced by a given endogenous promoter, is much associated with the level of transgene expression and not affected by any other factor such as insertion sites or the deletion of the authentic ORFs of these endogenous promoters.

Ideally, this conclusion should be tested by making rMVA deletion mutants that lack the ORFs of the tested promoters, with ectopic promoters utilised at the TK. However, because we did not see any difference, here, between the compared rMVAs, we could conclude that the deletion of *B8R*, *E3L*, or *F11Lper se* does not contribute to the improved immunogenicity. This is not surprising in the case of *B8R* and *F11L* as they are already fragmented and not active genes. So, their deletion should not affect MVA. The *E3L*, however, is an essential gene in MVA and involved in immunosuppression mechanisms, and the *E3L*-deletion MVA mutant does not replicate in CEFs, but replicates in BHK-21 cells [92,200]. Furthermore, E3-deleted MVA mutant, encoding the pb9 epitope, was not able to improve the pb9-specific $CD8^+$ immune responses as compared to non-deletion rMVA, encoding pb9 as well (Cottingham, M., unpublished data). This indicates that the improved immunogenicity of E3-MVA could be mainly due to the pE3 activity. So, as the *E3L* is essential for producing MVA in the GMP standard CEFs, the use of the ectopic pE3 promoter should overcome any

manufacturing concern since eE3-MVA(ΔTK) grew in CEFs (observation, no data). Furthermore, the E3-MVA(ΔTK) and eE3-MVA(ΔTK) viruses showed more scattered values for immunogenicity (Figure 4.8), which made it an interesting virus, discussed further in chapter 5.

Regarding the insertion sites, most of the studies that compare insertion sites were performed on VACV rather than MVA, which clearly has a different phenotype. It has been reported that the insertion of the same transgene into the TK or into another locus did not have any significant difference in terms of both transgene expression and *in vivo* virulence or immunogenicity [131,169]Although the second study [131], only showed the immunogenicity was similar but did not measure the transgene expression. In such studies, two viruses are made with the same antigen driven by the same promoter and expressed at the TK or other loci, and deletion of the TK gene in both viruses.

In addition, Coupar *et al* [170] attempted to compensate for the absence of the TK gene by inserting the herpes simplex virus thymidine kinase (HSV-TK) at the F7L locus, this method was also used for recombinant selection. Then, inserted a p7.5-driven luciferase at either the TK (virus 1) or adjacent to the HSV-TK at the F7L site with deleting the native VACV thymidine kinase (VACV-TK) by insertion of influenza haemagglutinin (virus 2). So, both viruses have their native TK deleted with the HSV-TK inserted. Their result showed that the luciferase expressed at the F7L was in higher level *in vitro* and *in vivo*, though only in lungs. However, when the virus 2, which showed improved expression, was compared a rVACV that expresses the same luciferase at the TK (virus 3) with no other modifications, i.e. does not have HSV-TK at the F7L, the luciferase expression was similar between virus 2 and 3 *in vitro* and *in vivo*. Although this was confusing, the authors suggested there might be a role of the F7L that was present in virus 3. In spite of that, the insertion of HSV-TK could not be enough to control for the TK activity. The TK from HSV and VACV are two different

kinases, and belong to two different categories of TK families. VACV-TK is also a small size protein with different structure. So, a TK gene from poxvirus strain should be used to control the experiment in a more efficient way. As an example, the TK gene from FPV has been inserted into the VACV genome although it was conducted to improve recombinant selection process and not for examining the effect of the TK activity or insertion sites [201]. The TK effect will be discussed in depth in chapter 5. Finally, others have reported increased transgene expression from the VP37 locus than the TK, but used different promoters (p7.5 and SSP) in each site [202]. The work presented in this chapter attempted to determine the effect of insertion site on transgene expression and immunogenicity. Several rMVAs were derived with the different promoters at different sites versus the same promoters at the TK locus, and with deactivating the TK gene in all rMVAs (e.g. E3-MVA(ΔTK) vs. eE3-MVA(ΔTK)). The result showed that the insertion loci of *B8R*, *E3L*, or *F11L* as compared to the TK locus did not impact on the transgene expression and immunogenicity. Overall, the enhanced immunogenicity with the pB8, pE3, and pF11 promoters is independent from the deletion of their ORFs and their insertion sites, and seems mainly due to their *in vivo* activity.

4.4 Recommendation for future research

For future vaccines, it would be important to test a promoter *in vivo* despite its expression level *in vitro*. If any of the endogenous promoters is used at an external site, such as the TK, then the minimal sequences of these promoters should be determined to avoid any concern of homologous recombination between the two sequences of the same promoter. The pF11 promoter seems a very useful promoter for cellular immune responses and more antigens should be tested with the pF11. The pE3 can be used at an external site, like the TK, because *E3L* is essential in CEFs, the cGMP standard cells for MVA-based vaccine manufacturing.

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Chapter 5

The Effect of Inactivating Thymidine Kinase Gene on the Expression and Immunogenicity of the Transgene in Recombinant MVA

5. The Effect of Inactivating Thymidine Kinase Gene on the Expression and Immunogenicity of the Transgene in Recombinant MVA

5.1. Introduction

The Thymidine Kinase (TK) gene is a conserved gene in vaccinia virus, encodes a homotetrameric complex of 20 kDa monomers, 80 kDa total [203] that plays a role in DNA synthesis by phosphorylating the 2'deoxy-thymidine (dThd) to produce thymidine 5'-monophosphate (dTMP), which is then converted by thymidylate kinase to thymidine 5'-diphosphate (dTDP), leading eventually to pyrimidine deoxyribonucleotide triphosphate (dTTP) and DNA synthesis [204]. When the TK gene was deleted from VACV, the mutant grew normally *in vitro*, but showed less virulence in immunised mice [47,205]. The TK locus has been used to insert transgenes into VACV and MVA [48]. This was developed originally, in the 1980s, to allow for more efficient selection of recombinant viruses. It involves growing TK-disrupted recombinant viruses in TK-deficient cells while providing Bromodeoxyuridine (BrdU), thymidine analog. Only the wild type viruses would phosphorylate BrdU, affecting their DNA replication and halting their life cycle whereas the TK-disrupted recombinants could grow and be selected for. Bromodeoxyuridine could affect the TK-disrupted viruses only at higher concentration [206]. For cGMP MVA manufacturing, there are no TK-deficient chickens to produce TK-deficient CEFs. Therefore, a marker would be inserted with a transgene at the TK locus and then removed before producing the final cGMP MVA-based vaccines.

In addition to the TK locus, there is a range of insertion sites in MVA, such as deletion II and deletion III [20] regions and any non-essential [3] or non-functional gene [207] could also be used to insert transgenes. However, all of the MVA-based vaccines that have been developed in the Jenner institute, Oxford University, many of them reaching clinical trial phase I and II, utilise the TK ORF as an insertion locus for the recombinant antigens. This was not performed in the interest of selection as these vaccines were selected based on fluorescent markers, which are removed from the final products.

Deletion of the TK gene from VACV reduced replication of the virus *in vivo* [208]. The TK deletion in MVA, which is replication-deficient, should not have a deleterious impact in many TK-producing cells (e.g. CEF and BHK-21) whereas *in vivo*, the TK deletion does not seem to inhibit MVA viral gene expression[169]. As discussed in chapter 4, transgene expression and immunogenicity of MVA were not different when the transgene was inserted at the TK locus or other loci with a disrupted TK. However, transgene expression and immunogenicity of rMVA that have an active TK gene could be different. Kunke *et al* inserted the HBsAg at the TK locus and the HBcAg at the K1L locus in one rVACV, and reversed their locations in a second rVACV, and showed that the insertion site did not influence their mouse immune responses or virus virulence. The immunogenicity of each antigen was also similar to rVACV with singly inserted antigens at the TK. However, this could not be used to conclude the effect of the TK as the TK was inactivated in all viruses [169]. In addition, human IL-2 was used to attenuate the virulence of rVACV *in vivo*, and was inserted into three different loci of rVACV, including the TK ORF. The attenuation effect of IL-2 was similar in all three viruses, and was not altered by the presence or absence of the TK. [131]. So, from this

early study, the TK inactivation did not seem to influence rVACV immunogenicity although it was performed on VACV rather than MVA, which clearly has different phenotypes. Most early studies were performed on VACV, aiming at attenuating the virus to produce safer vaccines as well as inducing immune responses to the recombinant antigens [209]. When the TK phenotype was the focus of a study on VACV, the presence of the TK allowed for more virus replication and stimulated higher antibody and cellular immune responses [208], higher than TK-deleted VACV or TK-deleted VACV with HSV-TK inserted (TK gene from HSV inserted into VACV). Although MVA, unlike VACV, does not complete its replication cycle in mammals or mammalian cells, the presence of the TK in MVA could at least allow for viral DNA replication, which should lead to at least more copies of the genome and potentially higher transgene expression. More genome copies could also be packaged into more virions, and even though these would be immature virions, they could stimulate more immune responses.

In the light of this, we hypothesised that TK inactivation in mH5-MVA or p7.5-MVA led to a lower cellular immunogenicity (observed in chapter 4) as compared to rMVAs with endogenous promoters, for example, F11-MVA, despite the strong activity of mH5 and p7.5 promoters *in vitro*. So, keeping the TK gene intact could contribute to improved immunogenicity, presumably, in addition to the strong activity of endogenous promoters. Alternatively, disrupting the TK in rMVAs with endogenous promoters could reduce their improved immunogenicity.

In support of this hypothesis, the results in chapter 4 showed that all rMVAs with endogenous or ectopic MVA promoters (like pB8, pE3, and pF11) contributed to similar or better cellular immunogenicity as compared to the mH5 promoter (Figure 4.8).

Apart from the promoter used, the differences between these rMVAs were that in rMVAs with endogenous promoters, the TK gene was intact (active); and in rMVAs with ectopic promoters, the ORFs of these promoters were intact. We have previously noticed that the deletion of *B8R*, *E3L*, and *F11L* ORFs from rMVA did not result in enhanced immunogenicity, in BALB/c mice (Alharbi *et al*, in prep and Cottingham M., unpublished data). Therefore, the presence of an active TK gene in rMVAs with endogenous promoters was the only possible explanation for improved immunogenicity of rMVA with endogenous promoters (Figure 4.5).

To test this hypothesis, in this chapter, recombinant MVAs with the endogenous pB8, pE3 or pF11 promoter (in the viruses: B8-MVA, E3-MVA, and F11-MVA) were used as parental viruses to derive three recombinant MVAs with the mCherry gene (red fluorescent marker) [191] inserted at the TK locus, resulting in viruses: B8-MVA(ΔTK), E3-MVA(ΔTK), and F11-MVA(ΔTK), see Figure 4.1. The mCherry was chosen to also facilitate faster plaque picking and purification of the rMVAs. Inserting mCherry in the middle of the TK gene instead of replacing (deleting) the whole TK gene was intended to mimic the way of inserting vaccines into this locus. A schematic representation of the method used to insert mCherry into TK locus of the B8-MVA virus was included in figure 4.1 (chapter 4). The resultant viruses, B8-MVA(ΔTK), E3-MVA(ΔTK), and F11-MVA(ΔTK), were then tested and compared to their parents in terms of transgene expression and *in vivo* immunogenicity.

5.2. Results

5.2.1. The effect of TK gene inactivation on rMVA transgene expression

Pairs of rMVAs, derived with the TK gene intact or disrupted, using pB8, pE3, or pF11 promoters to drive the transgene at their respective endogenous loci were tested for *in vitro* expression profile. These six rMVAs were tested in luciferase assay. BHK-21 cells infected with the rMVAs were treated with AraC to study the early expression only, or left untreated to show the overall expression profile of these promoters. Every pair of rMVAs with the same promoter showed very similar expression level when TK was intact or disrupted despite the AraC treatment (Figure 5.1). Again, this showed a reproducible result of that in chapter 4 as pF11 promoter continue to show lower expression than pE3 or pB8. Overall, this result showed that the transgene expression as measured by this *in vitro* assay was not affected by the TK gene inactivation.

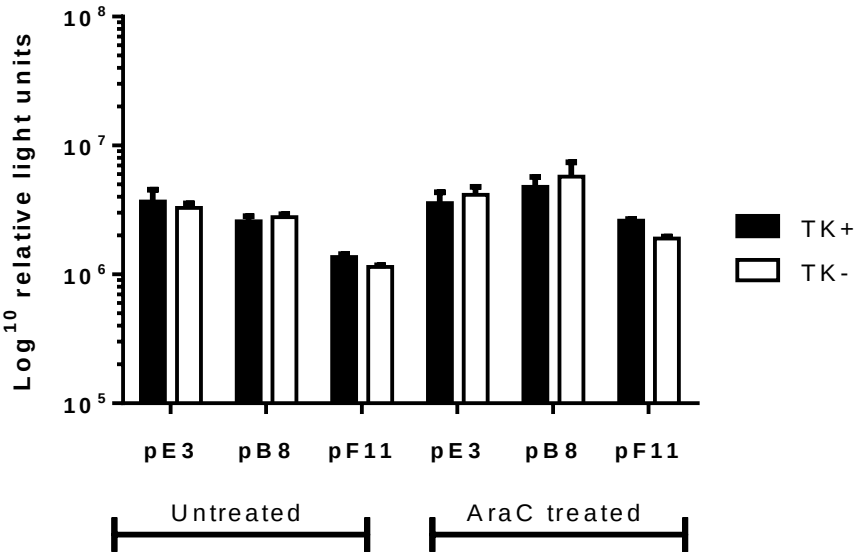


Figure 5.1: The effect of TK gene deletion on the transgene expression in rMVAs.

BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of endogenous promoters at their natural loci with TK locus intact (closed bars), or disrupted (open bars) at MOI of 1. Cells were either treated with AraC (to assess the early expression) or left untreated (for the overall promoter activity). 24 h.p.i, cells were lysed, and added to supernatants and the total level of luciferase expression was measured to determine the promoter activity, using *Renilla* luciferase system. The data, shown in logarithmic scale, represent the mean of 4 wells with SD error bars. Data is representative of three independent experiments.

5.2.2. The effect of TK gene inactivation on rMVA immunogenicity

As the inactivation of the TK gene did not alter the expression profile of the transgene. It was then sought to determine the effect of the TK gene inactivation on the cellular immunogenicity of rMVAs, which is the main aim of this chapter. The *in vivo* immunogenicity elicited by each pairs of rMVAs with TK gene intact or disrupted was tested in BALB/c mice. Four female mice per group were immunised, i.m., with 10^6 pfu rMVA. The mH5-MVA was also included as an external control. Mice were sacrificed a week later, and spleens were collected. Splenocytes were then re-stimulated *ex vivo* with the pb9, F2(G), or E3 peptides and the cellular immune responses were measured by ICS and showed an interesting result. The disruption of TK gene resulted in a reduced transgenic peptide-specific cellular immunogenicity as compared to keeping TK intact using pB8 or pF11 promoters (Figure 5.2.A). Not only that, but MVA-vector specific immune responses were also reduced upon TK disruption, indicating the importance of the TK enzyme in providing more DNA replication that leads to prolonged "generic" gene expression. The reduction in pb9-specific responses was clearer in groups immunised with viruses with pF11 promoters, even though this reduction was not statistically significant. This mice immunization experiment was repeated independently three times and showed the same pattern of reduction with pB8 and pF11 promoters after disrupting the TK gene but without statistically significant difference. The mH5-MVA was included as a control, which has mH5 promoter driving the transgene at the TK locus, inactivated the TK gene. rMVAs with the pB8, pE3, or pF11, and with intact TK gene enhanced higher immunogenicity as compared to the mH5-MVA control. However, pF11 or pB8 utilising rMVAs, but not pE3, which have disrupted TK gene enhanced lower immunogenicity that could be comparable to that of mH5-MVA.

In contrast to the B8-MVA and F11-MVA, the E3-MVA was the opposite by enhancing the cellular immunogenicity when TK was disrupted (Figure 5.2.A), which makes it a very interesting virus. In addition, I detected the same pattern with the MVA vector-specific cellular immunogenicity. Inactivating the TK gene resulted in lower F2(G) or E3 peptide-specific $CD8^+$ T cell responses with the use of pB8 and pF11, but not with the pE3 (Figure 5.2 B&C). The E3-MVA(ΔTK) showed increased responses similar to that of pb9-specific $CD8^+$ T cell responses. Moreover, E3-specific responses were not detected (Figure 5.4 B), which confirms the deletion (replacement) of the *E3L.*

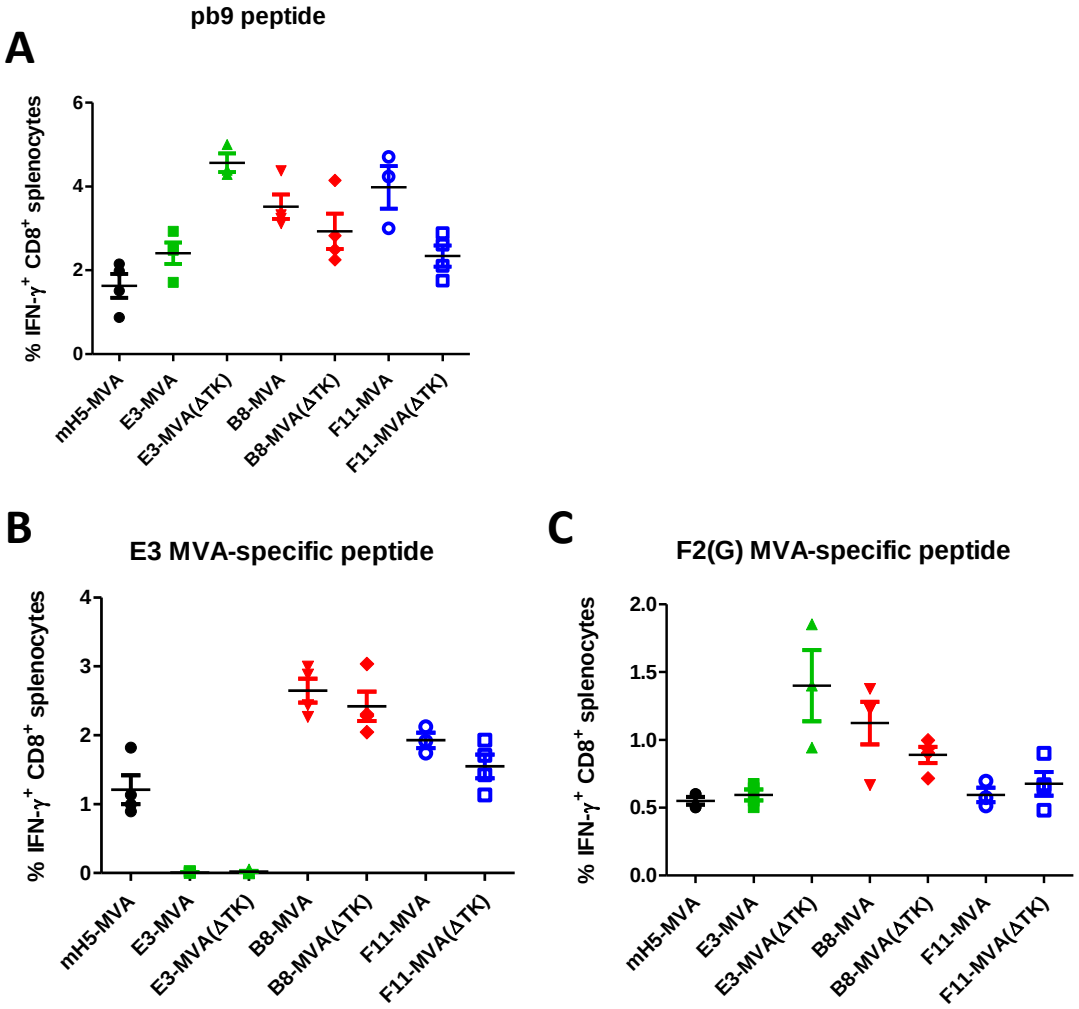


Figure 5.2: The effect of TK gene deletion on *in vivo* cellular immunogenicity of rMVAs with different endogenous promoters.

Seven groups of female BALB/c mice (n=4) were immunised with rMVAs with different endogenous promoters driving tPA-pb9-rLuc at their endogenous loci, in the presence or absence of TK gene as indicated. mH5-MVA has the TK gene disrupted and used as a control. Seven days post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in response to *ex vivo* re-stimulation with pb9 peptide **(A)**, or with MVA vector-specific peptide **(B**and**C)**. All values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with SEM error bars are shown. Data is representative of three independent experiments. Looking at the pb9 responses for each pair of rMVA with the same promoter, such as F11-MVA versus F11-MVA(ΔTK), no statistical significane were detected, using One-way ANOVA (withBonferroni's test).

5.2.3. The effect of TK gene inactivation on rMVA transgene expression in non-permissive cells

Disrupting the TK gene did not affect the transgene expression *in vitro*, but slightly reduced transgene immunogenicity *in vivo.* This reduction pattern was not significant, but it was consistent in three independent experiments. Therefore, rMVAs were tested in non-permissive cells. The cells were used to mimic *in vivo* conditions, in which MVA does not replicate, but the presence of its TK enzyme might allow for "little more" genome copies and more transgene expression that could enhance immunogenicity.

Thus, monolayers of HEK 293 cells in 6-well plate were infected with F11-MVA and F11-MVA(ΔTK) at MOI of 1. The inoculum was not changed to mimic *in vivo* conditions. A sample of 100 µl was collected at different time points for two days. The time-course luciferase assay did not reveal any difference in the transgene expression profiles, except at 48 h.p.i. where the F11-MVA(ΔTK) produced significantly lower level of transgenic luciferase (Figure 5.3). This could illustrate the importance of the TK enzyme in producing more MVA genome copies, which result in more transgene expression.

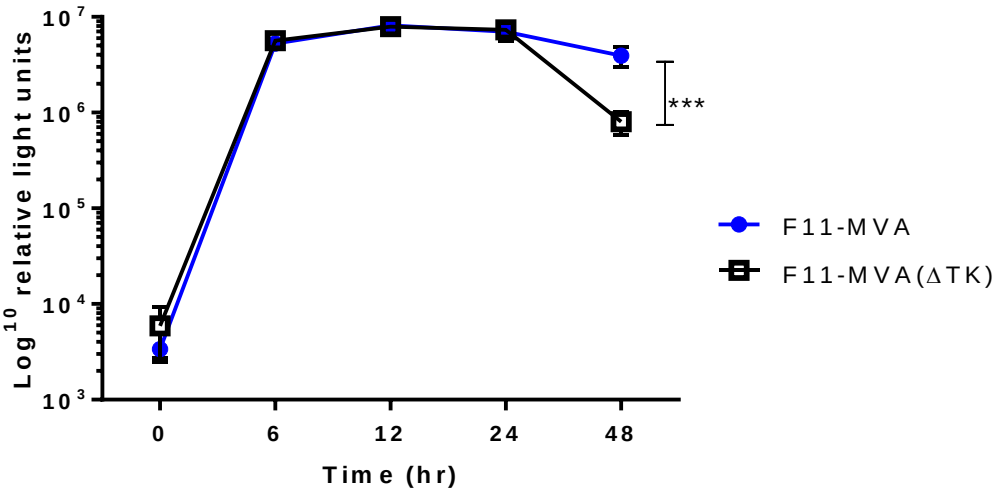


Figure 5.3: The effect of TK gene inactivation on the transgene expression of rMVAs in non-permissive cells.

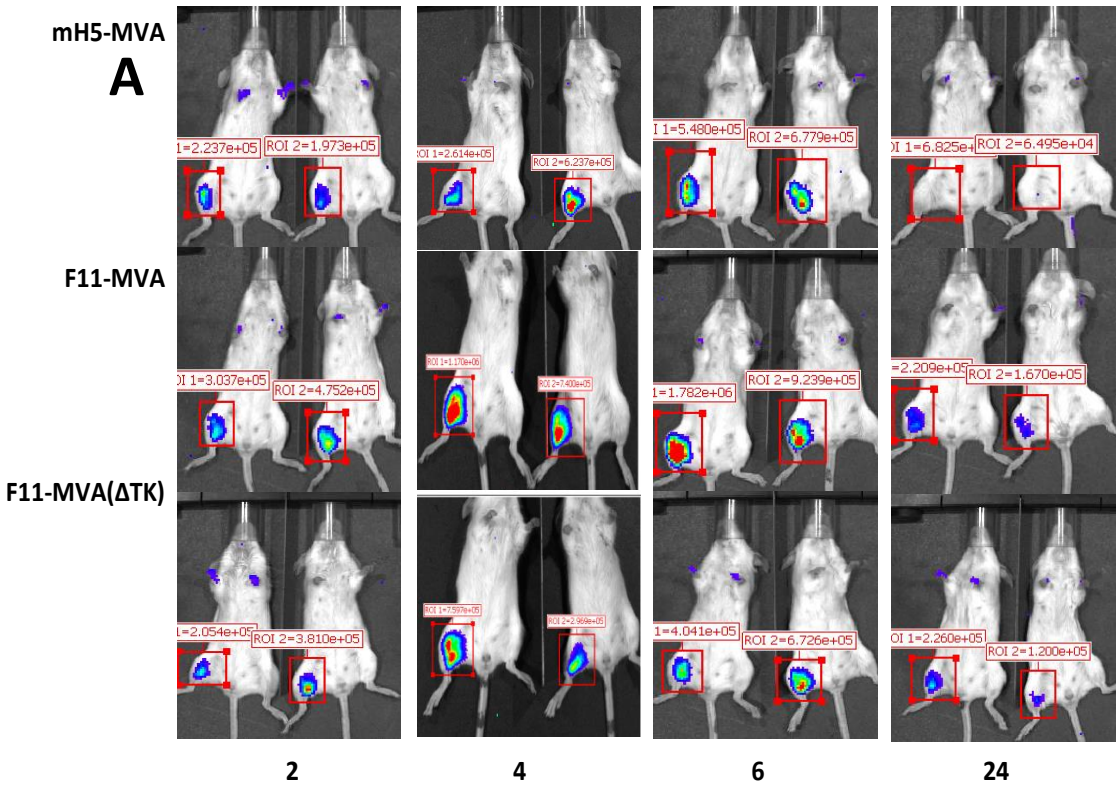
Confluent HEK 293 cells in 6-well plate were infected with F11-MVA and F11-MVA(ΔTK) at MOI of 1, in a volume of 1 ml 2% sera DMEM media. The plates were incubated for 2 days, and a sample of 100 µl was collected at different time point. The values of 4 wells with SD error bars are plotted. Data, shown in logarithmic scale, represent two independent experiments. *** $p<0.001$ (by *t*-test)..

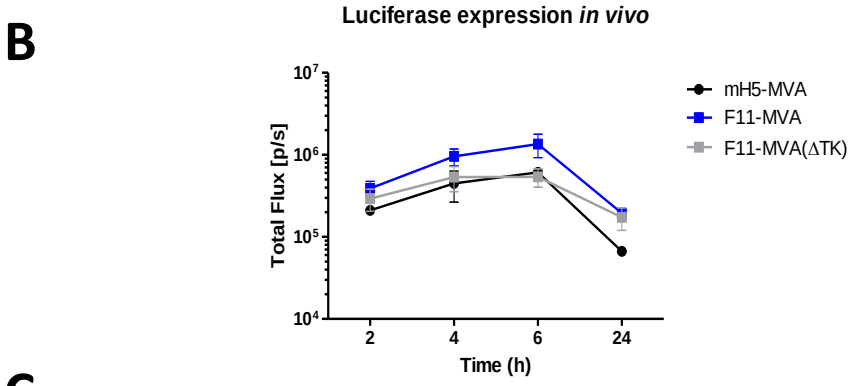
5.2.4. The effect of TK gene inactivation on rMVA transgene expression and immunogenicity *in vivo*

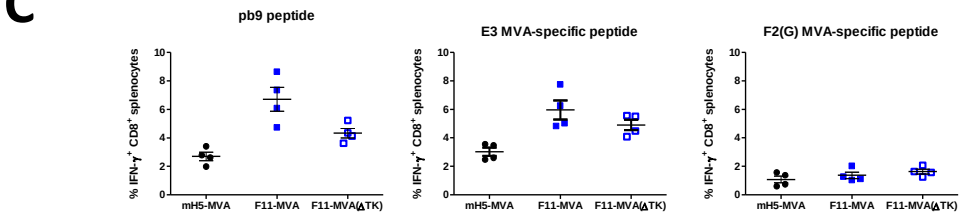
It was demonstrated that rMVA with endogenous promoters, especially F11-MVA, enhanced higher immunogenicity than mH5-MVA, but this immunogenicity did not correlate with *in vitro* expression profile of rMVAs (Chapter 4). In fact, F11-MVA *in vitro* expression was the lowest as compared to that of mH5-MVA. Therefore, the TK gene, which is present in F11-MVA but not in mH5-MVA, was thought to play a role in the improved immunogenicity. The TK disruption in F11-MVA(ΔTK); however, reduced the immune responses but not to a significant degree. In addition, F11-MVA(ΔTK) showed reduced transgene expression only at 48 h.p.i. in non-permissive cells.

As the TK role was not significant, the experemint setting was thought to have a role in thisdiscrepancy. Therefore, *in vivo* expression system was set up using *in vivo* imaging technology to detect whether the pF11 promoter is truly stronger than mH5. Thus, three groups of eight female BALB/c mice were immunised intramuscularly with 10^6 pfu of mH5-MVA, F11-MVA, or F11-MVA(ΔTK). Next, at 2, 4, 6 or 24 hours post immunisation, two mice from each group were injected with 100 µl of 0.150 mM VivRen® substrate (Promega), anaesthetised immediately, and imaged using an IVIS machine within a minute, with 2 minutes exposure time. The total flux of photon per second per centimeter per seridian were used to calculate the luciferase activity. Four mice per group were housed for seven days and sacrifices for spleen collection. Splenocytes were then re-stimulated *ex vivo* with the pb9, F2(G), or E3 peptides and the cellular immune responses were measured by ICS.

Surprisingly, the *in vivo* imaging showed for a first time that the F11-MVA expressed transgenic luciferase to a higher level than that of mH5-MVA at all tested time points. The F11-MVA(ΔTK) also expressed higher level of luciferase as compared to mH5-MVA, but lower than F11-MVA. This clearly suggests that the pF11 promoter is stronger than mH5 (Figure 5.4B, virus F11-MVA vs. mH5-MVA) and that the expression profiles were reduced by the inactivation of the TK virus (Figure 5.4B, F11-MVA vs. F11-MVA(ΔTK)). The hierarchy of immune responses to the pb9 peptide correlated very agreeably with the *in vivo* expression data, especially at 2 and 4 hours (Figure 5.4C).


mH5-MVA
A
F11-MVA
F11-MVA(ΔTK)
2
4
6
24


B
Luciferase expression in vivo
Total Flux [p/s]
Time (h)
mH5-MVA
F11-MVA
F11-MVA(ΔTK)


C
pb9 peptide
E3 MVA-specific peptide
F2(G) MVA-specific peptide
% IFN-γ+ CD8+ splenocytes
mH5-MVA
F11-MVA
F11-MVA(ΔTK)

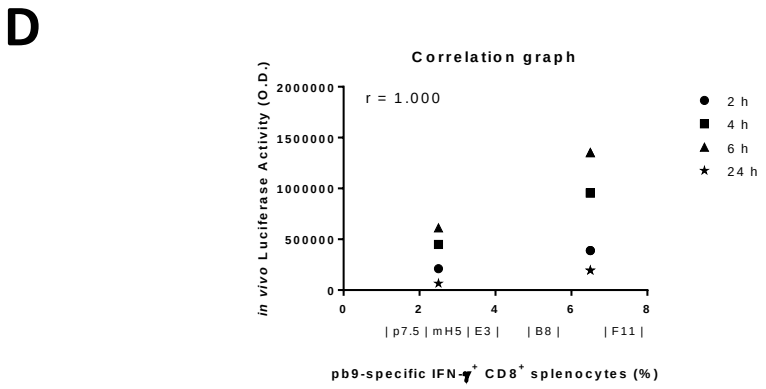

D
Correlation graph
r = 1.000
in vivo Luciferase Activity (O.D.)
2 h
4 h
6 h
24 h
| p7.5 | mH5 | E3 | | B8 | | F11 |
pb9-specific IFN-γ+ CD8+ splenocytes (%)

Figure 5.4: The effect of TK gene inactivation on rMVA transgene expression and immunogenicity *in vivo.*

A: Three groups of female BALB/c mice (n=3) were immunised with $1X10^6$ rMVA, i.m. Two mice were then injected intravenously with 100 µl of 0.295 mM VivRen substrate, at 2, 4, 6, or 24 hours post immunisation, anaesthetised immediately and imaged using IVIS machine within one minute, with 2 minutes exposure time. rMVA used for immunisation and imaging time points are indicated **B:** The luciferase expression is presented by plotting he total flux of photon per second against the time (h) post immunisation. The difference in luciferase expression between mH5-MVA and F11-MVA at either 4 or 6 h was significant (p <0.05) by t-test. **C:** Four mice that were imaged at 2 and 4 h.p.i. were housed for 7 days, and then splenocytes were harvested and used to assess cellular immunogenicity, exactly as performed in figure 5.2. . **(D):** Mean of values from graph B are plotted against mean of values from graph C (pb9-specific responses) to test the correlation between promoter activity and cellular immunogenicity *in vivo*. The correlation coefficient (r) is 1.000, which indicates perfect correlation using nonparametric Spearman correlation test.

5.3. Discussion

Disrupting the thymidine kinase (TK) gene does not seem to deleteriously affect the replication of rMVA in tissue culture and many rMVA-based vaccines have been produced in a large quantity for clinical trials. All the rMVA-based vaccines produced by the Jenner institute used the TK locus as an insertion site by disrupting, rather than deleting, the TK gene. The role that the TK plays in inducing more cellular immune responses of these vaccines has not been clearly addressed. So, it was important to determine the effect of inactivating the TK gene on the cellular immunogenicity of rMVA-based vaccines, using the T cell-specific malaria epitope pb9, which is part of the reporter transgene (tPA-pb9-rLuc). We noticed that all rMVAs with endogenous promoters driving the expression of the tPA-pb9-rLuc transgene at the endogenous loci of these promoters with the TK gene intact induced higher cellular immunogenicity than when mH5 was used to drive the same transgene at the TK, despite the fact that the activity of these endogenous promoters, *in vitro*, was similar or lower than mH5 promoter. Thus, there was no correlation, which was not due to the insertion site nor it is related to the endogenous ORFs deleted from MVA as a result of using their endogenous promoters. Therefore, in this chapter, we hypothesised that the TK gene present in all of the rMVAs with endogenous promoters could enhance their *in vivo* cellular immunogenicity and could also explain the lack of correlation between *in vitro* expression in BHK-21 cells and *in vivo* mouse cellular immunogenicity.

After testing the rMVAs with endogenous promoters in the presence or absence of active TK gene, I showed that TK disruption led to a reduction in the immunogenicity

level of B8-MVA and F11-MVA. This reduction was not statistically significant, but it made CD8+ T cell responses comparable to that achieved using mH5-MVA (which has inactive TK gene) although it is still not correlated with *in vitro* expression profile. This marginal reduction might be due to the absence of thymidine kinase, which is involved in phosphorylating the deoxythymidine, leading eventually to the deoxythymidine triphosphate (dTTP), which helps in building more genomic DNA copies as MVA virus does not replicate in mouse model or most mammalian cells. In support of this interpretation, the presence of an intact TK gene in rVACV increased the viral replication *in vivo*, and stimulated higher antibody and cell-mediated responses than a TK negative rVACV [208]. Not only the response to the transgenic pb9 epitope dropped in the absence of the TK, but also the overall immunogenicity, measured by responses to the vector-specific epitopes. This suggests that the MVA genome could undergo more rounds of replication when the TK is active, which could increase the gene expression, including the transgenic expression. Hence, this could result in eliciting more $CD8^+$ T cell responses.

Nevertheless, these endogenous promoters stand as strong promoters in eliciting cellular immune responses, *in vivo*, as presented in chapter 4 and inactivation of the TK could not be the only factor behind improved immunogenicity. The lack of correlation in the hierarchy of promoters between *in vitro* expression and *in vivo* immunogenicity could be more complex. The *in vitro* expression experiments was carried out in BHK-21 cell lines that come from hamsters whereas *in vivo* immunogenicity was done in inbred BALB/c mice. Moreover, *in vitro*, rMVAs could either be replication competent in BHK-21 cells or stopped at the early expression by treating cells with AraC to block its DNA

replication, whereas in mouse models, rMVAs does not fully replicate and halt after the expression of its early, intermediate, and late genes. Therefore, rMVA were tested in non-permissive cells to mimic *in vivo* conditions. Luciferase expression in HEK 293 cells was not different between rMVA with or without active TK gene, except at 48 h.p.i., indicating the role of TK enzyme in building more genomic DNA and more gene and transgene expression.

However, *in vivo* expression would be the ideal test to show the correlation of a promoter activity with its enhanced immunogenicity. So, the transgene (tPA-pb9-rLuc) enabled the evaluation of luciferase expression level via *in vivo* imaging while assessing immunogenicity of pb9 epitope in mouse model. This experiment, strikingly, revealed a true correlation between immune responses and transgene expression, especially at 2 hours post immunisation. It showed that mH5 promoter is not stronger than pF11 promoter, which explained the enhanced immunogenicity of F11-MVA, supporting previous evidence that early expression improved cellular immunogenicity. The transgene expression in the presence or absence of active TK also correlated well with the observed immune responses, and having the same promoter, pF11, showed the role of the TK in enhancing gene and transgene expression, *in vivo*, although not significantly.

Additionally, our results showed that the E3-MVA(ΔTK) is an interesting virus. It was the opposite of B8-MVA(ΔTK) and F11-MVA(ΔTK) and enhanced cellular

immunogenicity when the TK gene was disrupted. Unlike *F11L* and *B8R* ORFs, *E3L* is an active gene in MVA, which could explain why E3-MVA(ΔTK) enhanced rather than reduced immunogenicity when the TK was disrupted. This improved immunogenicity of E3-MVA(ΔTK) over the E3-MVA, which lacks the E3L gene, suggests that this enhancement cannot be only because of E3L deletion. This enhancement could not either be a result of deleting the TK gene, because of, first, the TK gene deletion did not enhance the immunogenicity of B8-MVA(ΔTK) or F11-MVA(ΔTK) over their TK-positive counterparts, which implies an effect of the double deletion of E3L and TK. Second, the TK deletion in eE3-MVA (from chapter 4), which has the pE3 promoter driving the transgene at the TK locus with the native *E3L* gene intact (i.e. E3L intact but TK disrupted) did not show significant increase in cellular immunogenicity as compared to E3-MVA(ΔTK). This suggests that the *E3L* gene presence or absence does not affect MVA immunogenicity. Moreover, the single deletion of *E3L* from rMVA did not improve pb9-specific or vector-specific immunogenicity (Matt Cottingham, personal communication and unpublished data). Again, this strongly suggests that only the double deletion of both TK and *E3L* genes could enhance the cellular immunogenicity, presumably by a synergistic effect. E3-MVA(ΔTK) did not only enhance the immunogenicity of the transgenic pb9, but also the responses to the vector-specific epitope of F2(G) (Figure 5.4), suggesting the overall immunogenic profiles were enhanced by a generic mechanism. This mechanism might be involved in enhancing viral replication.

The Deletion of *E3L* from MVA resulted in a virus that is replication-deficient in HeLa cells, CEF cells, and other cell types, but replicates normally in BHK-21 [92]. The

inability of E3-deleted MVA to replicate in CEFs was linked to the level of PKR (protein kinase R) being higher in CEF than BHK-21. Moreover, the high level of accumulated type I interferon in infected CEF cells also prevented the MVA mutant whereas VACV lacking *E3L* replicated in CEF. VACV replication in CEFs could be due to its interferon binding proteins, which are deleted in MVA[92]. The *E3L* product is a multifunctional protein that binds to ds-RNAs and inhibits them from activating PKR, which plays an important role in activating apoptosis pathway. It also phosphorylates the eIF2A, which leads to the shutdown of viral and cellular protein synthesis; also phosphorylates the inhibitory subunit of NFκB, thus, inhibiting its involvement in IFN-α and β production.

Overall, E3 protein is an apoptosis and interferon inhibitor [92] and despite being an immunosuppressive protein, E3 was reported to be involved in DNA synthesis and late protein expression [92]. Del-E3-MVA reduced the level of viral DNA replication and inhibited late protein synthesis in CEFs, but not in BHK-21 [210].

This might provide insights on how the double deletion of E3 and TK proteins could act in a synergetic way, in mouse model, but not in BHK-21 cells, to enhance the cellular immunogenicity. If both E3 and TK proteins involved in DNA synthesis, their deletion could impair DNA replication and could potentially give persistent early gene and transgene expression that leads to higher cellular immunogenicity. However, E3 absence could result in more apoptosis and less immunogenicity whereas TK absence would result in the opposite (reduced immunogenicity as a result of lower viral DNA replication and transgene expression). Therefore, MVA lacking E3, TK, or both of them

would induce different mechanisms with major effect on immunogenicity. So, the question of why the double deletion in rMVA resulted in enhanced immunogenicity requires careful assessment and is open for future experimentation.

5.4. Recommendations for future research

The pB8, pE3, and pF11 are very strong early promoters that enhance cellular immunogenicity. This enhancement is not related to the insertion site or to the deletion of their endogenous ORFs as presented in chapter 4. In most cases the presence of an active TK gene marginally enhances immunogenicity, by enhancing more DNA synthesis and gene expression; however, disrupting the TK gene has a modest effect on the immunogenicity of rMVA. The double deletion ΔE3-ΔTK-MVA has strong immunogenic profile that was not observed in single deletion mutants (ΔE3-MVA or ΔTK-MVA). This improved profile might be due to the effect of the double deletion on DNA replication and the persistence of early expression. Thus, this hypothesis needs to be studied in a more comprehensive testing in the future. For vaccine applications; however, one does not need to delete the E3L and TK genes to achieve high level of immunogenicity, because similar level of immune responses were achieved with the use of pF11, in its either form of endogenous or ectopic. In addition, E3 deletion MVA mutant replicates in a limited range of cells, which are not currently available for cGMP manufacturing.

Finally, for better vaccine design, promoter activity should always be tested *in vivo.* If *in vivo* expression is not plausible, I found that the immunogenicity data of an antigen is more informative than its *in vitro* expression. Otherwise, at least a more relevant cell line that represents *in vivo* model should be used.

Chapter6

Modifying the pB8 Endogenous Promoter to Enhance the Transgene Expression and Immunogenicity in Recombinant MVA

6. Modifying the pB8 Endogenous Promoter to Enhance the Transgene Expression and Immunogenicity in Recombinant MVA

6.1. Introduction

Utilising stronger promoters to drive transgene expression was shown in many studies to enhance MVA immunogenicity. In the two previous chapters, the use of a stronger conventional promoter, like mH5, in rMVA induced higher immune responses. The search for a stronger poxviral promoter was focused on either finding a better native (endogenous) promoter, or optimising an existing promoter. The mH5 promoter is an example of optimised promoter; it is a modified version of the endogenous H5 promoter that has been used in rVACV before [153,154,211]. Wyatt *et al* introduced the mH5 promoter by modifying two nucleotides in the H5 promoter core regions; one nucleotide in each element of the early and late elements. These two nucleotide changes were based on the consensus sequence of early [162] and late [163] vaccinia virus promoters. Another example of promoter optimisation came from a study on the p7.5 promoter where four tandem sequences of the early region of the p7.5 were fused to one late sequence of the p7.5 to enhance transgene expression, presumably by recruiting more transcription factors, a promoter called pHyb. However, the pHyb enhanced the immunogenicity of rMVA only after four homologous immunisations [155]. The short synthetic promoter (SSP) could be considered a modified promoter because it was made by combining the consensus sequences of the late and early promoter core regions [159,160]. A recent study also reported a modified version of the synthetic promoter by swapping the early and late elements, and adjusting for the spacer sequence between the early sequence and the transcription start site (the LEO promoter). The LEO promoter enhanced the cellular immune responses to the transgenic GFP protein [165]. Moreover, others optimised the intermediate element of

the I3 promoter to make it active only at the early transcription stage, before DNA replication [164]. Therefore, MVA could be improved in terms of its transgene expression by optimising promoters in many ways, which could then enhance its immunogenicity.

In vitro mutagenesis was conventionally used to define promoter core sequences and transcription start site of some early promoters in VACV [152]. A nucleotide substitution approach has also been used to manipulate poxviral promoters. In two studies reported by Davison and Moss [162,163], the p7.5 early and late core regions were manipulated to detect whether nucleotide substitution would enhance or inhibit transgenic protein expression. In these studies, three VACV mutants were derived with three different nucleotides at every residual nucleotide in the core regions of the p7.5 promoter. The early core region of the p7.5 promoter was around 33 nucleotides, and by replacing every nucleotide to the three other possible nucleotides, there was a range of around 99 VACV mutants [162]. All these mutants drove the *LacZ* transgene and the β-gal activity was measured *in vitro*. As a result, some mutants did not express β-gal, suggesting that the original nucleotides in the core region of p7.5 are critical, for example the underlined G and A in table 6.1. On the other hand, the expression was higher than the original p7.5 in some other substitution mutants. For example, when the lowercase "A" (table 6.1) was replaced with T the β-gal activity was significantly enhanced, suggesting that the p7.5 promoter, in particular, and other vaccinia virus promoters could be optimised. Davison and Moss has concluded a consensus sequence for VACV early promoters' core region based on this study as well as some previous related studies. The consensus sequence in this early study (1989) was still valid and in agreement with two recent studies that used more advanced technologies: microarray

study, reported by Assarsson *et al* [19], and deep RNA-Sequencing, reported by Yang *et al*[198]. Both studies reported sequences of early promoters as well as the classification of vaccinia promoters based on the stage of genome expression, i.e., early, intermediate, and late. Yang *et al*[198] have reported a consensus sequence for early promoters' core region (table 6.1) similar to that showed by Davison and Moss [162].

The mH5 promoter, mentioned above and studied in chapter 4, is in fact another example of poxviral early promoter modification. It was modified by the same approach (substitution of the crucial nucleotides) although mH5 has early and late elements and each one of the elements was modified by one nucleotide substitution (Wyatt *et al* [20]). The mH5 early element was made closer to the consensus even though it did no lack the two crucial nucleotides in its original sequence (see H5 vs. mH5 in table 6.1). However, mH5 was not compared to its original H5 promoter, and it was not clear whether the modification in the early promoter region enhanced the early activity of the mH5 over its original version of H5 promoter. On the other hand, Davison and Moss [162,163] compared any mutated promoter to the original p7.5 promoter.

Therefore, based on the vaccinia early promoter core sequences, from these studies, we hypothesised that changing the most crucial nucleotides, mentioned above, in the core region of the MVA early promoter of *B8R* ORF could increase transgene expression. Table 6.1 presents a comparison on the sequences of the endogenous

early promoters that were studied in this thesis. It clearly shows that the pB8 promoter lacks the most crucial nucleotides (G and A, in red). This makes the pB8 promoter a suitable candidate for optimisation.

The pB8 promoter is a strong early promoter (as shown in chapter 4) and was selected for modification not only because it lacks the most crucial nucleotide in its core region, but also because the *B8R* ORF is inactivated, fragmented, and non-essential gene, producing a truncated protein [24]. Early promoters are preferred to drive transgenes intended to induce cellular immune responses as discussed in chapter 4. In vaccinia virus, the *B8R* gene product is an IFN-γ receptor [24], and was reported to have the most immunogenic epitope in MVA in C57BI/6 mice (MHC-II-restricted H-2^b-haplotype mice [113]). It is also one of the most immunogenic epitope in human vaccinees [196]. Therefore, removing B8 protein from MVA vectored vaccine could presumably reduce the vector antigenic complexity, reducing the anti-vector immunity even though its protein is truncated. The deletion of *B8R* does not impair MVA ability to grow in CEF and BHK-21 cells, and it would also create an insertion site into the MVA genome linked to the endogenous pB8. So, the rationale for modifying the pB8 promoter is to create a new insertion site in the MVA genome with an optimised pB8 endogenous promoter. This could help in constructing multivalent MVA vector with two strong promoters, mH5 or p7.5 at the TK locus and this modified pB8 promoter at its own locus. If a modified version of the pB8 proved stronger, it might also be useful to utilise it into a different insertion site, i.e. an ectopic modified pB8 promoter. Multivalent MVA vectors are needed, for example, to have both blood- and liver-stage malaria vaccine, or to have a multi-antigen flu vaccine.

Therefore, this chapter presents the generation of three MVAs with three modified version of pB8 promoter and testing their expression profile *in vitro* and their cellular immunogenicity *in vivo* using the same transgene as in the previous chapters, the tPA-pb9-rLuc. The three modified versions of the pB8 (mB8 promoters) were made using PCR mutagenesis with different substitutions as presented in table 6.2. Each of these promoters was linked to the transgene tPA-pb9-rLuc at the native ATG start codon of the *B8R* ORF, inserted into MVA using MVA-BAC recombineering technology. The rMVAs with mB8 promoters were then tested for their *in vitro* transgene expression and *in vivo* cellular immunogenicity.

promoters	promoter core region*	Spacer**	promoter sequence	Note
pE3	AAAAATGATAAAATA	**39**	aaaaatgataaaataaattagttttattgctggttgtgtt agttctctctaaaaATG	
pB8	ATTATTCAAAATATG	**60**	atattattcaaaatatgatttttaaaAatttaaaatatatt atcacttcagtgacagtagtcaaataacaaacaacacc ATG	
pF11	AAAAGTGAAAAACAA	**28**	aaaaaagtgaaaaacaatattattttTatcgttggttgtt *t*cactATG	
pC11	ATTACTGAATTAATA	**67**	atattactgaattaataatAtaaaattcccaatcttgtcat aaacacacactgagaaacagcataaacacaaaatcca tcaaaaATG	
pA44	AAAATAGAATAAGTA	**22**	gtaaaatagaataagtagtctgatattaTgagtggca gcaATG	
H5	AAAAATGAAAATAAA			The original promoter of mH5
mH5	AAAA**t**TGAAAATAAA			**t** indicates mH5 early element modification.
SSP	TAAAAATTGAAATTTTA	**25**	taaaaattgaaattttattttttttttttTgaatataaataa ---MCS	
p7.5	AAAGT*a*GAAAATATA	**45**	taaaagtagaaaatatattctaatttatTgcacggtaag gaagtagaatcataaagaaca*c*gt---MCS	Underlined G and A were shown as crucial nucleotides to *LacZ* expression. Lowercase "a" when replaced with "T" gave the highest expression level of β-gal
Consensus	AAAAATGAAAAAAAA			Davison and Moss's consensus by *in vitro* mutagenesis
Consensus	AAAA-TGAAAA-ATA			Yang *et al* consensus by Deep RNA sequencing

Table 6.1: Early promoter sequences, information taken from Yang, *et al* (2010).

The underlined G and A in the p7.5 promoter were shown as crucial nucleotides to *LacZ* expression, in Davison and Moss [162]. The p7.5 gave the highest expression level of β-gal when the lowercase A was replaced with T. Red coloured indicate nucleotides that can be substituted to match the consensus sequence. H5 and mH5 are included to show the modification in mH5 early element (bold **t**). SSP was used in the literature and it is very close to the best nucleotide substitutions achieved in p7.5 study. * Based on Yang *et al* study. ** The spacer sequence between the promoter core region and the ATG.

Virus	Promoter	Promoter core sequence
B8-MVA	pB8	ATTATTcAAAATATg
mB8.1-MVA	mB8.1	ATTATTgAAAATATG
mB8.2-MVA	mB8.2	ATTATTCAAAATATa
mB8.3-MVA	mB8.3	ATTATTgAAAATATa

Table 6.2: The modified versions of pB8 promoter (mB8 promoters).

The shaded lowercase letters represent the modified residues by nucleotide substitution compared to their original residues (lowercase, not shaded) in the pB8 promoter.

6.2. Result

6.2.1. Generating three recombinant MVAs with modified versions of the pB8 promoter

The generation of recombinant MVA with different modified pB8 (mB8) promoters was achieved using MVA-BAC recombineering followed by BAC DNA rescue into viruses as explained in the material and methods, chapter 2. Briefly, recombineering primers of 70 nucleotides long were designed to introduce the *B8R* right and left homologous sequences. Because the core region of the pB8 promoter was beyond the 70 nucleotides, i.e. more than 70 base pair upstream of the ATG start codon, It was necessary to carry out two rounds of PCR to reach the pB8 core sequence and to introduce the intended nucleotide substitution(s) in the forward recombineering primers (e.g. Rec-mB8.1-F, see chapter 2). The product of the first-round PCR was used as a template for the second PCR. As the second PCR primers cover the core region of the pB8 promoter, three different primers were used to introduce the three different nucleotide substitutions shown in table 6.2 and presented in the schematic representation (Figure 6.1.1). These three different second PCR products were cloned into MVA-BAC, using the standard MVA-BAC recombineering. The positive clones were confirmed by sequencing the insert (tPA-pb9-rLuc) along with the introduced modified promoters. In general, ten clones sequenced for each modified version of the pB8 promoter, three clones out of ten had the correct modification. The rest did not have the nucleotide substitution, which indicates that the recombination occurred downstream of the introduced nucleotide somewhere in the rest of the promoter or the spacer sequences, as screened by Seq-PCR and DNA sequencing. Next, three recombinant MVAs were derived, purified, and the viral DNA was used to confirm the

introduced modified pB8 promoters by sequencing again. These were given the names of mB8.1-MVA, mB8.2-MVA, and mB8.3-MVA. All were confirmed of having the correct introduced nucleotide changes. The sequencing data were aligned to the original pB8 promoter, shown in figure 6.1.2, and any mismatch indicates a correct intended change.

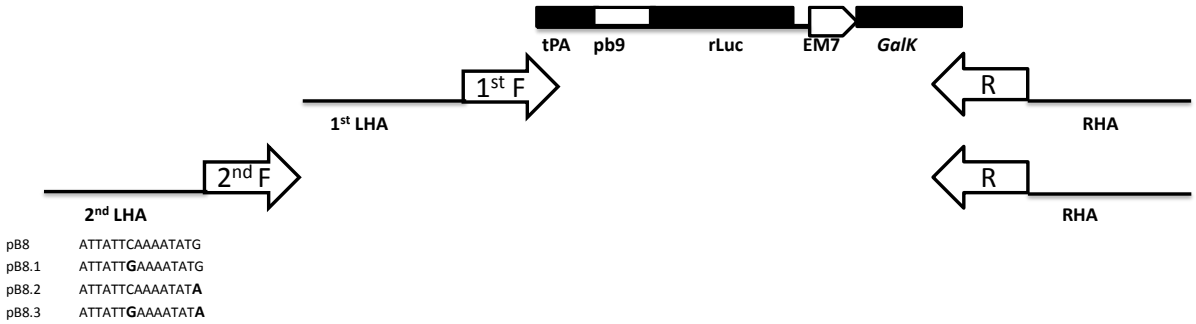


Figure 6.1.1: Schematic diagram of pB8 promoter modification.

The homology arm sequences of *B8R* locus (1st LHA and RHA) were added to the transgene (tPA-pb9-rLuc) by PCR using 1st forward primer (arrow: 1st F) and reverse primer (arrow: R). Then a second left homology arm sequence (2nd LHA) was added to the left side of the construct by a second PCR introducing nucleotide substitutions (in bold) in the pB8 core region using the first PCR product as a template with a second forward primer (2nd F) and the same (R) primer. Three 2nd F primers were used to amplify produce three 2nd PCR products, which were then used to derive three rMVAs: B8.1-MVA, B8.2-MVA, and B8.3-MVA. Each virus has different modified pB8 promoter.

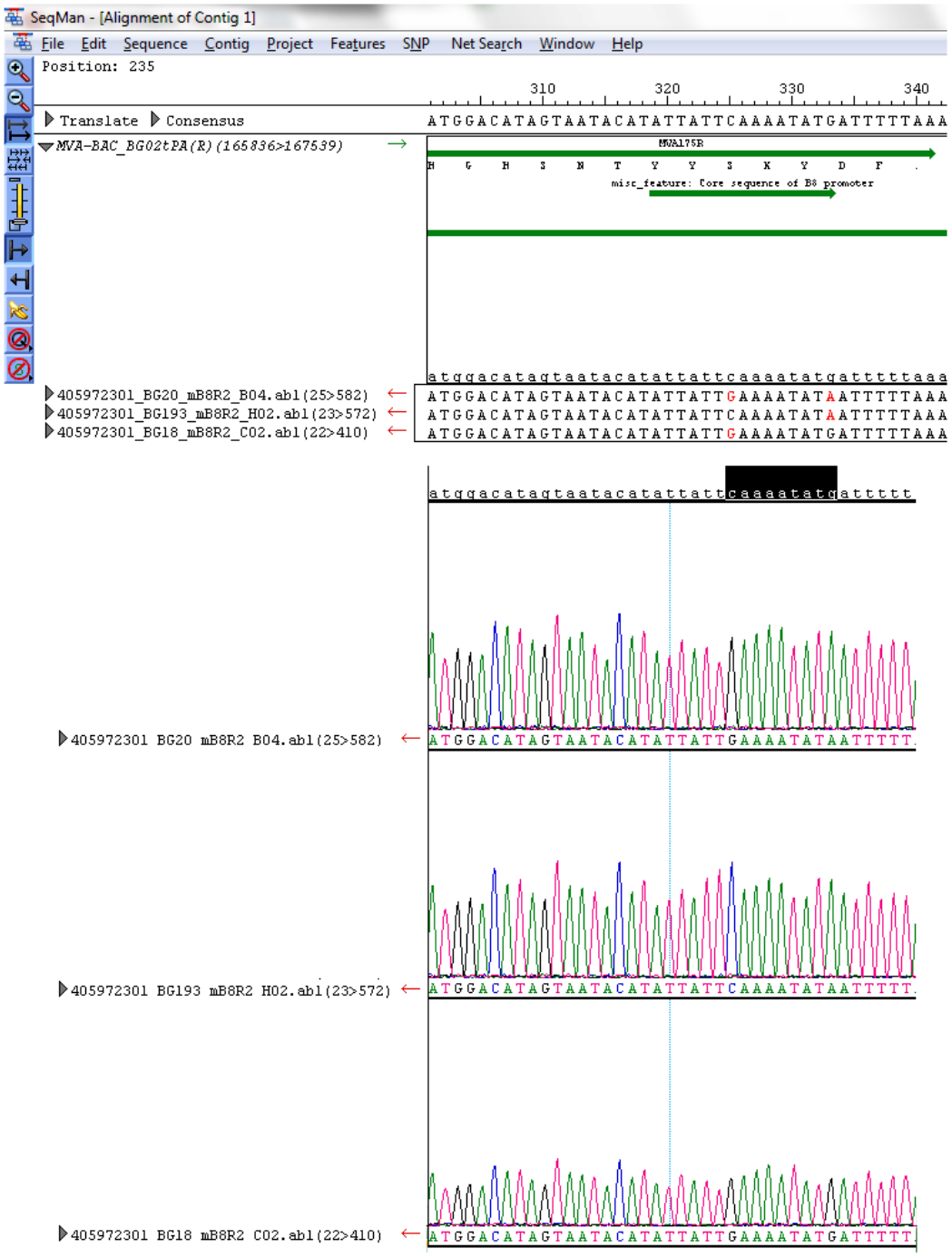


Figure 6.1.2: Sequencing data.

Three rMVAs were made with three pB8 promoter modifications. Here, a snap-shot of sequencing results of the three modified B8 promoters (m**B8.1: TgAAAATATG, mB8.2: TCAAAATATa, and mB8.3: TgAAAATATa;** lowercase letter indicate the modifications) are aligned against the natural pB8 promoter (**TCAAAATATG)**. The red coloured nucleotides indicate modifications.

6.2.2. The luciferase expression profile controlled by modified pB8 promoters in rMVAs

The rMVAs with three modified versions of the pB8 promoter were tested in the luciferase expression assay to determine the effect of the modifications on the activity of pB8 promoter. BHK-21 cells were infected with B8-MVA, mB8.1-MVA, mB8.2-MVA, or mB8.3-MVA. The cells were either treated with AraC to show the effect of modifications on the early activity of the pB8 promoter, or left untreated to determine the overall activity. The expression profile of the modified promoters, after 24h, was similar to that of the native pB8 promoter (Figure 6.2). However, the mB8.1 showed lower expression than the rest of the promoters. In a time course assay, the expression did not change over three time points for 24 hours (Figure 6.2). The expression profile was similar among all modified pB8 promoters in the early expression condition, controlled with the AraC treatment. This indicates that these modifications did not enhance the early pB8 promoter activity although they were based on optimising the early core region, relative to the consensus sequence of vaccinia early promoters core region. However, the mB8.3, which has double nucleotide substitution, was selected for further investigations, as this version was much similar to the native pB8 promoter in terms of the luciferase expression profile, and much closer to the consensus sequences.

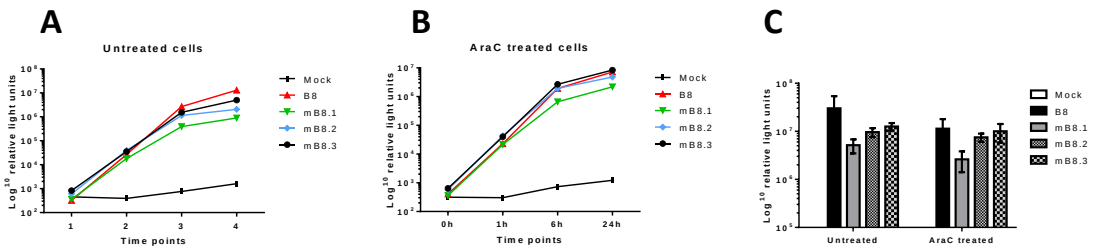


Figure 6.2: Luciferase expression of rMVAs with different modified versions of pB8 promoter.

BHK-21 cells, in 96-well plate, were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of three modified versions of the endogenous pB8 promoter (mB8.1, mB8.2, and mB8.3) as compared to the native pB8 promoter at MOI of 1. Supernatant samples (20 µl) were taken at different time points from cells that were either left untreated for the overall promoter activity **(A)** or treated with AraC to assess the early promoter activity **(B)**. 24 h.p.i, the cells were lysed, and added to the supernatant and the total level of luciferase expression was measured to determine the promoter activity **(C)**, using *Renilla* luciferase system. The data, shown in logarithmic scale, represent the mean of 4 wells with SD error bars. Data is representative of two independent experiments.

6.2.3. The transcription of rLuc transgene controlled by a modified pB8 promoter in rMVAs

Promoter function, in general, is to control the transcription of their respective genes and it would be relevant to test the modified pB8 promoters at the transcription level rather than the protein expression. Here, RT-qPCR using ΔΔCt method was performed as described in chapter 2 (material and methods) and as used in chapter 3 (IRES insertion study) to determine whether any of the nucleotide substitution influenced the transcription of the rLuc.

Confluent monolayers of BHK-21 cells were infected with B8-MVA or mB8.3-MVA and either treated with AraC or left untreated and incubated for 24 hours. Next, supernatants were removed and cells were harvested. The total RNA was extracted from cell lysates, and cDNA samples were prepared. The RT-qPCR was then performed on cDNA samples as explained in the material and methods. The samples were all harvested at 24 hrs and this time point would not reveal the difference in the very early expression; however, the AraC treatment should control for the early expression. The result of the ΔΔCt qPCR showed no difference in the relative fold change of the transgene expression (i.e. the transcript level) between the native pB8 promoter and the modified version mB8.3 promoter. This is reported as the expression change fold of the rLuc gene in figure 6.3. This experiment was repeated twice with similar results.

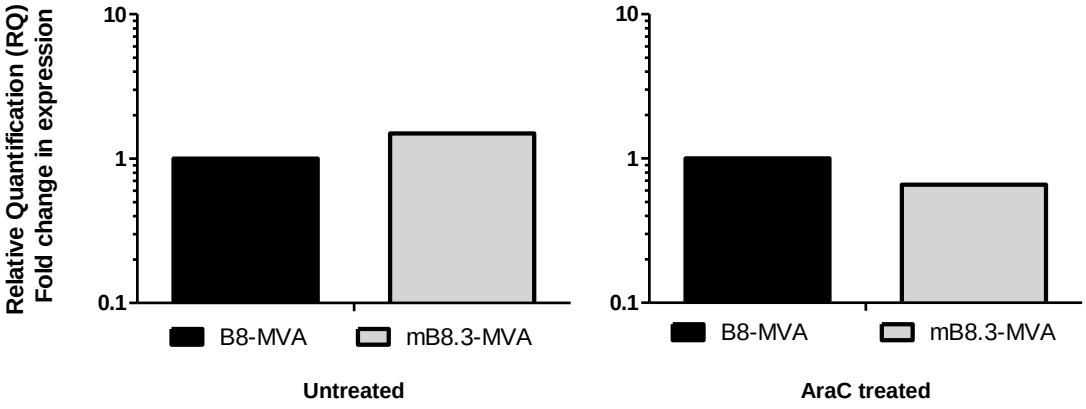


Figure 6.3: Luciferase transcripts level of rMVA with a modified pB8 promoter.

BHK-21 cells were infected with rMVAs, which contain the tPA-pb9-rLuc transgene under the control of pB8 endogenous promoter, or modified pB8 promoter (mB8.3) at MOI of 1. Cells were either treated with AraC (to assess the early transcription) or left untreated (for the overall transcription). 24 h.p.i, the cells were lysed, and the total RNA was extracted and used to make cDNA for the qPCR. The ΔΔ Ct method qPCR was used to determine the relative change fold in the rLuc gene expression, which reflects promoter strength. The Data is representative of two independent experiments.

6.2.4. *In vivo* cellular immunogenicity of rMVA with a modified pB8 promoter

Although there was no higher expression or transcription driven by the modified versions of pB8 promoter, the study was completed by performing an *in vivo* cellular immunogenicity experiment. Two groups of female BALB/c mice were immunised with either B8-MVA or mB8.3-MVA, and a third group was immunised with mH5-MVA as a control. We, continue to include mH5-MVA in mouse immunogenicity studies as the benchmark control although the mB8.3-MVA would be compared to its original copy of pB8 promoter in B8-MVA. First, compared to mH5-MVA, B8-MVA induced slightly more transgenic peptide-specific $CD8^+$ T cells, which reproduces findings reported in chapter 4. Second, there was not any difference between B8-MVA and mB8.3-MVA groups (Figure 6.4). The MVA vector-specific cellular immune responses, using MVA-specific F2(G) and E3 peptides, were also similar between rMVAs with pB8 and mB8.3 promoters.

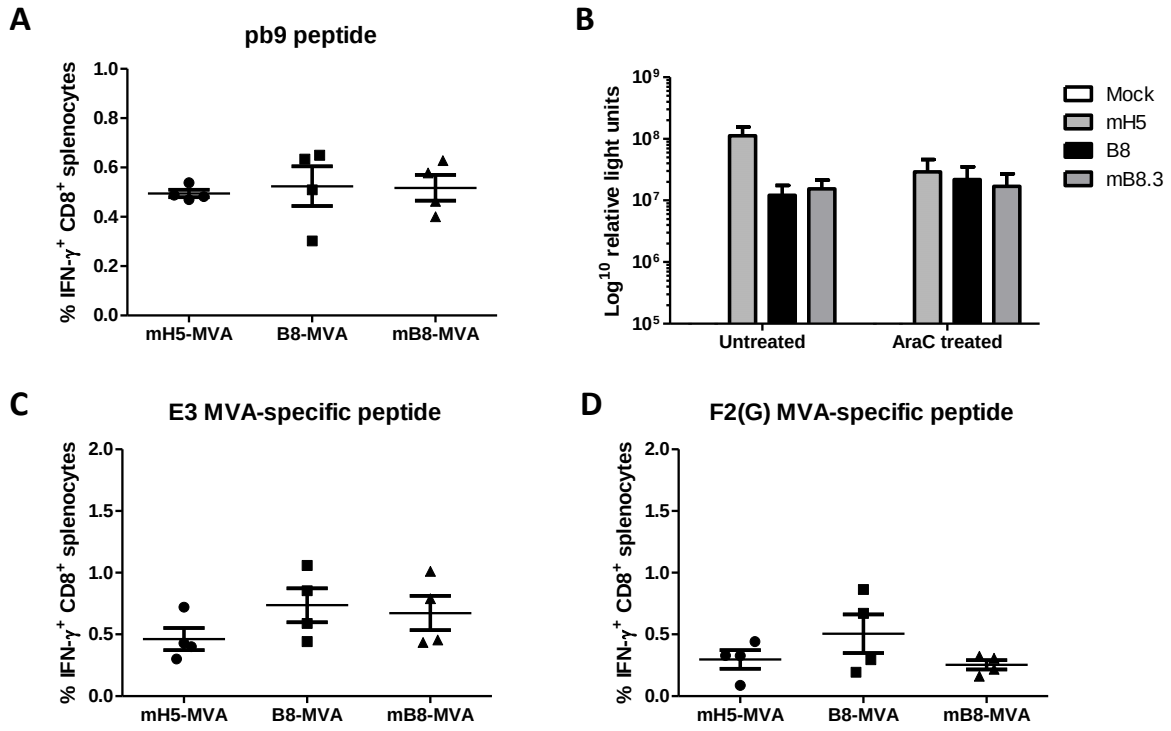


Figure 6.4: *In vivo* cellular immunogenicity of rMVAs with a modified pB8 promoter.

Three groups of female BALB/c mice (n=4) were immunised with B8-MVA, mB8-MVA (which has the mB8.3 promoter), or mH5-MVA (as an external control), all contain the tPA-pb9-rLuc transgene. Seven days post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in responses to *in vitro* re-stimulation with pb9 peptide **(A)**, or MVA-specific peptide **(C** and **D)**. These values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with the SEM error bars are shown. Data is representative of two independent experiments. The expression profile of the same rMVAs was determined in vitro using *Renilla* luciferase system **(B)**.

6.3. Discussion

In this chapter, we attempted to make an improvement to the pB8 promoter, which is one of the strong early promoters as described by Orubu *et al* [156] and was also confirmed in this thesis, chapter 4. The choice of pB8 was based on many criteria; the *B8R* ORFs is non-essential to the growth of MVA; the *B8R* gene encodes an IFN-γ receptor although this gene is fragmented and does not express an active protein [24]; the truncated B8 protein harbours one of the most immunodominant epitopes of MVA vector, both in C57BI/6 mice [113] and in humans[196]. So, *B8R* ORF deletion by using its endogenous promoter could reduce the vector antigenic complexity. More importantly, the promoter of this gene is a strong early promoter in spite of lacking two nucleotides that are dominant in the consensus sequence of early promoter core region [19,156,198]. In addition, substituting one of those two optimal nucleotides in the early element of the p7.5 promoter stopped the recombinant protein expression completely, which also supports their importance. Furthermore, optimising the early promoter core sequence based on the consensus sequence enabled others to optimise the H5 promoter, resulting in the improved and stronger mH5 promoter. Taken together, we hypothesised that changing one or two of those nucleotides in the core region of pB8 promoter would improve the transgene expression. The hypothesis was tested by deriving three rMVAs with the tPA-pb9-rLuc transgene driven by different version of modified pB8 promoter (mB8); in each virus we introduced one or two nucleotide substitution(s), making the pB8 core region much similar to the consensus sequence of vaccinia virus early promoters, as summarised in table 6.2.

The three viruses, mB8.1-MVA, mB8.2-MVA, and mB8.3-MVA, were tested in luciferase assay to determine promoter activity in driving the protein expression. This assay showed no difference between the three modified pB8 promoters and the control, which was the native pB8 promoter expressing the same transgenic protein. The lack of improvement was also observed over time and when the early expression was studied by AraC treatment. I therefore attempted to ensure that the transcript level, controlled by the modified promoters, is consistent with protein level in the luciferase assay. Thus, a real-time relative quantitative PCR (qPCR) was set up to determine the change in the transgene expression of mB8.3-MVA as compared to the control. However, the transcript level and the protein expression data were consistent in showing the similarity of pB8 to mB8 promoters. The lack of improvement could be because that the modification were based on data from optimising the p7.5 promoter that has early and late elements unlike the pB8 promoter, which is an early promoter. Although the modifications were also designed to mimic the consensus sequence of early promoters, the original pB8 was strong promoter [156] despite being different from the consensus sequence, especially at the most crucial guanine residue. So, it is likely that the pB8 is already an optimal promoter and could not be enhanced by further modification. These data could also imply that promoter activity is not based only on their core region sequences and other factors like the spacer region between the core sequence and the transcription start site could be important for promoter optimisation as has been suggested for other poxviral promoter [165]. However, prolonging the spacer of the pB8 promoter by inserting the EMCV IRES did not have any impact on its activity, as tested in chapter 3. Furthermore, the similar approach to modifying pB8 is the H5 promoter optimisation, which was achieved by one nucleotide substitution at the early element and one at the late element, resulting in the mH5

promoter. However, the mH5 was compared to the p7.5, SSP, but not to the original H5, so the presented work could not benefit from the mH5 study in this respect. Thus, the study of Davison and Moss [162] is the only published work suggesting that modifying nucleotides of vaccinia virus early promoters enhance their activities.

In vivo immunogenicity was performed to test whether these modification have an effect on early transgene expression *in vivo*, and would therefore presumably enhance the $CD8^+$ T cell-specific pb9-specific responses. However, the $CD8^+$ T cells induced by mB8.3-MVA were not significantly different from that of B8-MVA or mH5-MVA control groups. The dose used for mice immunisation was 10^6 pfu, which might be a high dose, inducing strong immune responses in all groups. These strong immune responses might reach immune "break-point" preventing detection of real differences that are associated with the used promoter.

6.4. Recommendations for future research

Overall, the modification of the pB8 promoter core region did not yield any improvement on either protein expression or transgene transcription. Thus, it seems that the pB8 has already reached its optimal activity or that the early promoter sequence is not the key to optimise early promoters. Also, it is likely that the crucial nucleotides for pB8 are not the modified A and G, but different from the crucial nucleotides in the consensus sequence.

Chapter 7

The Effect of Deleting Immunomodulatory and Non-Essential Genes on Immunogenicity of Recombinant MVA

7. The Effect of Deleting Immunomodulatory and Non-Essential Genes on Immunogenicity of Recombinant MVA

7.1. Introduction

The original attenuation of MVA resulted in the loss of almost third of its parental genome, deleting or mutating the majority of immune evasion and host range genes [3]. By losing many immunomodulatory genes, MVA has become a strong immune activator, infecting a range of immune cells and eliciting a wider range of chemokines and cytokines as compared to the parental VACV [88,93]. However, it has retained some genes that are involved in host-virus interactions and immune evasion such as *B15R,* encoding the IL-1β binding protein [123], or *A41L*, encoding the CC-chemokine binding protein [122]. Deleting some of these immunomodulatory genes that play a role in reducing immune responses to poxviruses might potentially increase anti-transgene responses. Moreover, since the number of MVA antigens is also reduced when some genes are deleted, there may be a second desirable effect of reducing anti-vector immunity. This immunity can be a challenge as discussed in the thesis introduction, and it is in many cases immunodominant over the transgenic antigens of a vaccine [196]. For example, deletion of VACV vector antigens boosted the transgenic antigen-specific immune responses in an experimental setting, suggesting the feasibility of reducing anti-vector immunity and boosting recombinant vaccine immunogenicity by vector-specific gene deletion [117].

Deleting some of the retained immunomodulatory genes improved innate, adaptive cellular or humoral immune responses to the vector (MVA) and to encoded

recombinant antigens, as discussed below. It also enhanced the protection efficacy of MVA following a challenge with VACV WR [122,123]. The deletion of ORFs *A41L*[122], *B15R* [123], or both of them [212], showed around 2-fold increase in memory $CD8^+$ T cell responses upon stimulation with VACV-infected cells or MVA-specific peptides. In these studies, the immune responses to recombinant antigens were also increased with a similar fold.

However, previous studies showed some variable results of the improved immunogenicity. In the study where both *A41A and B15R*[212] were deleted from MVA-B, rMVA encoding HIV-1 clade B immunogen, the use of different peptide pools for mice splenocytes re-stimulation revealed a shift in the increased $CD8^+$ T cell responses to one of the pools (GPN-pool) as compared to non-mutated MVA-B (control). The MVA-B mutant also induced more $CD8^+$ T cells than $CD4^+$ T cells only in one peptide pool (Env-pool). In another study, the deletion of *C6L* gene from MVA-B (ΔC6-MVA-B) also showed increased memory $CD4^+$ and $CD8^+$ T cell responses, and although this mutant did not shift the immune responses towards some peptide pools, the increased $CD4^+$ or $CD8^+$ T cells were only observed in one peptide pool (GPN-pool), but not in either Env- or Gag-pool [125]. Although the ΔC6-MVA-B was not tested for the peak (acute phase) adaptive immunity, a recent study from the same research group testing the effect of a double-deletion MVA-B, lacking *C6L* and *K7R* (ΔC6/K7-MVA-B) showed that the ΔC6-MVA-B, included as a control, had no impact on the peak immune responses [126]. This is not surprising as the majority of the previous studies reported small (around 2-fold) or no enhanced immunogenicity of MVA deletion mutants at acute adaptive immune responses. Therefore a possible explanation for the

variability seen between experiments is that the size of the effect is small, and when the lack of precision in defining the infectious titre of poxvirus preparations is taken into account, it is not surprising that results may be difficult to interpret. The double-deletion ΔC6/K7-MVA-B showed increased memory $CD8^+$ T cells, but not $CD4^+$ T cells; and upon comparison with ΔC6-MVA-B, the increase seemed to be mainly an effect of C6 absence and not due to the K7 deletion [126]. Furthermore, in more recent studies, increased memory $CD8^+$ T cells specific to MVA vector or to encoded antigens were also reported upon the single deletion of *F1L [128]*, *N2L [127]*, or *C12L* (MVA008L) [35] in rMVA with around 2-fold increase. Overall, it seemed that the increased cellular immunogenicity is antigen-specific, or rather epitope specific, which could make it difficult to apply the same deletion in a different MVA-based vaccine before carefully assessing the immunological outcome.

In terms of improving humoral immunogenicity in these studies, while the deletion of *A41L*, *B15R*, or the double deletion of both had no impact on the antibody induction at the memory phase, the deletion of *C6L* or *N2L* increased the memory antibody-mediated responses by around 2-fold in the total anti-recombinant antigen antibody titres. Of note, the *C6L* deletion enhanced the anti-gp120 antibody titres [125], but the re-testing of *C6L* deletion mutant, in a later study, along with the double deletion *C6L/K7R* mutant showed no significant difference in the anti-gp120 antibody titres by those two mutants compared to the control (MVA-B vaccine) [126]. The humoral responses were not tested in the *F1L* and *C12L* deletion studies.

Finally, the deletion of *A35R* ORF showed small non-significant increase in cellular immunogenicity but increased anti-VACV antibody titres by 2-fold with isotype

switching to more IgG and its subclasses IgG1 and IgG2a [124]. Overall, in mice studies, it seems that all reported increases in MVA mutant immunogenicity are small, with 2- to 3-fold at most. In these reports there is a large number of important variables such as the used dose and route of administration, vaccination regimens, recombinant antigens, immunological readouts (e.g. ELISpot vs. ICS), mice strains, time and type of stimuli used in the *ex vivo* re-stimulation (e.g. VACV-infected cells vs. E3 or F2(G) peptides), the memory date set for harvesting the spleens (i.e. varies from 10, 21, 56, or 180 days post-MVA injection).

On the other hand, in macaques monkeys, a study reported that deleting four genes; *C12L* (MVA008L), *B15R* (MVA184R), *A41L* (MVA153L), and *A46R* (MVA159R) from MVA encoding HIV-1 clade C consensus Gag and Env immunogens, given twice with 9 weeks interval, resulted in increased $CD4^+$ and $CD8^+$ T cells either after the prime (4-fold) or after the boost dose (5-fold); and around 25-fold increase in the binding anti-gp120 antibody titres [213]. In this report, the deletion of uracil-DNA-glycosylate (*udg*) gene, in addition to those four genes, resulted in a five-gene deletion mutant that did not further enhance the improved immunogenicity of the four-gene deletion mutant. The deletion of *udg* was expected to improve the immune responses, especially knowing that a previous report by the same group showed that the deletion of *udg* gene alone in rMVA inhibited the late MVA gene expression, reduced the antigen complexity of MVA, and elicited improved $CD4^+$ and $CD8^+$ T cells (2-fold) in a similar regimen, but with Gag gene from HIV clade B, which is a different recombinant antigen [121].

Previously, Cottingham *et al* reported that individual deletions of *C12L, A44L, A46R, B7R,* or *B15R*, from MVA did not enhance the cellular immunogenicity of the TIP model antigen (described in material and methods and [174]) using 7 immune dominant MVA epitopes in two strains of mice at the peak cellular responses. At the memory responses (day 56 post MVA injection), only ΔB15R-MVA showed a small (1.5-fold) but significant increase in the $CD8^+$ T cell frequencies [129].

In this chapter, I extended Cottingham and colleagues' study using MVA-BAC recombineering technology to examine the effect of deleting two clusters of fifteen genes (described in table 7.1) on cellular immunogenicity of rMVA. These deleted genes have different immunomodulatory functions or, in some few instances, unknown functions (table 7.2). The derived recombinant MVA mutants (rMVA mutants) encode either the 85A antigen of *M. tuberculosis*[175] or the TIP model epitope string-based antigen [174] that comprises an immune dominant H2-K^d-restricted murine malaria epitope (pb9) from the *Plasmodium berghei* circumsporozoite protein [214]. The MVA-BAC system was shown previously to have no altering effect on the immunogenicity of rMVA, and elicited anti-vector immunogenicity was similar to the conventionally derived rMVA [129].

Here, I constructed rMVA mutants that lack fifteen genes and encoding either the 85A or TIP transgenes (Δ15-MVA-85A and Δ15-MVA-TIP), in addition to one rMVA mutant, lacking the *A35R* ORF and encoding 85A (ΔA35-MVA-85A).These mutants were tested and compared to various control viruses such as MVA*wt*, MVA85A, MVA-BAC-85A,

MVA-TIP, which are all explained in the results section, and in table 7.1. The rMVA mutants and controls were used to assess cellular immunogenicity of these two different transgenes in different vaccination regimens. Mutants were tested in prime only or prime-boost vaccinations, and they were also assessed at different time points, sometimes also determining the memory $CD8^+$ T cell phenotypes.

MVA	Deleted ORFs	Notes
MVA*wt*	None	Not recombinant not modified virus
MVA85A	None	Conventionally derived MVA with 85A at the TK* locus. Markerless virus. Control for 85A responses
MVA-BAC-85A	None	BAC recombineering-derived MVA with 85A at the TK* locus. Control for 85A responses. Expresses GFP.
MVA-TIP	None	Encodes TIP antigen at the TK* locus. Contains BAC DNA and expresses GFP. Control for TIP responses.
ΔA35-MVA-85A	*A35R*	Encodes 85A antigen at the TK* locus. Contains BAC DNA and expresses GFP. Control for 85A responses.
Δ15-MVA-TIP **Δ15-MVA-85A** (i.e. Δ *A41L* to *46R*&*B7R* to *B15R*)	*A41L, A42R&A43R, SalF6R, A44L, A45R, A46R, B7R, B8R, B9R, B10R, B11R, B12R, B13R+14R,* and *B15R.* All mentioned above.	Contain BAC DNA. Express GFP. Encode either 85A or TIP at the TK*.

Table 7.1: MVA deletion mutants, lacking different clusters of genes, with the 85A or TIP antigen.

* TK: Thymidine kinase locus, used as an insertion site for recombinant antigens. BAC DNA is part of the MVA-BAC clone that was used to construct different mutants. GFP: Green fluorescent protein that is part of the MVA-BAC clone.

7.2. Results

7.2.1. Construction of rMVA deletion mutants

Deletion MVA mutants have been made previously by Cottingham *et al* [129] who showed the fast and efficient approach of deleting MVA non-essential genes by the MVA-BAC recombineering technique. Here, one of these MVA mutants, named Δ15-MVA-TIP, was selected for further investigations such as memory CD8+ T cell phenotyping and the effect of prime-boost vaccination regimen on immunogenic outcomes of this MVA mutant. The Δ15-MVA-TIP encodes the TIP antigen, which is an 82 amino acid epitope string from **T**uberculosis, **I**mmunodeficiency virus (HIV) and **P**lasmodium), including the protective H-2K^{d}-restricted pb9 epitope from *Plasmodium berghei* circumsporozoite protein [174].

The MVA-TIP, which is non-mutated rMVA with the TIP antigen inserted at the TK locus, was also used as a comparative control for immunogenicity experiments. The Δ15-MVA-TIP was selected, in particular, as it lacks 15 genes; some of which are immunomodulatory or unknown but non-essential genes as illustrated in table 7.2. Next, the mutant Δ15-MVA-TIP and a MVA mutant lacking the *A35R* gene, encoding the TIP antigen (ΔA35-MVA-TIP) were re-engineered to replace the TIP antigen with the antigen 85A to generate Δ15-MVA-85A and ΔA35-MVA-85A as explained in the materials and methods (chapter 2). Genomic sequencing was performed on viral DNA from the Δ15-MVA-85A mutant and revealed no genetic change, except the antigen replacement. This therefore confirmed that the genetic sequences of MVA-BAC clones are preserved despite passing through four rounds of MVA-BAC recombineering in bacterial cultures.

In addition, various non-mutant MVAs were also derived to serve as controls. First, MVA with only 85A antigen inserted (MVA-BAC-85A) was used as a comparative control for 85A immunogenicity studies. Second, another comparative control was the MVA85A vaccine (MVA85A), which has been to clinical trial phase IIb [175]. The difference between these two controls (MVA-BAC-85A and MVA85A) is that the MVA-BAC-85A was derived using the bacterial system, MVA-BAC recombineering, and retains the BAC DNA in its genome. This MVA-BAC was then rescued to an infectious virus and was then amplified in BHK-21 cells whereas the MVA85A vaccine was derived using homologous recombination to corporate 85A transgene into parental wild type MVA in chicken embryo fibroblast (CEF) cells. Last, wild type MVA (MVA*wt*) was included, in some of the immunogenicity studies, as a negative control for 85A responses. It served also as a positive comparative control for the vector-specific immune responses. All viruses are summarised in table 7.1.

Gene	Function in VACV	Status of Gene and [protein] in MVA	Reference
B7R	CC chemokine binding protein	Mutated by 15bp [Truncated]	[215]
B8R	IFN-γ soluble receptor	Pseudogene [Truncated]	[216]
B9R	Not involved in virulence	Pseudogene [Truncated]	[217]
B10R	Unknown	Mutated by 24bp [Truncated]	
B11R	Unknown	Conserved	
B12R	Kinase, not involved in virulence	Conserved	[218]
B13R/B14R	SPI-2 (serine protease inhibitors) Inhibits IL-1β converting enzyme and therefore blocks apoptosis	Fragmented pseudogene. Already fragmented in VACV	[219]
B15R	Virulence factor	Mutated by 18bp	[220]
B16R	IL-1β receptor (binding protein)	Conserved	[123]
A41L	CC-Chemokine binding protein	Conserved	[122]
A42R / ***A43R***	Profilin homolog / Unknown	Mutated by 15bp / Mutated by 12bp	[221] /
SalF6R	Unknown	Conserved. Similar to VACV WR	
A44L	Hydroxysteroid dehydrogenase Involved in VACV synthesized steroid hormones	1 aa substitution. [Slight change]	[222]
A45R	Inactive superoxide dismutase-like protein	Mutated by 12bp	[223]
A46R	TLR signalling inhibitor	Conserved	[224]

Table 7.2: Genes deleted in Δ15-MVA-TIP and Δ15-MVA-85A mutants.

7.2.2. Immunogenicity of pb9 epitope in rMVA deletion mutants in a prime only vaccination regimen with memory $CD8^+$ T cell phenotyping

Two groups of ten BALB/c mice were immunised with 10^6 Δ15-MVA-TIP or MVA-TIP intramuscularly. Seven days after the immunisation mice were bled and PBMC (Peripheral blood monocyte cells) were isolated and a blood ELISpot assay was performed. The results showed that both groups have similar interferon γ secreting splenic cells in responses to the *ex vivo* re-stimulation with MVA vector-specific peptides (E3 and F2(G), or to the pb9 peptide, which is part of the TIP antigen (Figure 7.1.1). However, there was a slight increase (not statistically significant) in the E3-specific responses with Δ15-MVA-TIP.

One month post immunisation (day 28), five mice from each group were sacrificed, and spleens were collected, to perform intracellular cytokine staining assay (ICS). This assay showed similar $CD8^+$ T cell responses to the pb9 or F2(G) peptides (Figure 7.1.3 A, and C). However, E3-specific $CD8^+$ T cell responses in the Δ15-MVA-TIP group were significantly higher than the control MVA-TIP group (Figure 7.1.3 B). Moreover, samples from these mice were stained for markers of memory $CD8^+$ T cell phenotypes: CD127 and CD62L using the staining panel presented in table 7.3. Then, samples were run on flow cytometry to determine central memory (T_{CM}), effector memory (T_{EM}), or effector ((T_{EEF}) $CD8^+$ T cell type using the gating strategy illustrated in figure 7.1.2. As a result, there was no difference in either the effector or effector memory $CD8^+$ T cells in responses to pb9 or F2(G) peptides (Figure 7.1.3 D and F). However, in responses to the E3 peptide, both effector and effector memory $CD8^+$ T cell showed slight increase in the 15ΔMVA-TIP group compared to the control group (Figure 7.1.3 E). Interestingly,

there was no detectable level of central memory $CD8^+$ T cell at this time point (28 days post MVA).

Marker	Fluorophore
Live/dead	AmCyan
CD8	PerCPCy5.5
CD4	Qdot655
INF-γ	APC
CD127	Pacific Blue
CD62L	PE-Cy7

	CD127	CD62L
T_{CM}	+	+
T_{EM}	+	-
T_{EFF}	-	-

Table 7.3: Markers used to identify the subsets of $CD8^+$ T cells.

Top: Staining panel used to identify subset populations of INF-γ^+ $CD8^+$ T cells. **Bottom:** T_{CM}: central memory, positive for both CD127 CD62L markers. T_{EM}: effector memory, positive only for CD127 marker. T_{EFF}: effector $CD8^+$ T cell, negative for both markers.

Three months post immunisation (day 84), the remaining five mice from each group were sacrificed, and spleens were collected, to perform intracellular cytokine staining assay (ICS). This assay showed similar $CD8^+$ T cell responses to the pb9, E3, or F2(G) peptides (Figure 7.1.4 A, B, and C). On the other hand, $CD8^+$ T cell phenotyping showed interesting results. Although the percentage of effector memory (T_{EM}) $CD8^+$ T cells was similar between groups in response to each peptide, the effector (T_{EEF}) $CD8^+$ T cells were detected only in the Δ15-MVA-TIP group, in responses to pb9 and E3 peptides (Figure 7.1.4 D, E, and F). This could imply the ability of Δ15-MVA-TIP virus to prime $CD8^+$ T cells for longer time than the non-mutant MVA (the control group). At this time point (3 months post MVA), both Δ15-MVA-TIP and MVA-TIP viruses had induced central memory $CD8^+$ T cells although this cell population did not show differences between the groups in responses to any of the three examined peptides (pb9, F2(G), and E3).

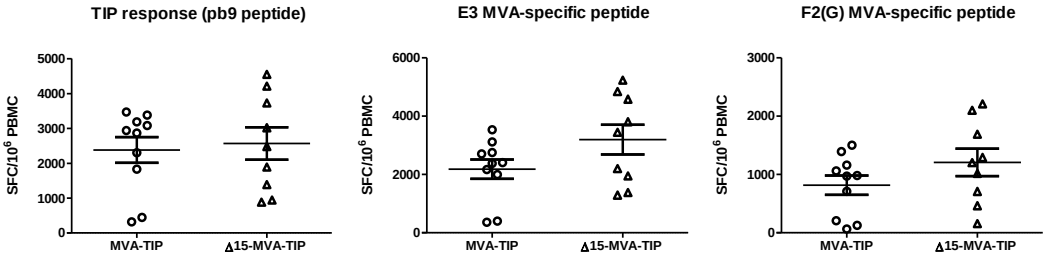


Figure 7.1.1: *In vivo* cellular immunogenicity of MVA deletion mutants with TIP antigen (7 days post immunisation).

Two groups of female BALB/c mice (n=10) were immunised with non-mutated MVA with TIP antigen (MVA-TIP), or MVA deletion mutant (lacking 15 genes) with TIP antigen (Δ15-MVA-TIP) at a dose of $1x10^6$pfu. **Seven days** post immunisation, *ex vivo* ELISpot was performed to determine the percentage of IFN-γ-secreting PBMC in responses to *in vitro* re-stimulation with pb9 peptide or with MVA vector-specific peptides (all three peptides are $CD8^+$ T cell specific). These values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with SEM error bars are shown. Data is representative of two independent experiments.

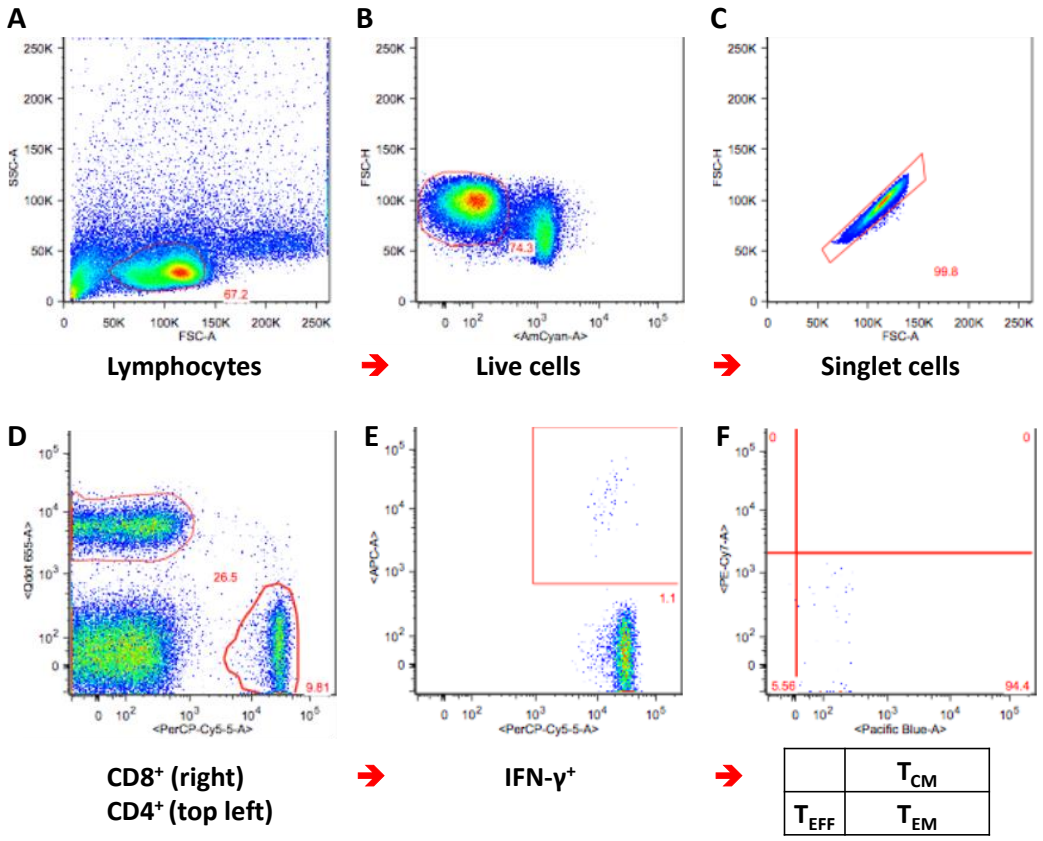


Figure 7.1.2: Gating strategy for intracellular cytokine staining assay (ICS).

A: Lymphocytes were gated following the standard cells dot plot. **B:** Live lymphocytes were not stained with AmCyan, which stains only dead cells**. C:** Singlet cells were then gated from live lymphocytes using area and height of forward scatter FSC-H and FSC-A. **D:** From C, PerCP-Cy5.5 and Qdot labeled antibodies were used to stain and gate for $CD8^+$ and $CD4^+$ T cell populations, respectively. **E: Using** APC labeled antibody INF-γ-secreting $CD8^+$ T cells were detected and gated for. **F:** quadrants plot based on INF-γ^+ $CD8^+$ T cells (E) using pacific Blue labeled Anti-CD127 marker and PE-Cy7 labeled Anti-CD62L marker. The table below indicates T cell subset populations in each quadrant.

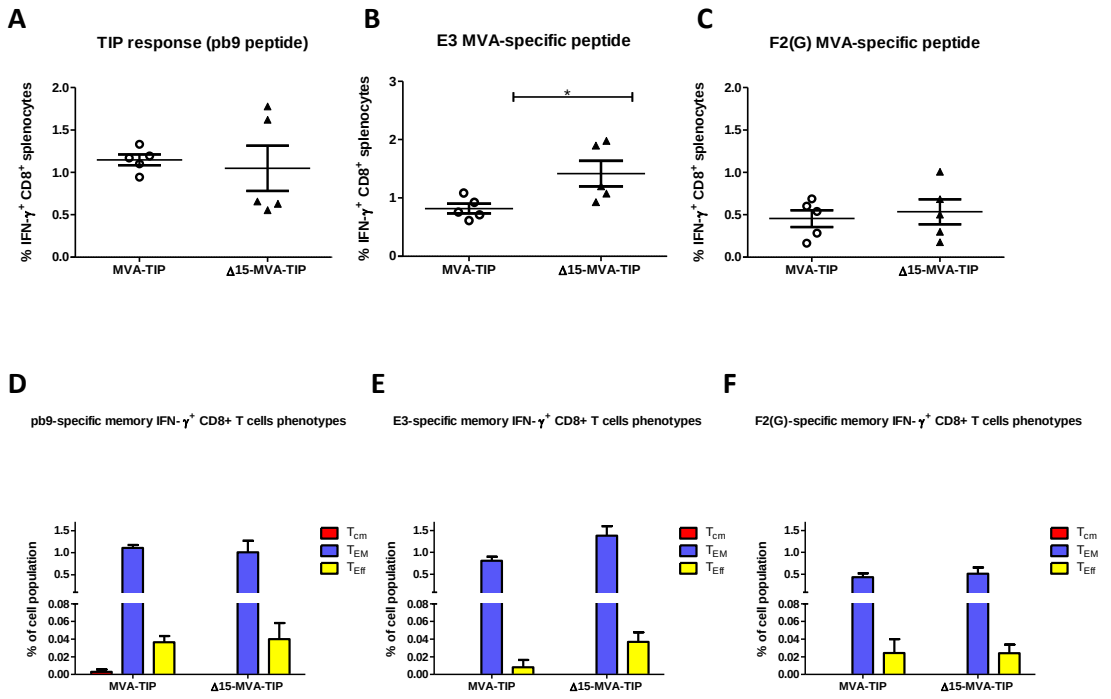


Figure 7.1.3: *In vivo* cellular immunogenicity of MVA deletion mutants with TIP antigen (28 days post immunisation).

Two groups of female BALB/c mice (n=10) were immunised with non-mutated MVA with TIP antigen (MVA-TIP), or MVA deletion mutant (lacking 15 genes) with TIP antigen (Δ15-MVA-TIP) at a dose of $1x10^6$pfu, as in figure 7.1.1. **28 days** post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in responses to *in vitro* re-stimulation with pb9 peptide **(A)**, or with MVA vector-specific peptides **(B and C)**. The memory T cell phenotypes was determined for every peptide response and presented at the bottom **(D, E, and F)**. T_{CM}: central memory T cells (red bars), T_{EM}: effector memory T cells (blue bars), and T_{EFF}: effector T cells (yellow bars). All values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with SEM error bars are shown. Data is representative of two independent experiments. * $p = 0.0331$, by *t*-test.

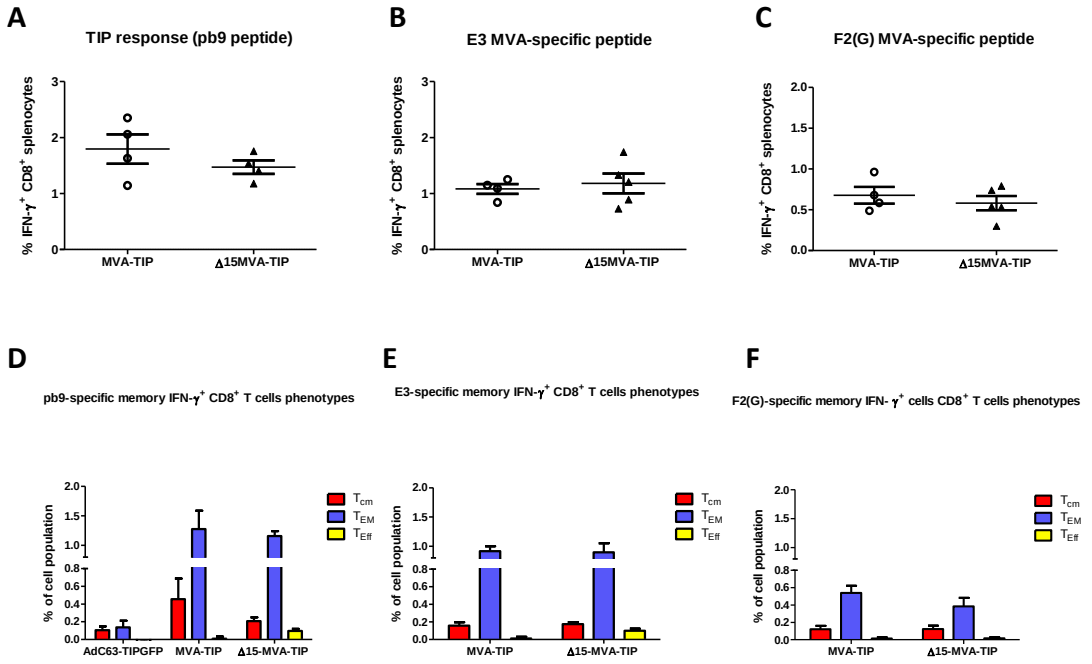


Figure 7.1.4: *In vivo* cellular immunogenicity of MVA deletion mutants with TIP antigen.

Two groups of female BALB/c mice (n=10) were immunised with non-mutated MVA with TIP antigen (MVA-TIP), or MVA deletion mutant (lacking 15 genes) with TIP antigen (Δ15-MVA-TIP) at a dose of $1x10^6$pfu as in figures 7.1.1 and 7.1.3. **84 days** post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in responses to *in vitro* re-stimulation with pb9 peptide **(A)**, or with MVA vector-specific peptides **(B and C)**. The memory T cell phenotypes was determined for every peptide response and presented at the bottom **(D, E, and F)**. T_{CM}: central memory T cells (red bars), T_{EM}: effector memory T cells (blue bars), and T_{EFF}: effector T cells (yellow bars). All values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with the SEM error bars are shown. Data is representative of two independent experiments. The T_{EEF} population was detected only in the Δ15-MVA-TIP group (D and E) but there was no statistical significance by One-way ANOVA.

7.2.3. Immunogenicity of pb9 epitope in rMVA deletion mutants in a prime-boost vaccination regimens

The Δ15-MVA-TIP mutant showed a slight increase only when E3 peptide was used for re-stimulation although this was performed in a single MVA shot immunisation regimen. In previous reports, the deletion of two (*B15R* and *A41L*) of these fifteen genes, which are deleted in Δ15-MVA-TIP, was reported to enhance MVA-B vaccine immunogenicity when used in DNA-prime MVA-boost vaccination regimens [212]. Therefore, a similar regimen was then carried out to test Δ15-MVA-TIP immunogenicity. Two groups of ten BALB/c mice were immunised intramuscularly with pSG2-TIP DNA, and boosted with intraperitoneal injection of 10^7 pfu of MVA-TIP or Δ15-MVA-TIP two weeks later. Five mice were sacrificed from each group at day 7 and day 56 after MVA boost. Intracellular cytokine staining (ICS) was performed on splenocytes that were re-stimulated with pb9, F2(G), or E3 peptides. The peak adaptive (at day 7) or the memory (at day 56) $CD8^+$ T cell responses did not show any significant difference between the MVA-TIP and Δ15-MVA-TIP groups (Figure 7.2.1 and Figure 7.2.2).

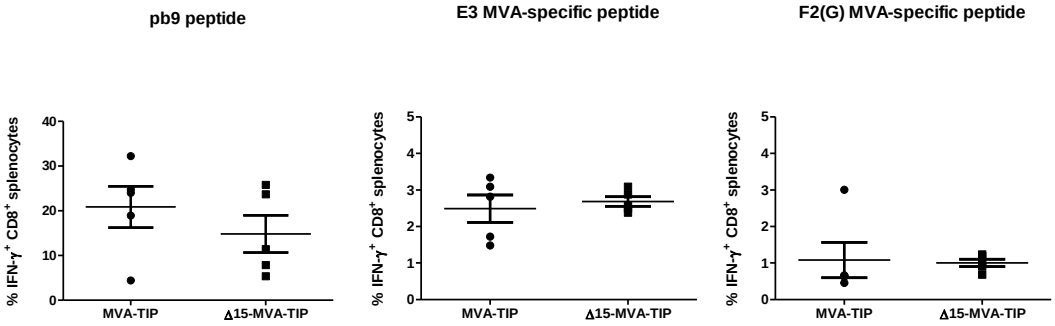


Figure 7.2.1: *In vivo* cellular immunogenicity of MVA deletion mutants with TIP antigen in a prime boost regimen (7 days post immunisation).

Two groups of female BALB/c mice (n=10) were immunised i.m. with 100 µg of pSG-TIP DNA, then boosted with i.p. injection of non-mutated MVA with TIP antigen (MVA-TIP), or MVA deletion mutant (lacking 15 genes) with TIP antigen (Δ15-MVA-TIP) a dose of $1x10^7$ pfu/ml for MVAs. **7 days** post boost immunisation, the intracellular cytokine staining and flow cytometry were performed on five mice from each group to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in responses to *in vitro* re-stimulation with pb9 peptide or with MVA vector-specific peptides. All values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with the SEM error bars are shown. Data is representative of two independent experiments.

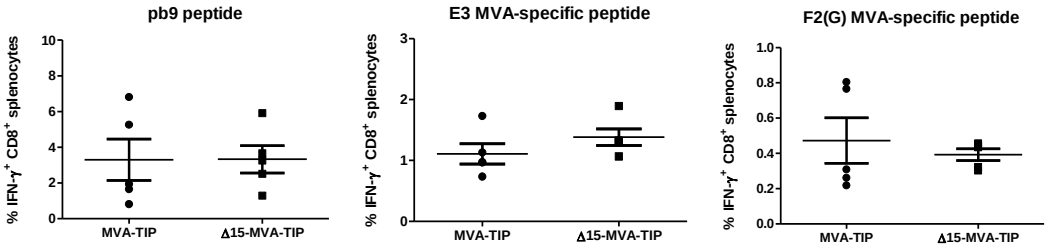


Figure 7.2.2: *In vivo* cellular immunogenicity of MVA deletion mutants with TIP antigen in a prime boost regimen (56 days post immunisation).

The remainder of five mice per group from the experiment above were sacrificed at day **56 days** and immunogenicity was determined exactly as in figure 7.2.1.

7.2.4. Cellular and humoral immunogenicity of 85A antigen in rMVA deletion mutants

The deletion of fifteen genes from MVA (Δ15-MVA-TIP) did not show any effect on the peak, memory, or memory phenotype of $CD8^+$ T cells in the tested animals. However, previous reports have presented the effect of deleting immunomodulatory genes on MVA immunogenicity, especially the deletion of *B15R* and *A41L* ORFs, but these reports used different recombinant antigens. This therefore encouraged the change of the TIP to the *M. tuberculosis* 85A antigen, which is known for its immunogenicity. The 85A antigen has defined $CD8^+$ and $CD4^+$ T cell-specific epitopes that can be used to measure both $CD8^+$ and $CD4^+$ T cell responses. Moreover, the 85A is the antigen in the MVA85A, the leading vaccine candidate in the TB vaccine field.

Replacing the TIP with 85A antigen in Δ15-MVA-TIP resulted in the same mutant but different recombinant MVA (named Δ15-MVA-85A). The 85A antigen was also inserted into a MVA mutant that lacks *A35R* gene, resulting in ΔA35-MVA-85A. In addition, non-mutated recombinant MVA with 85A was derived, using MVA-BAC recombineering technique, to serve as a comparative control (MVA-BAC-85A). The TB vaccine, MVA85A was also used as a second comparative control (named MVA85A). MVA wild type (MVA*wt*) was used as a negative control for 85A immune responses as well as a positive control for the vector-specific immune responses (table 7.1).

Next, five groups of mice were immunised with $2x10^6$ pfu of 1) MVA *wt*, 2) MVA85A, 3) MVA-BAC-85A, 4) ΔA35-MVA-85A, and 5) Δ15-MVA-85A. Seven days post

immunisation, splenocytes were collected and re-stimulated *ex vivo* using vector-specific peptides (F2(G) and E3, which are both $CD8^+$ T cell epitopes) and 85A peptide pool, which contains $CD8^+$ or $CD4^+$ T cell-specific epitopes. ICS was performed on these cells and showed similar levels of cellular immunogenicity between the groups, as measured by IFN-γ producing $CD8^+$ or $CD4^+$ T cells (Figure 7.3.1). This experiment was repeated with $1x10^6$ pfu, instead of $2x10^6$ pfu and showed no significant differences between the groups. Interestingly, the immune responses were similar between group 2 and 3 (MVA85A and MVA-BAC-85A) despite that the latter was derived using MVA-BAC recombineering and retains some bacterial DNA. ELISpot was thought to be more sensitive than the ICS, given that the cells are not treated with different antibodies, and was carried out on the splenocytes using $CD8^+$ T cell-specific epitope (named p11 peptide) and $CD4^+$ T cell-specific epitope (named p15 peptide). However, ELISpot data confirmed the ICS result and there were no significant differences in the $CD8^+$ or $CD4^+$ T cell responses (Figure 7.3.1). The MVA85A showed magnitudes of responses, in both ICS and ELISpot that were comparable to the usual responses, reported by the TB vaccine team in the Jenner institute. For example, in a similar regimen, they detect a group average of 23 SFU/million cells for the p11 peptide, or 70 SFU/ for the p15 peptide, in ELISpot. Here, I detected an average of 16 and 50 SFU/million cells, respectively (Figure 7.3.1).

Sera were collected from vaccinated mice, seven days after the vaccination, and ELISA was set up to determine whether a single MVA shot of these rMVA mutants was able to induce any detectable level, or differences, of anti-85A antibodies. Serum from mice that were vaccinated three times with MVA85A-IMX313 (IMX313 is a molecular

adjuvant [135]) was used as a positive serum, and serum from a naïve BALB/c mouse was used as a negative serum. However, ELISA showed no detectable level of serum anti-85A antibodies in all groups, as compared to the positive control serum (Figure 7.3.2). ELISA was performed with serum dilution factor of 1:100 (Figure 7.3.2) or with 1:10 (not shown) but with a similar result.

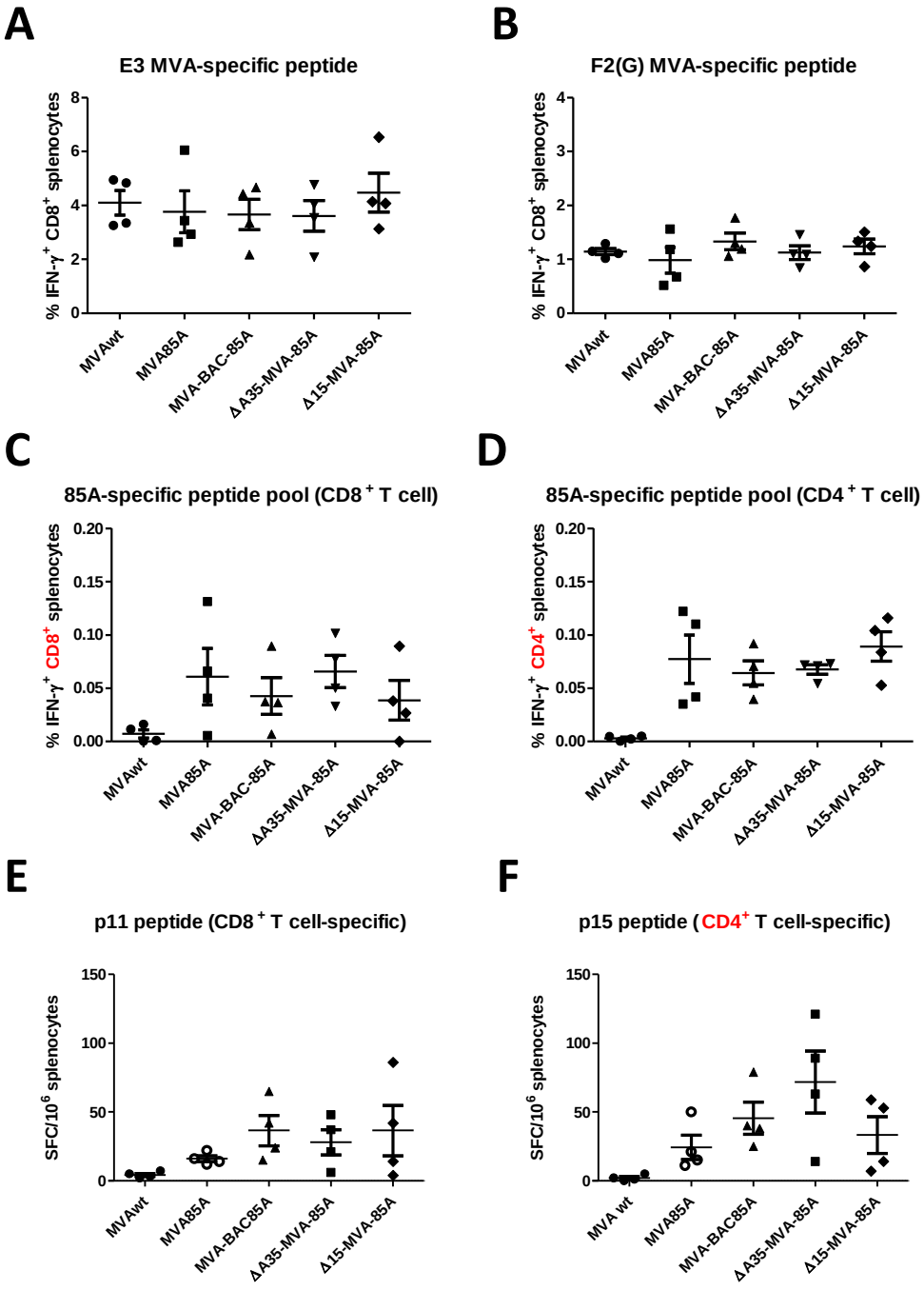


Figure 7.3.1: *In vivo* cellular immunogenicity of MVA deletion mutants with 85A antigen.

Five groups of female BALB/c mice (n=4) were immunised with the respective MVAs at a dose of $2x10^6$pfu/ml. Seven days post immunisation, the intracellular cytokine staining and flow cytometry were performed to determine the percentage of IFN-γ-secreting $CD8^+$ T splenocytes in responses to *in vitro* re-stimulation with MVA vector-specific peptide (**A** and **B**), with $CD8^+$ T cell **(C)** or $CD4^+$ T cell **(D)** specific 85A peptide pool. These values are presented after subtracting the values of unstimulated cells for every mouse (sample). The mean of each group with SEM error bars are shown. Data is representative of two independent experiments. **MVA*wt*:** non-recombinant non mutated MVA, **MVA85A:** MVA expressing TB 85A antigen, **MVABAC85A:** MVA, containing BAC DNA and expressing TB 85A antigen, **ΔA35-MVA-85A:** MVA mutant, containing BAC DNA and expressing TB 85A antigen with the *A35R* ORF deleted, and **Δ15-MVA-85A:** MVA mutant, containing BAC DNA and expressing TB 85A antigen with 15 genes deleted. **p11:** $CD8^+$ T cell-specific peptide [EWYDQSGLSVVMPVGGQSSF]. **p15:** $CD4^+$ T cell-specific peptide [TFLTSELPGWLQANRHVKPT].

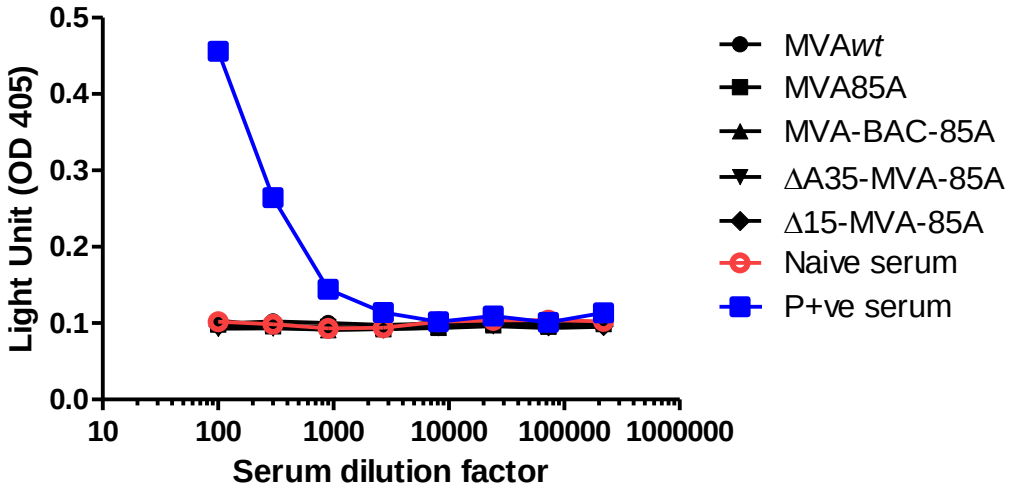


Figure 7.3.2: *In vivo* humoral immunogenicity of MVA deletion mutants with 85A antigen.

Sera were collected from mice immunised with different MVA deletion mutants with 85A antigen (same experiment as figure 7.3.1, above) and used to assay mice sera anti-85A antibodies. ELISA was performed to measure antibody immune responses in mice from the respective groups. The values are the mean of duplicates for every dilution of every sample, and then the mean of 4 mice's mean values is presented here. Data is representative of two independent experiments. Serum from unvaccinated mouse (naïve serum) was used as a negative antibody control. The positive antibody control (P+ve serum) is serum from a mouse that received three shots of 10^7 pfu MVA85A-IMX313 (IMX313 is a molecular adjuvant). **MVA*wt*:** non-recombinant non mutated MVA, **MVA85A:** MVA expressing TB 85A antigen, **MVABAC85A:** MVA, containing BAC DNA and expressing TB 85A antigen, **ΔA35-MVA-85A:** MVA mutant, containing BAC DNA and expressing TB 85A antigen with the *A35R* ORF deleted, and **Δ15-MVA-85A:** MVA mutant, containing BAC DNA and expressing TB 85A antigen with 15 genes deleted.

7.3. Discussion

This chapter presented an investigation on the effect of deleting some immunomodulatory or non-essential genes from MVA, in different combinations (table 7.1), on the cellular immunogenicity. Deleting either one (*A35R*) or fifteen genes from rMVA showed no significant differences in $CD8^+$ T cell responses to the murine malaria epitope pb9 or to the vector-specific E3 and F2(G) epitopes. The use of TIP model antigen, which harbours the pb9 as a strong immune dominant epitope, enabled us to follow the $CD8^+$ T cell responses for three months, but there was no significant difference at this long-term memory phase. Many previous studies showed that the single deletion of *C6L* [125], *N2L* [127] or *C12* [35]*;* or the double deletion of *A41L*/*B15R (REF)* and *C6L*/*K7R (REF)* from MVA vectors enhanced the cellular immunogenicity in prime-boost vaccination regimens. This type of regimen is also relevant to MVA clinical testing since MVA is used as a boosting agent in many vaccine clinical trials [72]. Thus, I applied this regimen priming with DNA via i.m. route, and boosting with MVA given i.p. Moreover, some of these reported genes, such as *A41L* and *B15R* are deleted in the Δ15-MVA-TIP mutant, and should elicit stronger cellular immune responses, especially following this regimen. Nevertheless, our results did not show any improved immunogenicity with the Δ15-MVA-TIP in prime-only or prime-boost vaccinations.

Garber *et al* (2012) reported that the deletion of four immunomodulatory genes enhanced MVA cellular immunogenicity in macaques, but the deletion of a fifth gene did not extend this improved immunogenicity [213]. Yet, when this fifth gene was deleted singly, the immunogenicity was enhanced in the same experimental setting

[121]. It could be that the deletion of some genes could compensate for the absence of other deleted genes and the overall immunogenicity is not improved, but the unimproved immunogenicity with our large deletion mutant (Δ15-MVA-TIP), which lacks *A41L* or *B15R,* was noted even when the *A41L* or *B15R* were singly deleted, as previously published by Cottingham *et al*[129]. Moreover, these two genes were deleted in a different cocktail of deleted gene-clusters in different MVA mutants, in the Jenner institute (Alharbi, *et al*, in preparation, from Matt Cottingham's work) and none of these mutants showed any enhanced immune responses. This could rule out the possibility that the enhancement by some deletions could be rendered useless by other deleted genes in the same mutant. The reports from other groups on the deletion of *B15R*, *A41L*, or both of them showed enhanced immunogenicity, but this enhancement was only 2-fold increase at the best [122,123,212]. This small increase might be due to technical or biological details such as the titration error. In the Jenner institute, researchers used to repeat titration experiments to define infectious titre of individual batches of MVA vaccine before clinical trials, and usually detect 2-3 fold differences in average, sometimes even higher. These data are not published but documented and kept in the trials files, in the Institute. In addition, FDA accepts a difference of up to 0.5 log on titration of viral vaccines, using a validated assay.

In a similar discrepancy, although the *C12L* and *C6L* were reported to enhance cellular immunogenicity when singly deleted from rMVA [35,125], the deletion of many genes, including the *C12L* and *C6L,* from MVA-TIP did not enhance cellular immune responses (Alharbi, *et al*, in prep). Moreover, in this manuscript, the single deletion of

C12L[129]or *C6L* from MVA-TIP did not enhance the TIP immune responses or reproduce what have been reported before [35,125].

Nevertheless, the concern over the use of non-natural antigen like the TIP should be disregarded by using a "real" immunogenic antigen. The TB 85A antigen is one of the best candidates that reached phase IIb clinical trial and showed strong $CD4^+$ T and $CD8^+$ T cell immunogenicity [175]. Therefore, I replaced the TIP with 85A antigen in the large MVA deletion mutant (deriving Δ15-MVA-85A from Δ15-MVA-TIP), and the viral DNA was extracted and sent for genomic-sequencing. The sequencing data revealed that the viral genome remained unchanged, except the replacement of the TIP with 85A antigen, despite the fact that this MVA-BAC has been through four rounds of bacterial recombineering before the insertion of the 85A transgene. The sequencing result showed the robustness and genetic preservation of the MVA-BAC recombineering system (See genomic sequencing data, Figure S7.1, Appendices).

The Δ15-MVA-85A mutant was then tested in mice, measuring both $CD4^+$ and $CD8^+$ T cell responses to the 85A peptide pool in ICS or to two strong individual peptides (specific for $CD4^+$ or $CD8^+$ T cells) in ELISpot. Here, three control viruses were included; the MVA85A vaccine that is used for clinical testing against TB, the MVA-BAC-85A (which is similar to MVA85A, except it was derived via MVA-BAC recombineering), and the MVA wild type (MVA*wt*), which is non-recombinant and non-mutant control. One reason behind the inclusion of MVA85A vaccine was to relate these modifications to the clinical testing, in case we achieved any improvement.

The results did not show any improved immunogenicity even with this known immunogenic antigen. Although I was able to test $CD4^+$ T cell responses, there was no difference in this type of T cells between the mutant group and any of the control groups. In addition, I used serum samples from vaccinated mice to assess the antibody responses, and there was no detectable level of Anti-85A antibody titres. Because the positive control sera were obtain from mice that were vaccinated three times with MVA85A-IMX313 (IMX313 is a molecular adjuvant), both the dose and the regimen might be very weak for inducing antibody responses, explaining the lack of detectable antibody titres in our MVA deletion mutants.

It could be speculated that the MVA-BAC system or deriving MVA in BHk-21 cell lines had some effect on these mutants, which could prevent them from enhancing the immune responses. The BHK-21 cell line, in particular, was reported to allow for the replication of some MVA mutants lacking *E3L*, which is difficult to grow in CEFs [92]. This was because BHK-21 cell line expresses low concentration of INF-α/β upon the infection of the E3-deleted MVA mutant, unlike CEFs that express high concentration of INF-α/β, leading to abortive replication of the mutant [92]. The BHK-21 cells have also a low level of PKR (double-stranded RNA-dependent protein kinase), compared to the CEFs, which allowed for the growth of the E3-deleted MVA mutant [92]. The PKR is an important immune mediator in the NFκB activation, so the growth of MVA mutants in BHK-21 cells may have less immune pressure. However, any proposed effect of MVA-BAC system or BHK-21 cells could be ruled out by comparing the vaccine MVA85A to the MVA-BAC-85A; the MVA85A was derived in the conventional method of constructing vaccinia virus recombinants and was propagated in CEFs whereas the

MVA-BAC-85A was derived using MVA-BAC bacterial system and has been through multiple rounds of MVA-BAC recombineering, then it was rescued using fowlpox virus and propagated in BHK-21 cell lines. Both viruses induced similar $CD4^+$ and $CD8^+$ T cell responses to the 85A-specific peptide pool in ICS or to the individual strong peptides in ELISpot as well as similar $CD8^+$ T cell responses to the vector-specific E3 and F2(G) peptides. This was also supported by the result of MVA*wt* as compared to either MVA85A or MVA-BAC-85A that showed similar responses to the vector specific E3 and F2(G) peptides. The MVA-BAC system was also shown to have no altering effects on the immunogenicity of MVA [129]. Taken altogether, producing rMVA using MVA-BAC system and BHK-21 cell line should not have any effect on MVA immunogenicity or testing vaccines in future.

In conclusion, none of the derived rMVA mutants showed improved immunogenicity either to the MVA antigens or to the encoded TIP antigen. Focusing on the large deletion Δ15-MVA-TIP mutant, neither prime only nor prime-boost regimens showed any improved $CD8^+$ T cell responses. The memory responses at 28 or 84 (long-term) days post MVA immunisation showed no improved memory $CD8^+$ T cell frequencies, except to one peptide (E3) at day 28. At day 56, in prime-boost regimen, this increase was also observed but it was very marginal and non-significant. Even replacing TIP with the TB 85A "real" antigen (in Δ15-MVA-85A) and testing $CD8^+$ as well as $CD4^+$ T cell responses, there was no significant difference as compared to various control viruses. This study suggests that the previously reported immunogenicity increases in MVA deletion mutants, which were small increases in many cases (2-fold), could not be replicated for different antigens.

7.4. Recommendations for future research

The approach of gene deletion to improve MVA-vectored vaccines should be carefully assessed for every recombinant antigen and epitope (peptide), and may not be taken as a generic approach for improving MVA immunogenicity. The enhanced immunogenicity reported by other groups was small increases, which might come from unavoidable technical errors. So, extra care should be taken when setting up these studies, especially over defining infectious titre. We, in the Jenner institute, used to see 2-3 fold differences in the titre of the same batch of an MVA vaccine, which was manufactured by external supplier following cGMP standards. Moreover, The mouse group size was small in many of these studies in the literature.

Ideally, to detect a real biological difference, the mutant that enhanced immunogenicity should be shared with other researchers who could replace the transgene and determine whether the same increase can be detected.

Chapter 8

Concluding remarks

8. Concluding remarks

Modified Vaccinia virus Ankara has been extensively used as a viral vector for a variety of candidate vaccines. Its excellent safety profile has been well documented in many clinical trials. MVA can induce robust innate immune activation, partially due to its loss of many immunomodulatory genes. For adaptive immune responses, rMVA can induce recombinant antigen-specific T cells in a higher magnitude than rVACV. MVA has been shown in many studies to enhance T cell immunity when used as a boosting agent in heterologous prime boost regimens, priming with DNA or adenoviral vectors. It expands the primed antigen-specific T cells without priming a high level of anti-vector T cells, inducing lower vector-specific T cells than either rVACV or nrMVA, which gives MVA an exquisite advantage as a vaccine viral vector. Although MVA is a potent T cell inducing vector, it has also been shown to elicit protective antibody-mediated responses when used in the right combination of dosage, vaccination regimens, and a suitable antigen for antibody induction. Therefore, enhancing MVA immunogenicity would improve an already versatile tool and assist vaccine development.

This thesis was planned to test different hypotheses concerning novel approaches, all aimed at improving MVA immunogenicity in the context of vaccine vector. Fifteen rMVAs were produced to test these hypotheses, which encode either a murine malaria strong epitope (pb9) or the TB antigen 85A. The transgene in the majority of these rMVAs is the tPA-pb9-rLuc that has a *Renilla* luciferase gene (rLuc) fused to the pb9 epitope to determine the transgene expression level *in vitro*, with the tPA leader sequence to help in detecting secreted transgenic protein in supernatants.

First, an internal ribosome entry site (IRES) from endomyocarditis virus (EMCV) was inserted into a rMVA between the ATG start codon of the transgene and the pB8 promoter at the *B8R* locus. The insertion of the EMCV IRES did not improve transgenic protein expression or transgene transcription *in vitro*, or the cellular immunogenicity in BALB/c mice. This first part of the project also involved *in trans* overexpression of the D10, the main decapping enzyme in vaccinia virus, which revealed that this mechanism occurs in MVA and reduced viral growth and transgene expression. The EMCV IRES, however, did not protect transgene transcripts from the effect of the excess amount of the D10 protein. Second, the core region sequence of the pB8 promoter was modified by nucleotide substitutions to make it close to the consensus sequence of VACV early promoter core region. This approach did not, however, result in any improvement in transgenic protein expression or transgene transcription *in vitro*, or *in vivo* cellular immunogenicity. Third, finding a stronger endogenous promoter was attempted by replacing non-essential or fragmented ORFs in the MVA genome, such as *B8R, E3L, and F11L*, with the transgene. Replacing these ORFs exactly at the ATG start codon allows for assessing activity of promoters in their natural context whilst simultaneously creating new insertion loci in the MVA genome. This approach revealed that the pB8, pE3, and pF11 endogenous promoters have slightly lower or similar *in vitro* activity to that of the p7.5 or mH5 conventional promoters. Next, inserting the pB8, pE3, or pF11 promoters at the TK locus, called ectopic promoters, showed similar activity to their endogenous counterparts. For *in vivo* cellular immunogenicity, all of the three promoters at either endogenous or ectopic locations resulted in higher T cell magnitudes than those induced by p7.5-MVA or mH5-MVA. This enhanced cellular immunogenicity was statistically significant in the case of pF11 promoter despite the absence of correlation between cellular immunogenicity and *in vitro* transgene

expression of the mH5-MVA and F11-MVA. This lack of correlation was not observed when the two viruses were tested for *in vivo* transgene expression using *in vivo* imaging. In fact, the pF11 activity *in vivo* was statistically significantly higher than that of mH5 promoter and that correlated with *in vivo* immunogenicity. Fourth, the effect of disrupting the TK gene (by using it as an insertion site) on MVA immunogenicity was tested in pairs of rMVAs. Each pair of recombinant MVAs has the same endogenous promoter (pB8, pE3, or pF11) driving the transgene with disrupted or intact TK gene. Disrupting the TK by inserting *mCherry* gene (a fluorescent marker) did not affect transgene expression *in vitro*, but resulted in lower cellular immunogenicity in the case of the pB8 and pF11 promoters. The F11-MVA viruses with disrupted or intact TK gene were selected for further testing because the reduction in cellular immunogenicity was clearer than with the pB8 promoter. They were tested in a non-permissive cell line but showed no difference in transgene expression, except at a late time point, indicating the role of the TK in providing more DNA synthesis and presumably more template DNA for transgene expression. Therefore, *in vivo* imaging was performed to determine *in vivo* promoter activity. Strikingly, pF11 promoter activity *in vivo* was reduced when the TK was inactive. This expression profile was correlated with the *in vivo* cellular immunogenicity as the F11-MVA with inactive TK induced lower T cell responses than the F11-MVA with an active TK. These results showed the importance of testing both promoter activity and immunogenicity in the same system (e.g. BALB/c mice).

The approach of utilising stronger promoters such as the ectopic pF11 in MVA could be readily applied to other poxviral vectors such as NYVAC or FPV. This is because poxviral promoters are recognised only by poxviral transcription machinery, which is packaged

into poxvirus virions. This approach however is limited to poxviral vectors. Other viral vectors such as adenovirus could not benefit from the promoters presented in this thesis. Therefore, viral vectors other than poxviruses require new studies that focus on the search for stronger promoters to enhance transgene expression.

On the other hand, disrupting the TK gene in E3-MVA (which utilises the pE3 promoter to drive the transgene expression) resulted in rather higher or similar immunogenicity to the E3-MVA with intact TK. The E3 protein is essential for MVA replication, acts as anti-apoptotic factor, and one of the immunosuppressive factors, its deletion has been shown to impair DNA replication and late gene expression [210]. So utilising the pE3 promoter in E3-MVA resulted in E3-deletion mutant and further disrupting the TK in E3-MVA also resulted in a double E3/TK-deletion mutant. However, the TK inactivation did not reduce the immunogenicity of E3-MVA (as with the case of pB8 and pF11 promoters). One explanation for this is that the E3-MVA, which is an E3-deletion mutant, affects the DNA replication of MVA *in vivo* and halts its late gene expression, presumably reducing its immunogenicity so that the inactivation of the TK would not result in a further reduction of the immunogenicity. Another possibility is that the enhanced immunogenicity observed with E3-MVA is a result of the pE3 promoter and is not due to the absence of the *E3L* ORF; this is supported by the similar immunogenicity results of rMVA with endogenous or ectopic pE3, excluding any effect that might come from deleting the *E3L* ORF. Therefore, by excluding the effect of ORF deletion, disrupting the TK in E3-MVA should result in reduced level of immunogenicity as occurred with the pB8 and pF11 utilising viruses. As this was not the case, the explanation here could be that the TK inactivation does not affect transgene

immunogenicity when the *E3L* is deleted. This, however, needs to be elucidated by systematic virology experiments, and I could not continue because the work of this thesis was defined by the scope of vaccine development. Since the E3-deletion MVA does not replicate in cells that are cGMP certified for vaccine production such as CEFs, there is little to be gained by pursuing the development of E3-deleted MVAs for clinical vaccine development.

The use of fused tPA-pb9-rLuc transgene allowed for simultaneous assessment of transgene expression and immunogenicity, especially *in vivo* although this has its limitations. For example, the pb9 epitope is not suitable to study effects on peptide processing and presentation. Moreover, the differences detected between p7.5, mH5, SSP, pB8, pE3, or pF11 could be limited to the enzymatic activity of luciferase and may vary when a natural antigen is used. These differences could also vary considerably as a result of titration errors. In fact we have noticed that immunogenicity (potency assays) of the same MVA vaccine could vary because of repeating titration assays. This variance sometimes reached 2-3-folds. Moreover, the FDA accepts variation of up to 5-fold in viral vaccine titres.

The fifth approach was the deletion of immunomodulatory or unknown non-essential genes from the MVA genome and studying the effect on cellular immunogenicity. There is a growing body of literature showing that MVA immunogenicity could be enhanced by immunomodulatory gene deletion. However, the deletion of a single gene (*A35R* ORF) or two regions of multiple genes (15 genes in total) from the MVA

genome that encodes either the pb9 epitope or the TB antigen 85A did not result in any differences in cellular immune responses to rMVA, in different vaccination regimens. My results support earlier studies by Cottingham *et al* [129]showing that deleting immunomodulatory genes from rMVA did not enhance the cellular immune responses to either the vector or recombinant antigens. This suggests that gene deletion from rMVA encoding a certain antigen could not be applicable to other antigens due to many technical details that vary across laboratories. Therefore, gene deletion may not be taken as a generic application to enhance MVA immunogenicity and should be carefully assessed for individual antigens.

Overall, this thesis presents advancements in using MVA as a T cell inducing viral vector that might accelerate the search for effective vaccines against infectious diseases and cancer. First, the pB8 and pF11 promoters can be used in their natural loci, with the option of still using the p7.5 or mH5 promoter at other loci like the TK, to express multiple antigens from a pathogen or different pathogens. This should allow for a multivalent MVA vector, a vector that could bring about effective vaccines against complex pathogens. For example, malaria is caused by *Plasmodium ssp.* that consist of different forms in different stages of the life cycle, so different antigens are expressed on the parasite surface in different stages. Therefore, rMVA that encodes antigens from blood-stage merozoite, and liver-stage (pre-erythrocytic) sporozoite, and gametocyte (or other mosquito-stage forms) should enhance the block of the parasite from interacting with its targets in various stages.

Second, this thesis presents the use of the pF11 promoter at the *F11L* locus without disturbing the TK gene to increase *in vivo* transgene expression and immunogenicity. This might be useful to increase the immunogenicity of some current vaccines that are already in clinical testing. For instance, MVA85A is a vaccine against TB, which has been recently tested in humans as the first TB vaccine to enter clinical trial phase IIb since the BCG was widely introduced in the 1940s. The trial resulted in modest cellular immunogenicity and modest protection (17% efficacy, not significant) that would benefit from enhancing the vaccine immunogenicity [175]. However, a vaccine against a complex pathogen such as TB might require more than changing a promoter. A vaccine against TB might need to have multiple antigens, as suggested above, or a more immunogenic antigen. It would also surely benefit from a more effective vaccination regimen.

Vaccines against complex pathogens like TB and malaria or difficult pathogens like HIV and HCV might need more effective adjuvants or strategies. In terms of adjuvants, MVA by itself can be a potent adjuvant and non MVA-based vaccines could be enhanced by combining them with MVA-based vaccines, in preclinical and clinical trials. In terms of effective strategies, virus-like particles (VLP) have become a useful tool to generate strong antibody-mediated immunity against some viruses, therefore a multivalent MVA, discussed above, could encode multiple proteins that are able to form VLPs *de novo* in the vaccinated animals or subjects. This, if proved possible, would save the time and effort of making and purifying VLPs prior to vaccinations. Moreover, some of the recombinant protein produced intracellularly might not form a functional VLP, but would still induce immune responses.

In addition, MVA is now being manufactured and stockpiled as the third generation vaccine against any bio-threat of smallpox. Therefore, licensure and application of any MVA-based recombinant vaccines would allow for simultaneous vaccination of humans against smallpox and would reduce the risk of its bio-threat. MVA has also been used to vaccinate animals against other poxviruses, for example, vaccinating camels against camelpox virus (CMLV) [225]. Recently, camels have been reported as the reservoir for a new emerging coronavirus (MERS-CoV), so developing an MVA-based MERS-Cov vaccine would vaccinate against two infectious diseases, one is an animal pathogen (CMLV) and one is a zoonotic (MERS-CoV). In my view, these two diseases are in economic (in case of CMLV) and health (MERS-CoV) importance to one geographical area, like the Arabian Peninsula, and targeting them together might also attract more funding bodies, which is fundamental for developing a vaccine.

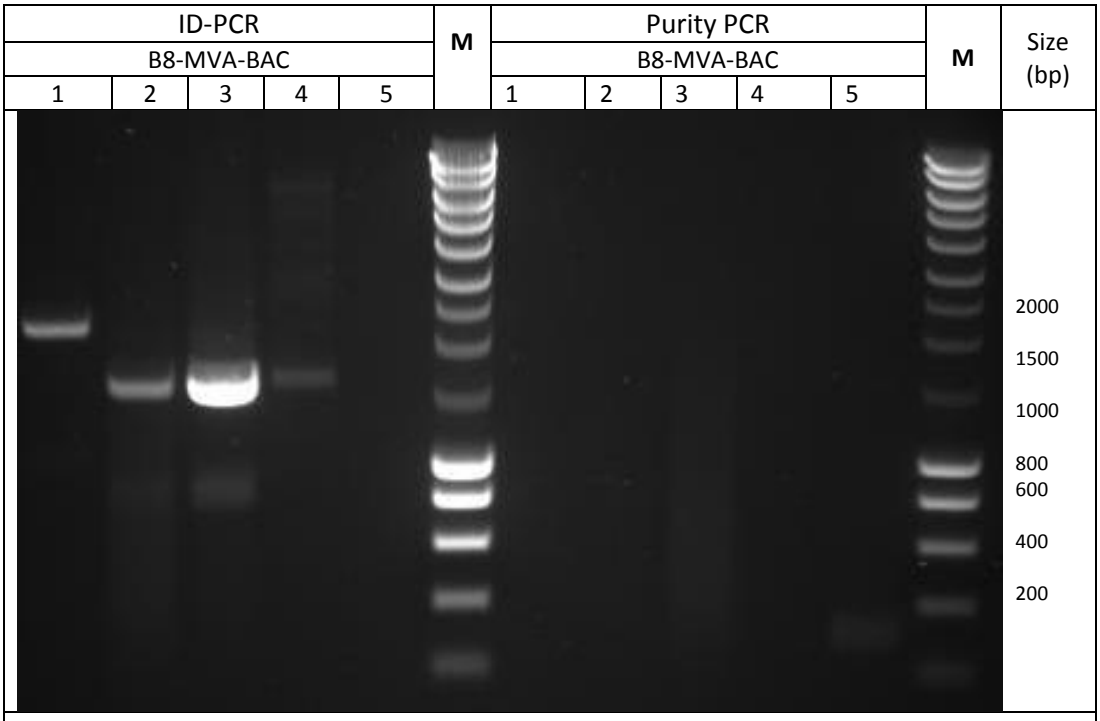
Another example of emerging disease is the recent (2014) outbreak of Ebola virus (EBOV) in West African countries such as Guinea and Sierra Leone with a mortality rate of around 50%[226]. As responses, there has been a number of candidate vaccines developed against EBOV. One candidate antigen is vectored by Chimpanzee adenovirus type 3 (ChAd3, by GSK) and is being tested in Phase I clinical trial in Oxford University (UK) and in the NIH (USA) [226]. An MVA vector encoding the same antigen will be tested as a boosting vaccine in the near future. This suggests that MVA is one of the first line platforms in response to emerging infectious diseases.

Vaccines for emerging viruses, such as MERS-CoV and EBOV, require a quick and immunologically robust platform to produce vaccines. MVA is a potent immunogenic vector, but its manufacturing requires a long and slow process based on isolating CEFs from chicken eggs. Therefore, to optimise this vector, it would ideally be produced in cell lines. I think, there are two ways of tackling this; first, by validating cell lines for cGMP standards, cell lines that are permissive for MVA replication such as the BHK-21 or other quail cell lines like EB66, AGE1.CR, or QOR2. Second, by mutating MVA to enable it to grow in the already cGMP certified cell lines such as VERO and MDCK.

Overall, MVA is a highly immunogenic and safe viral vector, and there is accumulating evidence of its safety and immunogenicity gained from many clinical trials. This makes it a vector of choice, especially as a booster. There are not many vectors with both advantages and any improvement to MVA would add to its excellent safety and immunogenicity. Therefore, MVA is likely to continue in clinical vaccine developments in the future. However, with the increasing use of MVA, scientists should be careful about the anti-vector immunity. This might become an obstacle if an MVA-based vaccine was approved for routine human vaccinations.

9. Appendices

Supplementary figures of chapter 2



Expected size:
ID-PCR:
B8-MVA: 1.5 kb
For parental virus: None

Purity PCR:
B8-MVA: None
For parental virus: 318 bp

From this example, colony #3 from B8-MVA-BAC was selected for more re-streaking and purification before amplifting the BACs for MVA rescue.
Note: Colony # 5 seemed to have parental MVA-BAC as it showed no bands for IR-PCR and a band of ~350 for Purity PCR. **M:** DNA ladder size marker.

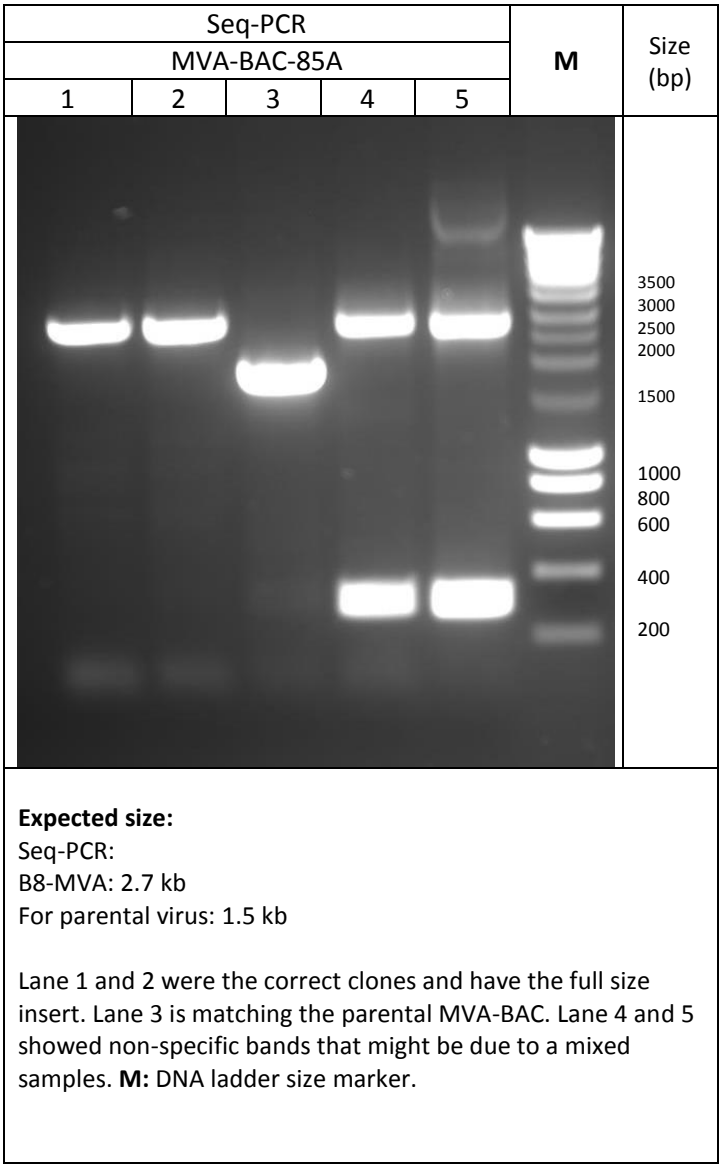
Figure S2.1: ID-PCR and Purity PCR.
Five colonies of the B8-MVA-BAC clone were selected for ID and purity screening. The expected correct sizes are indicated.

eF11-MVA-BAC clones

Expected sizes of 5 bands for integrated full size MVA-BAC:

232 bp
359 bp
444 bp
525 bp
592 bp

Figure S2.2: Multiplex PCR.
Seven colonies of the eF11-MVA-BAC clone were selected for MVA-BAC integrity screening by multiplex PCR.



Expected size:
Seq-PCR:
B8-MVA: 2.7 kb
For parental virus: 1.5 kb

Lane 1 and 2 were the correct clones and have the full size insert. Lane 3 is matching the parental MVA-BAC. Lane 4 and 5 showed non-specific bands that might be due to a mixed samples. **M:** DNA ladder size marker.

Figure S2.3: Seq-PCR.
Five colonies of the MVA-BAC-85A clone were selected for flank-to-flank PCR screening (a.k.a. Seq-PCR). The PCR product that had the correct size was purified and sent for DNA sequencing.

Supplementary figures of chapter 3

D10 peptide sequence alignment:

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VACV WR, 2   MNFYRSSIISQIIKYNRRLAKSIICEDDSQIITLTAFVNQCLWCHKRVSVSAILLTTDNK
    MVA, 1   MNFYRSSIISQIIKYNRRLAKSIICEDDSQIITLTAFVNQCLWCHKRVSVSAILLTTDNK
             ************************************************************

VACV WR, 62  ILVCNRRDSFLYSEIIRTRNMFRKKRLFLNYSNYLNKQERSILSSFFSLDPATADNDRID
    MVA, 61  ILVCNRRDSFLYSEIIRTRNMSRKKRLFLNYSNYLSKQERSILSSFFSLDPATTDNDRID
             ********************* ************* ***************** ******

VACV WR, 122 AIYPGGIPKRGENVPECLSREIKEEVNIDNSFVFIDTRFFIHGIIEDTIINKFFEVIFFV
    MVA, 121 AIYPGGIPKRGENVPECLSREIKEEVNIDNSFVFIDTRFFIHGIIEDTIINKFFEVIFFV
             ************************************************************

VACV WR, 182 GRISLTSDQIIDTFKSNHEIKDLIFLDPNSGNGLQYEIAKYALDTAKLKCYGHRGCYYES
    MVA, 181 GRISLTSDQIIDTFKSNHEIKDLIFLDPNSGNGLQYEIAKYALDTAKLKCYGHRGCYYES
             ************************************************************

VACV WR, 242 LKKLTEDD
    MVA, 241 LKKLTEDD
             ********
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Figure S3.1: Alignment of the vaccinia virus D10 peptide sequence.

Alignment of the D10 protein sequences in the vaccinia virus Western Reserve strain (VACV WR) and the modified vaccinia virus Ankara (MVA). The three amino acid substitutions are in italicised letters with lack of stars underneath. The active (NUDIX) motif is in bold letters. GenBank accession for *D10R* gene: KF866253.1. GenBank accession for D10 protein: AHB23554.1.

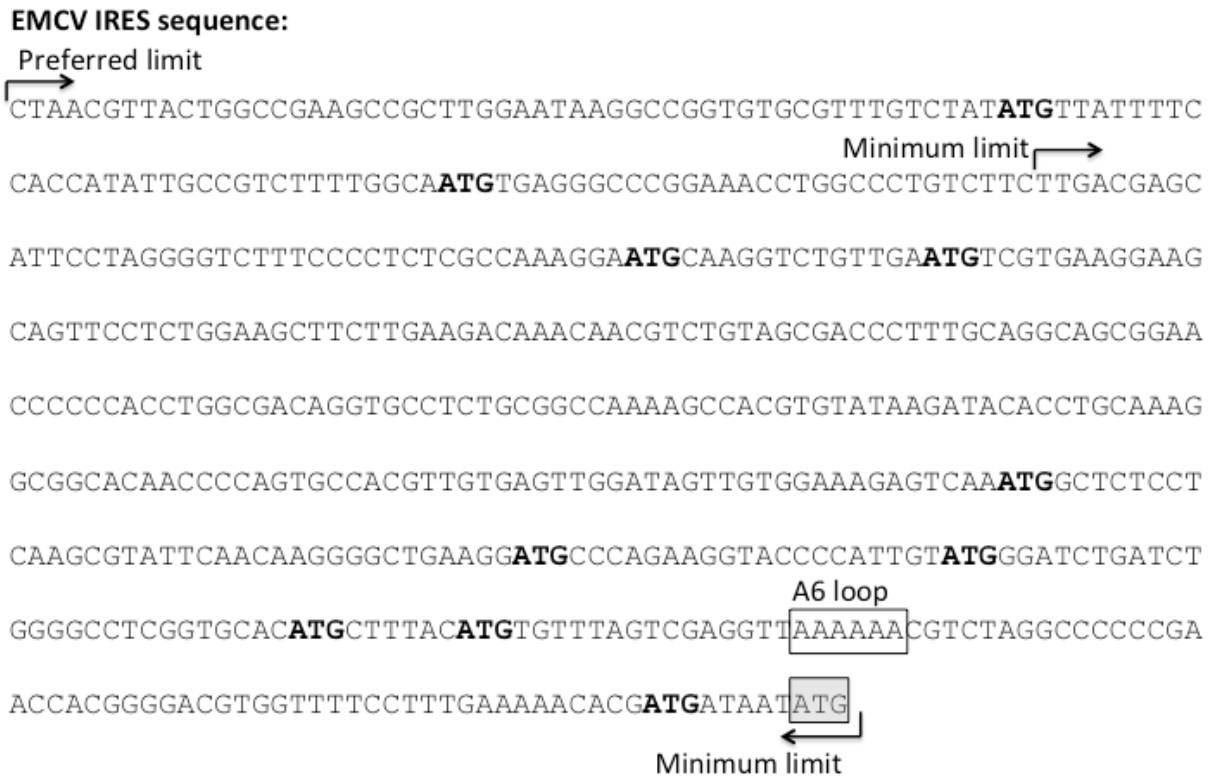

Figure S3.2: EMCV IRES nucleotide sequence.

The 557 nucleotide of the EMCV IRES sequence (GenBank Accession: KF836387.1), used in this study, was confirmed by DNA sequencing and presented here. The optimal EMCV IRES sequence according to Bochkov and Palmenberg [182] should have the A6 loop (boxed) and utilises the defined ATG start codon (in shaded box), not any of the upstream non-defined ATG (in bold). The minimum and preferred boundaries of the EMCV IRES are labelled with arrows. The IRES encompasses the minimum boundaries at both ends, but with the preferred boundary at the 5' end.

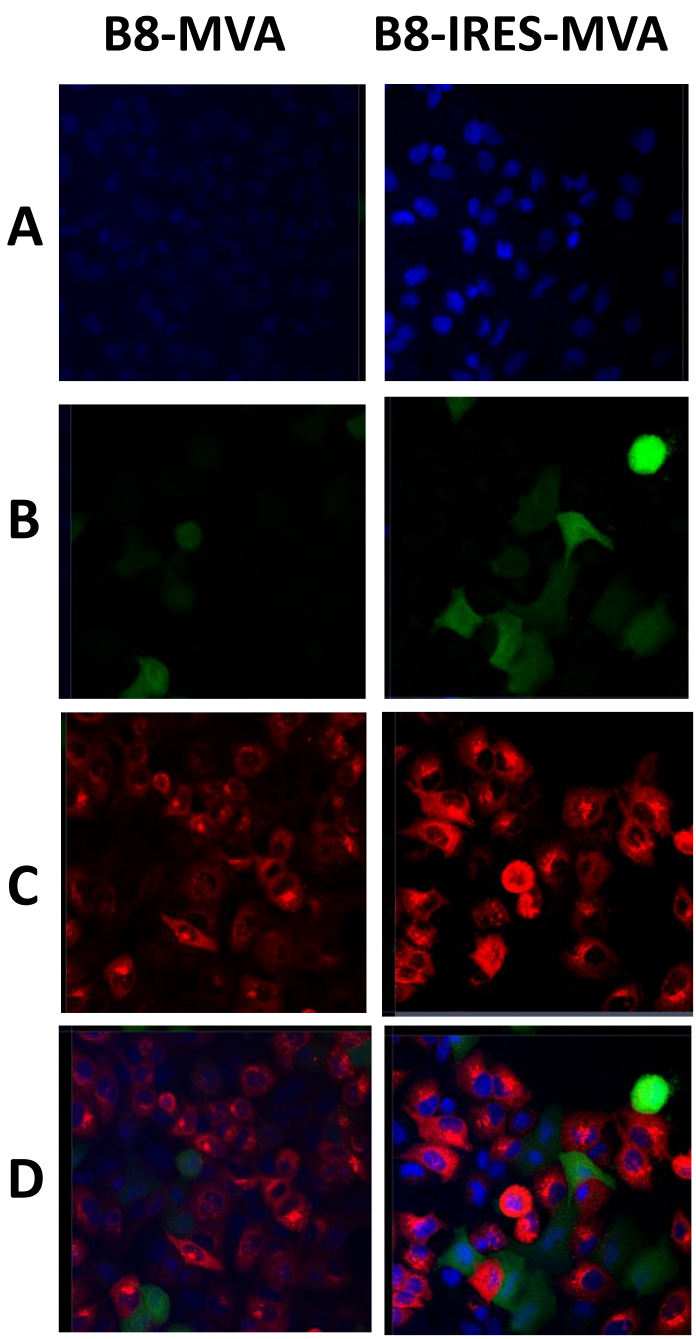

Figure S3.3: Immunofluorescence Assay

Confluent monolayers of A549 cells were infected with B8-MVA or B8-IRESMVA at MOI of 1 (similar to infection for Western blot). Cells were then fixed and stained, as explained in the materials and methods, and imaged using confocal microscope. **A:** DAPi dye staining nucleic acid in blue. **B:** GFP expressed by the recombinant MVAs. **C:** Alexa488 conjugated anti-mouse secondary antibody, binding to anti-rLuc monoclonal antibody, detected in the red channel. **D:** Merged images from A, B, and C.

Supplementary figures of chapter 7

Figure S7.1: Genomic sequencing result.

A snapshot of the sequencing reads of the Δ15-MVA-85A genome aligned against the sequence of MVA virus genome (GenBank Accession: U94848.1). Top: The sequence of the viral genome of the Δ15-MVA-85A. Middle: The sequence of the MVA-BAC clone that was used to derive the virus Δ15-MVA-85A. Bottom: The sequence of non-recombinant non-mutant MVA genome. Lack of signal (peaks) shows one of the two deletions, introduced in the Δ15-MVA-85A. The analysis was performed using the Integrative Genome Viewer (IGV) software.

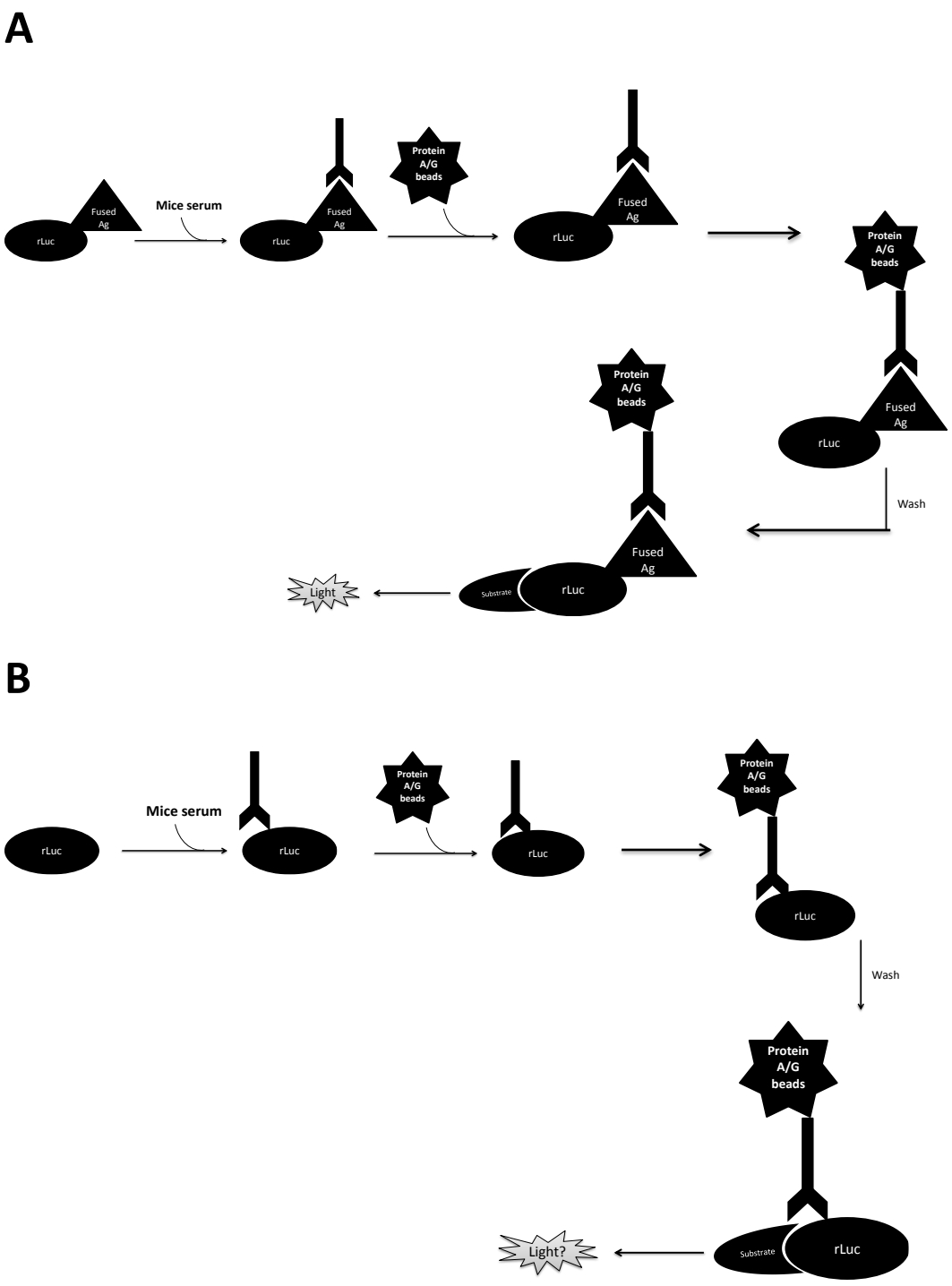

Figure S7.2: LIPS assay

A schematic representation of sequential steps in performing the luciferase immunoprecipitation system (LIPS) assay. **A:** The regular LIPS assay. **B:** The LIPS assay performed for chapter 7, it uses the *Renilla* luciferase as the detection agent as well as the antigen, which might reduce the sensitivity of the assay as both sera antibodies and the substrate bind to the same molecule.

10. References

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