HIV neutralising antibody delivered by gene therapy with a hybrid Vaccinia/retrovirus or BacMam/retrovirus expression systems

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LAYLA FAQIH

Infection, Immunity and Respiratory Medicine
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<td>Adeno-associated virus</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>AcMNPV</td>
<td><em>Autographa californica</em> multiple nucleopolyhedrovirus</td>
</tr>
<tr>
<td>Ad5</td>
<td>Adenovirus type 5</td>
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<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
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<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>ALVAC</td>
<td>Canarypox virus vector</td>
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<tr>
<td>AMP</td>
<td>Antibody mediated prevention</td>
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<td>BHK</td>
<td>Baby hamster kidney</td>
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<td>bNAbs</td>
<td>Broadly neutralising Abs</td>
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<td>Major Genomic Deletion region of MVA</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified Eagle medium</td>
</tr>
<tr>
<td>dNTP</td>
<td>degrades deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>EF</td>
<td>Elongation Factors</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope protein</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen-binding</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable region</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FIV</td>
<td>Feline immunodeficiency virus</td>
</tr>
<tr>
<td>FPV</td>
<td>Fowlpox virus</td>
</tr>
<tr>
<td>Gag</td>
<td>Gag polyprotein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMO</td>
<td>Genetically Modified Organisms</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practices</td>
</tr>
<tr>
<td>Gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>gpt</td>
<td>Xanthineguanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>Gus</td>
<td>E. coli β-glucuronidase A gene</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin gene</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HaCat</td>
<td>Human keratinocyte cell line</td>
</tr>
<tr>
<td>HaSNPV</td>
<td>Helicoverpa armigera single nucleopolyhedrovirus</td>
</tr>
<tr>
<td>HBsAg</td>
<td>HBV surface antigen</td>
</tr>
<tr>
<td>Term</td>
<td>Full Form</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervix adenocarcinoma cell line</td>
</tr>
<tr>
<td>HEV</td>
<td>Hepatitis E virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>hPGK</td>
<td>Human Phosphoglycerate kinase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HTLV</td>
<td>Human T-cell leukemia virus</td>
</tr>
<tr>
<td>IAVI</td>
<td>International AIDS Vaccine Initiative</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate Early</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IGR</td>
<td>Intergenic regions</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IN or Int</td>
<td>Integrase</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>LacZ</td>
<td>Gene for β-galactosidase</td>
</tr>
<tr>
<td>LAM-PCR</td>
<td>Linear - amplification mediated PCR</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>LV</td>
<td>Lentiviral vectors</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Media</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>MPA</td>
<td>Mycophenolic acid</td>
</tr>
<tr>
<td>MPER</td>
<td>Membrane proximal external region</td>
</tr>
<tr>
<td>MRC5</td>
<td>Human foetal lung fibroblast</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MVA</td>
<td>Modified Vaccinia Virus Ankara</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative regulatory factor</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleotide reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleotide reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>OB</td>
<td>Occlusion Bodies</td>
</tr>
<tr>
<td>ODV</td>
<td>Occlusion-Derived Virion</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>P</td>
<td>Promoter</td>
</tr>
<tr>
<td>pA</td>
<td>Polyadenylation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffer saline with Tween</td>
</tr>
<tr>
<td>PCE</td>
<td>Post-transcriptional control element</td>
</tr>
<tr>
<td>pCEF</td>
<td>Primary Chicken Embryo Fibroblast</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEC</td>
<td>Peritoneal Macrophages</td>
</tr>
<tr>
<td>PI/r</td>
<td>Ritonavir boosted protease inhibitor</td>
</tr>
<tr>
<td>PND</td>
<td>Principal neutralising domain</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>polh</td>
<td>polyhedrin gene</td>
</tr>
<tr>
<td>PPT</td>
<td>Polypurine tract</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>pvE</td>
<td>Post-vaccinial Encephalitis</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>rAAV</td>
<td>recombinant Adeno-Associated Virus</td>
</tr>
<tr>
<td>rBV</td>
<td>recombinant Baculovirus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>------------</td>
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</tr>
<tr>
<td>RCL</td>
<td>Replication-competent lentivirus</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of expression of virion proteins</td>
</tr>
<tr>
<td>rHA</td>
<td>recombinant Trivalent Hemagglutinin</td>
</tr>
<tr>
<td>rMVA</td>
<td>recombinant MVA</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev-responsive element</td>
</tr>
<tr>
<td>rSAP</td>
<td>recombinant Shrimp Alkaline Phosphatase</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>Rt</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SI</td>
<td><em>Spodoptera frugiperda</em></td>
</tr>
<tr>
<td>SHIV</td>
<td>SIV/HIV hybrid viruses</td>
</tr>
<tr>
<td>SIN</td>
<td>Self-inactivating LV</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
</tr>
<tr>
<td>SIV mac</td>
<td>SIV macaque species</td>
</tr>
<tr>
<td>SOC medium</td>
<td>Super Optimal broth with Catabolite repression</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
</tr>
<tr>
<td>SU</td>
<td>Receptor binding domain</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-activating protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated regions</td>
</tr>
<tr>
<td>V</td>
<td>Variable region of the HIV virion envelope glycoprotein</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney cell line</td>
</tr>
<tr>
<td>VH</td>
<td>Variable heavy chain</td>
</tr>
<tr>
<td>Vif</td>
<td>Virion infectivity factor</td>
</tr>
<tr>
<td>VIP</td>
<td>Vectored immunoprophylaxis</td>
</tr>
<tr>
<td>VL</td>
<td>Variable light chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>VSV-G</td>
<td>G glycoprotein of the vesicular stomatitis virus</td>
</tr>
<tr>
<td>VV</td>
<td>Vaccinia viruses</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>wtBV</td>
<td>wild type Baculovirus</td>
</tr>
<tr>
<td>wtMVA</td>
<td>wild type MVA</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>ψ</td>
<td>Packaging signal</td>
</tr>
</tbody>
</table>
General abstract

Production of an effective vaccine and long-term treatment against human immunodeficiency virus (HIV) is elusive. In this thesis two different techniques were used in an attempt to insert HIV-neutralising monoclonal antibody (IgG1b12) sequences into a simian retroviral gene therapy agent pseudo-typed with vesicular stomatitis virus glycoprotein. Genes were encoded in either a poxvirus split-vector system or a baculovirus expression system. Both systems aim to produce replication incompetent pseudotyped virus like particles with simian origin. It is believed that the resulting non-infectious artificial lentivirus particles enter neighbouring cells, penetrate the nucleus and insert genetic material (the antibody gene) into the mammalian genome.

The poxvirus split-vector system used in this project was a Vaccinia Retroviral Hybrid Vector, where recombinant modified vaccinia Ankara (MVA) is used to deliver the simian immunodeficiency virus (SIV) like particles into mammalian cells. However, the MVA system failed to express proteins of interest due to the instability of genetic insertion into the recombinant MVA genome. As an alternative strategy, two different BacMam systems were used to allow the production of VLPs, where mammalian cells are co-transduced with different recombinant baculoviruses (rBVs). VLPs were expressed either under the control of T7 RNA polymerase system or under the cytomegalovirus immediate early gene promoter. The results from the first BacMam system indicated that the T7 RNA polymerase system was not suitable to express detectable levels of proteins. The results indicated that translation of the produced mRNA by T7 promoter is inefficient, most likely because of the absence of RNA 5’ cap structure. To overcome this hybrid BV–T7 system limitation, a different system was developed. Proteins of interest from the second BacMam system were successfully expressed and detected using western blot analysis. VLPs were generated and visualised under electronic microscope. IgG1b12 was secreted in the supernatant of
the transduced mammalian cells. Mammalian cells were successfully transduced with multiple different recombinant BVs simultaneously.

The study establishes the feasibility of antibody gene transfer, and demonstrates the use of SIV like particles production to transduce mammalian cells using BacMam technology. The technique may have application for use as an immunotherapy of HIV infection as well providing long-acting prevention of HIV infection for those not yet infected with HIV.
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Acknowledgement

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I am grateful to my sibling Abdulhadi and Lama who have provided me through emotional support in my life. I am also grateful to my friends who have supported me along the way. Thanks you Lama, Nahla, Roba and Hammody for all your support, cakes and chocolates; I am sure that I could not do this without our adventurous trips.
Dedication

I want to dedicate this PhD thesis to my father Ahmed and my mother Elham the most amazing supportive parents, whose love and guidance are with me in whatever I pursue. You are the reason for who I am! Nobody has been more important to me in the pursuit of this PhD than you. Thank you my parents, you are the ultimate role models.
Conference paper and publications

Use of baculovirus expression system, utilising a CMV promoter, for generation of virus-like particles in mammalian cells


HIV neutralising antibody delivered by gene therapy, with a stable retroviral vector encoded in baculovirus expression system

- Poster at the Postgraduate Summer Research Showcase, the University of Manchester, 2017.

Baculovirus expression systems for virus like particles production in mammalian cells

- Poster at the Institute of Inflammation and Repair PGR Showcase, the University of Manchester, 2016.


Generation of marker-free rMVA by homologous recombination using a 50bp repetitive homologous sequence

- Poster at the London Vaccine conference, 2014.
1. Chapter 1: General Introduction

1.1. Human Immunodeficiency Virus (HIV)

1.1.1. Overview

In 1981, the first case of acquired immune deficiency syndrome (AIDS) was reported in the United Kingdom in a patient characterised by profound immune deficiency (Collier et al. 2011). Since then, a massive international effort has aimed to control HIV. HIV is the leading cause of death in sub-Saharan Africa where it is endemic causing almost 2 million new infections every year. It is the fourth leading cause of death worldwide (Girard et al. 2006; Collier et al. 2011; Greenwood et al. 2012; Esparza 2001). In Asia there are more than half a million new infections occurring every year. Significant progress has been made over the past thirty years, especially in antiretroviral treatment. However, the ability of the virus to develop drug-resistance during treatment is of major concern (Churchill et al. 2016; World Health Organization Guideline 2016; Seitz 2016).

HIV, together with the simian immunodeficiency virus (SIV) and the feline immunodeficiency virus (FIV) are enveloped ssRNA viruses belonging to the genus Lentivirus in the family Retroviridae. Lentiviruses possess a reverse transcriptase enzyme that uses the viral RNA genome as a template and produces a proviral DNA copy that integrates into the host cell chromosome. The provirus DNA is eventually transcribed into a set of mRNAs that encode the viral proteins and progeny genomic RNA (Klatt et al. 2012; Carter and Saunders 2013; Greenwood et al. 2012; Girard et al. 2006).

Lentiviruses produce characteristically slow progressive infections (prolonged clinical latency), which have the ability to cause immunosuppression. After initial contact, HIV is transported to secondary lymphoid organs where it infects and attaches to CD4 lymphocytes (T helper cells and macrophages) by using a viral envelope glycoprotein
(gp120). Fusion with the cell membrane is assisted by another glycoprotein (gp41). Infected CD4 cells then spread the infection, sometimes through cell-to-cell fusion, resulting in the formation of syncytia with other lymphocytes (Cho 2000; Levy 2007). Once HIV enters a host cell, it integrates into the cell’s DNA and may persist for a long and variable period before showing any pathogenic effect (Cho 2000; Girard et al. 2006). It was originally assumed that this latent period was characterised by a low level of viral replication. However it is now clear that high rates of viral replication actually occur early after infection.

HIV replicates in metabolically active cells (activated T lymphocytes and macrophages) but remains latent in inactive cells. Infected CD4+ T memory lymphocytes (CD45+ R0+) do not proliferate and are metabolically inactive, thus these cells do not transcribe the viral genome nor express the viral protein. As a result the cells remain unrecognised by CTLs. The immune system is unable to eradicate HIV, partly because of this latency phenomenon and partly because the virus continuously mutates its sequence and thus escapes neutralising antibodies and CTLs. These mutant variants are continuously selected in vivo by the pressure of the immune response. The infected patient may be asymptomatic or paucisymptomatic for several years, but have plasma viraemia and be infective. The phase of primary infection can be asymptomatic; however, 30–70% of infected individuals experience an acute syndrome, characterised by fever, fatigue, lymphoadenomegaly, maculopapular cutaneous eruption, and, in a few cases, neurological involvement. In most cases, this condition is misdiagnosed or unrecognised. As the infection progresses, the proportion of infected CD4+ cells and the quantity of circulating virus rise, until the patient becomes symptomatic (Levy 2007; Cho 2000; Giacca 2010).

HIV eventually damages the overall host immune response unless treated. The malfunction of the immune system leaves the host at a high risk of infection, particularly for opportunistic bacterial, viral, fungal and protozoan infections. The end stage of the disease caused by HIV infection is AIDS, in which CD4 cells are significantly depleted
(<200 cells/mL). At this stage, opportunistic infections are much more likely to occur 
(Girard et al. 2006; Collier et al. 2011; Giacca 2010; Capistrán 2010; Flint et al 2009).

1.1.2. Treatment

The major milestone in HIV treatment was the discovery of a multiple drug regimen 
(previously referred to as highly active antiretroviral therapy, or HAART, now known as 
combination antiretroviral therapy or cART) that has achieved remarkable success in 
slowing the progression of AIDS. Antiretroviral therapies aim to arrest and reverse the 
damage that results from the infection of the immune system, and thus reduce 
infectivity and prolong survival.

In 1996, the 11th International Conference on AIDS in Vancouver was followed quickly 
by sequential publications in The New England Journal of Medicine by Hammer & 
colleagues and Gulick & co-investigators suggesting that in order to maximise the 
effect of the treatment and to delay or to prevent the emergence of viral resistance, a 
three drug regimen should be prescribed from at least two different drug classes 
(Hammer et al. 1997; Gulick et al. 1997). This system of therapy allows continuous 
suppression of plasma viral RNA to a level below the limits of detection afforded by 
modern molecular assays (Greenwood et al 2012; World Health Organization Guideline 
2016; Collier et al. 2011). Generally a combination of two nucleoside/ nucleotide 
reverse transcriptase inhibitors (NRTI, e.g. tenofovir/emtricitabine, abacavir/lamivudine, 
zidovudine/lamivudine) plus either a non-nucleoside reverse transcriptase inhibitor 
(NNRTI, e.g. efavirenz, nevirapine) or a ritonavir boosted protease inhibitor (PI/r, e.g. 
darunavir/ritonavir, lopinavir/ritonavir, atazanavir/ritonavir) is currently recommended, 
where the two NRTI agents, often referred to as the backbone, are preferably given as 
a co-formulated preparation. A patient on combination therapy will have to adhere to a 
strict regimen. However, drug resistance may be present. It is now standard practice to 
test the patient's viral population for drug resistance before initiation of the therapy 
(Collier et al. 2011; World Health Organization Guideline 2016; Carter & Saunders 
2013).
1.1.3. Virus types

HIV comprises two types—HIV type 1 and HIV type 2—which appear to have indistinguishable clinical features and can both lead to the development of AIDS. Both have the same modes of transmission: they can be transmitted through sexual contact with an infected person; contaminated blood or blood clotting factor transfusions; sharing contaminated needles, syringes or other injection equipment with infected blood; and through maternal-fetal transmission (Collier et al. 2011; Levy 2007; Cho 2000). However, investigations indicate that HIV type 1 is more easily transmitted and much more virulent. On the other hand, the period between the initial infection and the appearance of symptoms is much longer in HIV type 2 (Carter & Saunders 2013). Worldwide, HIV type 1 is the predominant type; most of the current studies focus on it because HIV type 2 is relatively uncommon and geographically limited to West Africa and former Portuguese colonies where it is endemic and rarely found elsewhere. In general, when speaking of HIV without specifying the type of virus, HIV type 1 is being referenced (Collier et al. 2011; Girard et al. 2011; Seitz 2016).

1.1.4. Structure and genomic materials

As with all retroviruses, HIV contains two copies of a dsRNA genome. The capsid is cone-shaped with a diameter of 40-60 nm at the wide end, and about 20 nm at the narrow end. Each protein has multiple roles and together they control virus gene expression, modify the host’s immune response, and are involved in transporting virus components within the cell. Both HIV and SIV encode a unique set of accessory proteins that enhance viral replication in the host. HIV genome contains the three genes common to all retroviruses (Gag, Pol, and Env) and six additional accessory genes (Tat, Rev, Nef, Vpr, Vpu, and Vif) (Figure 1 and Figure 2) (Collier et al. 2011; Carter & Saunders 2013).

The HIV genome is about 9.3 kb in length. In addition to the standard retroviral genes gag, pol, and env, there are auxiliary genes. The genes encoding the virus proteins are
organised in three major regions of the genome; Gag (group specific antigen) internal structural proteins, Pol (polymerase) enzymes and Env (envelope) envelope proteins. The Gag gene codes for the Gag polyprotein (matrix, capsid, p2, nucleocapsid, p1 and p6), which is then processed by the viral protease to generate proteins associated with the viral mRNA and some may form a part of the viral capsid. The Pol gene (polymerase), translated into a Gag-Pol polypeptide thanks to a ribosomal frame-shift, codes for the viral enzymes reverse transcriptase RT (p55/p51), integrase IN (p32), RNase H, and protease PR (p11). The Env (envelope) gene codes for a precursor Env polyprotein which is cleaved by cellular proteases to generate TM (gp41) and SU (gp120) (Collier et al. 2011; Carter & Saunders 2013).

The accessory proteins are essential for viral replication and virion infectivity. Tat (transactivator of transcription) is a powerful activator of viral gene expression. The protein binds a structured region present at the 5’ end of the transcribed viral RNAs and recruits different cellular factors to the viral LTR (Long terminal repeat) promoter. Rev (regulator of expression of virion proteins) binds a highly structured RNA sequence (Rev-responsive element, RRE), present in correspondence of the Env gene and thus contained as a potential intron in the fully spliced mRNAs. The role of Rev is to mediate transport of these RRE-containing mRNAs outside the nucleus.

Figure 1: Lentivirus virion. The genome is made of two RNA molecules associated with the nucleocapsid (NC) protein. The nucleocapsid is enclosed by a core made of capsid (CA) protein, which is surrounded by a shell of matrix (MA) protein that associates to the virion envelope. The two subunits of the HIV-1 envelope are also indicated (SU and TM). In addition, other enzymatic (IN, RT, Protease) and accessory (Vpr, Nef) proteins are shown, which are incorporated into lentivirus particles. Reproduced from (Greene & Peterlin 2002).
Nef (negative regulatory factor) negatively regulates the expression of CD4, major histocompatibility complex (MHC) class I and II proteins, and the co-stimulatory molecules in the infected cells, thus blocking their recognition by cells of the immune system, besides regulating signal transduction. Vif (virion infectivity factor) is essential for proper virion infectivity since it blocks the function of a cellular enzyme, the RNA deaminase APOBEC, which would otherwise inactivate the viral genome by introducing mutations. Vpr (viral protein R) blocks progression of the cell cycle in the infected cells by accumulating them in the G2-M phase, is incorporated into virions, and contributes to nuclear transport of the reverse transcribed viral cDNA in the infected cells. Vpu (viral protein U), which is only present in the HIV-1 and SIVcpz genomes but not in HIV-2 and SIVmac (Figure 3), increases release of viral particles from the infected cells and contributes to CD4 degradation. Vpx (virion-associated protein) encoded by HIV-2 and most SIV strains counteracts a particular host restriction factor and facilitates reverse transcription in myeloid cells such as monocyte-derived macrophages and monocyte-derived dendritic cells. This mechanism of counteraction is similar to that of
the accessory proteins Vif and Vpu which antagonise other host factors. SAMHD1 a protein that functions as a restriction factor counteracted by Vpx. SAMHD1 degrades deoxynucleoside triphosphates (dNTPs), which are components of the viral genomic cDNA, in order to deprive viruses of dNTPs. Vpx has also been shown to have an apparent ability to enhance nuclear import of the viral genome in T lymphocytes (Collier et al. 2011; Carter & Saunders 2013; Seitz 2016).

Figure 3: Organization of lentiviral genomes. Orange boxes represent HIV-1 open reading frames, open boxes represent SIVMAC counterparts. Reproduced from (Ambrose et al. 2007).

1.2. Simian Immunodeficiency Virus (SIV)

1.2.1. Overview

HIV and SIV share many similar characteristics including genome organisation, cellular targets, receptors, and viral pathogenesis. Thus, studying SIV infections in various macaque species has helped scientists to understand lentiviral pathogenesis, in particular to understand the correlates of protection and vaccine production (Antoine et al. 1998; Sharp et al. 2005).

African monkeys are the natural hosts to a variety of SIV types, but do not appear to develop clinical signs following infection with these viruses. In contrast, Asian monkeys, especially rhesus monkeys from India, are highly susceptible to SIV. Infection of
macaque species causes a progressive immunodeficiency syndrome, which closely mimics human AIDS. In contrast, HIV does not usually infect Asian macaques and is non-pathogenic when it does. Thus, evaluating experimental vaccines in these animals requires a non-human analogue instead of the actual HIV vaccine candidate used in clinical trials in humans (Greenwood et al 2012; Esparza 2001; Antoine et al. 1998; Douglas et al. 1997).

SIV mac has been used extensively to model HIV in vaccine experiments. However, studies have shown that it is not possible to evaluate the protective efficacy of an HIV vaccine candidate using SIV mac. This is because there is a significant difference in the proteins produced by SIV and HIV (Douglas et al. 1997; Johnston 2000; Sharp et al. 2005). On the other hand, chimeric viruses can be used. SIV/HIV hybrid viruses (SHIVs), where the Env gene as well as other genes including Tat, Rev, Vpr, and Vpu, from an HIV-1 isolate were inserted in the backbone of an SIV genome (Ambrose et al. 2007; Johnston 2000) can allow the protective efficacy of vaccine candidates based on envelope glycoprotein to be tested. Insertion of more HIV-1 genes into SIV genome has been unsuccessful (Joag et al. 1997; Li et al. 1995; Shibata, Siemon, et al. 1997).

To varying degrees, SHIV can replicate in rhesus macaques causing no disease at the start. However, serial in vivo passages eventually lead to the emergence of highly pathogenic variants. These pathogenic SHIVs are capable of causing rapid depletion of the circulating CD4+ T-cell population in the infected host, generating a severe immunodeficiency syndrome and eventual death of the host within a year (Joag et al. 1996; Reimann et al. 1996; Igarashi et al. 1999; Johnston 2000). As such, SHIV could mimic HIV to some extent. However, the relevance of using these pathogenic SHIVs, in HIV vaccine protection experiments has been questioned. Studies argue that, compared to SIV, SHIV containing HIV-1 Nef replicates insignificantly in vivo (Shibata, Maldarelli, et al. 1997). Moreover, SHIV may demonstrate some host-range restriction of growth (Shibata et al. 1991). As such, SHIV has been used less widely in HIV vaccine experiments (Johnston 2000; Ambrose et al. 2007).
1.3. HIV and vaccine production

1.3.1. Overview

Despite ongoing international efforts to control the HIV/AIDS pandemic through individual action, behaviour modification and the screening of potentially contaminated blood and blood products, more than 15,000 people become infected with HIV every day. Most of them live in developing countries. The current studies of pandemics in Africa and Asia indicate that HIV transmission is mainly heterosexual or vertical in children (World Health Organization Guideline 2016; Greenwood et al 2012; Esparza 2001). The development of antiretroviral therapies to combat HIV infection has dramatically decreased the morbidity and mortality among HIV-positive patients (Churchill et al. 2016). However, HIV transmission has only been partially restricted by the advent of antiretroviral treatment and changes in sexual behaviour. Effective treatment of HIV-infected patients is limited by many factors, such as late diagnosis, appearance of resistant mutations and poor adherence to treatment. HIV treatment is expensive, and most HIV-infected patients cannot afford it as most live in low-income countries (Greenwood et al 2012; Churchill et al. 2016; Esparza 2001).

The importance of developing a safe, effective, and affordable HIV vaccine cannot be overemphasised as HIV is one of the world's deadliest scourges (Collier et al. 2011). Since the discovery of HIV as the causative agent of AIDS in 1983–84, hopes for the development of a HIV preventative vaccine have been raised, as the most potent defence against viral infections are vaccines (Sarngadharan et al. 1984; Johnston & Fauci 2007). A vaccine could be a valuable complement to other interventions that significantly alter the chain of HIV transmission, the course of the disease, and its infectiousness. Thus, this could provide positive health benefits for both infected individuals as well as the community at large. Also, a well-conceived HIV immunisation strategy could reach populations in which antiretroviral treatment and other interventions are not sufficiently effective, such as in low-income countries (World
Health Organization Guideline 2016; Carter & Saunders 2013; Esparza 2001). Studies suggest that a preventative HIV vaccine could possibly be used as a therapeutic intervention in association with antiretroviral therapies. This could lead to lower cost of the treatments and may increase the treatment’s long-term efficacy (Cho 2000; Di Nunzio et al. 2012).

1.3.2. HIV vaccine status and vaccine production challenges

Vaccines have been our best application to protect us from most of the world’s deadliest viral infectious diseases, including smallpox, polio, measles and yellow fever. However, there are many confounding issues that prevent the production of a sufficiently effective HIV vaccine (Morrow et al. 2012; Esparza 2001). Challenges include HIV extreme antigenic variability, lack of understanding of immune correlates for protection, limitations of available animal models, and the enormous constraints associated with the probable need for multiple large-scale clinical trials in different parts of the world.

The human body seems incapable of mounting an effective immune response against HIV infection as the virus has a unique way of evading the immune system. The fact that the virus infects the immune system itself presents a particular challenge. HIV attacks T helper cells as they have the HIV-specific receptor CD4. HIV is taken up by CD4+ T cells and macrophages following binding of viral glycoprotein (gp120) to CD4 and certain chemokine receptors (CXCR4 and CCR5). Thus, HIV specifically infects the very cells necessary to activate both B-cell and cytotoxic T-cell immune responses. T helper cells are a key part of the immune system; they help B-cells and effector T-cells to combat infection. CD4+ T cells have been identified as a major effector cell population in the response to many virus infections (Johnston & Fauci 2007; Levy 2007; Chhatbar et al. 2011; Cho 2000). However, HIV can also infect other cells of the immune system, including macrophages, memory cells in the lymph node, dendritic cells and brain microglial cells as well as its tendency to infect bone marrow-derived cells and lymphocytes. In addition, HIV can minimise its recognition by CTL through its
ability to down regulate MHC class I molecules (Cho 2000; Levy 2007). To date, HIV vaccine researchers have no human model with a full recovery from the infection and subsequent protection from reinfection (Ambrose et al. 2007). Moreover, scientists lack comprehensive information about the correlates of protective immunity to HIV (Esparza 2001).

HIV has a large genetic diversity as well as a high mutation rate. Inside the host, the virus continually mutates and recombines, evolving new strains of virus that differ slightly from the original infecting virus allows them to escape neutralising antibodies and evade the immune response. The mutation usually occurs in those viral peptides that bind to MHC class I molecules to which the initial T cell response arose which results in a failure of T cell surveillance. As a result, quasi-species of viral infection are usually present in HIV-infected patients. The extensive diversity of HIV poses a challenge to designing a long-lasting HIV vaccine as it would need to protect against the many different strains of the virus circulating throughout the world (Levy 2007; Cho 2000; Carter & Saunders 2013).

The lack of a suitable small-animal model to test vaccine candidates to predict the effectiveness of an HIV vaccine in people hampers HIV vaccine development. The only animals susceptible to experimental infection with HIV are chimpanzees and pigtail macaques. Both can be infected with HIV, however, no clinical signs arise as they tend to maintain low levels of persistent virus load and do not develop clinical manifestations of AIDS. Currently, researchers rely on experiments using non-human primate models infected with non-human lentivirus mainly SIV to somewhat mimic disease progression. Thus, evaluating experimental vaccines in these animals requires a non-human analog instead of the actual HIV vaccine candidate used in clinical trials in humans (Greenwood et al. 2012; Esparza 2001; Johnston & Fauci 2007; Ambrose et al. 2007; Johnston 2000).
1.3.3. Eliciting immune response using the current immunisation protocols

1.3.3.1. Live attenuated vaccines

In general terms, live attenuated vaccines are composed of a live, but substantially weakened pathogen through single or multiple mutations within its genome. The attenuated pathogen will replicate in host cells causing limited and non-life threatening infection. This vaccine strategy can elicit a strong and long-lasting immune response, which can mimic the disease in very mild form and prevent reinfection with the same pathogen (Niaid.nih.gov 2014; Cho 2000). However, in the case of HIV, serious potential risks in humans are an important concern when the correlates of protection are still largely unknown (Girard et al. 2011)

In favour of a live attenuated HIV vaccine approach, many studies have been conducted using attenuated HIV-2 or SIV. Attenuation of these viruses was achieved through induced deletions or mutations of the viral genome. Studies confirmed that the least attenuated viruses elicited a better immune response (Lohman et al. 1994; Johnson et al. 1999). However, as the virus integrates into the host genome following infection, it is essential that the administered strain does not regress to its original pathogenic form following vaccination.

Observations of several attenuated SIV or SHIV vaccine studies have produced confounding results where macaques infected with attenuated SIV or SHIV exhibited strong immune responses but various levels of protection (Girard et al. 2011). Although sterilising immunity has not been achieved, immunisation studies with SIV nef-deleted mutant indicate that SIV Δnef confer a persistent, life-long, low-grade viral infection in rhesus macaques that limits the development of AIDS. However, it does not protect the vaccinated monkeys against infection with wild-type virus. In contrast, another study indicated that infant monkeys developed AIDS when the SIV Δnef vaccine was administered orally. Prolonged observations of attenuated SIV with triple deletions,
where nef, vpr, and negative regulatory element was deleted from the virus genome, indicated potential reversion of the virus to become pathogenic in neonates as well as in adult macaques. Attenuated SIV with triple deletions is genetically unstable and can evolve into a fast replicating variant (Baba et al. 1999; Bordería & Berkhout 2009; Joag et al. 1998; Shibata, Maldarelli, et al. 1997; Shibata, Siemon, et al. 1997; Lohman et al. 1994).

Patients who were infected with naturally occurring attenuated HIV-1 isolates with a deletion in the nef gene remain healthy substantially longer than those patients infected with viruses with an intact nef gene. They have been shown to have high levels of CTL activity against HIV-1 as well as long-term, non-progressive infection (Deacon et al. 1995; Dyer et al. 1999; Kirchhoff et al. 1995; Bogers et al. 1995; Greenough et al. 1999). However, recent reports show a low gradual decline in the number of CD4+ T cells among those patients. As a result of these concerns, the live attenuated strategy has not been used in HIV vaccination experiments, as some can evolve to virulent virus over time (Girard et al. 2011).

1.3.3.2. Inactivated vaccines

An inactivated vaccine is achieved either by heat or chemical treatment to ensure that the virus has been killed and is no longer infectious. It is assumed that the native structure of the epitopes concerned with eliciting protective immune responses are preserved, despite the alteration of both the conformation and the antigenicity of the viral envelope that occurs as a result of the various treatments (Cho 2000; Levy 2007). A method has been developed to completely inactivate the infectivity of HIV or SIV while keeping the envelope epitopes intact and functional (Rossio et al. 1998). This was done by disrupting the two nucleocapsid protein zinc finger domains, which are essential for virus infectivity, through mild oxidation or alkylation (Arthur et al. 1998; Rossio et al. 1998).
Most of the vaccine studies using this approach used the macaque model with either inactivated SIV or HIV-2 due to the potential danger resulting from the incomplete inactivation of HIV-1. Since the virus has been killed and no longer replicates, this type of vaccination will not elicit strong cellular immunity as there is no intracellular protein expression, particularly affecting CTL responses. Immunisation studies using a whole inactivated SIV vaccine have shown varied protection results: some have demonstrated only partial protection, while others have reported complete protection (Desrosiers et al. 1989; Sutjipto et al. 1990; Girard et al. 2011). Although most of the vaccinated animals showed decreased viraemia and no significant depletion of CD4+ T cells, they could still be infected with the wild-type virus. However, most of these animals were protected from SIV grown in human cells, but not from the virus grown in macaque peripheral blood mononuclear cells (PBMCs) (Cho 2000; Girard et al. 2006; Girard et al. 2011).

In this regard, later studies indicated that it was likely that the protective immune response in vaccinated animals was directed against the xenoantigen rather than the viral antigens, as high levels of antibodies against human cellular proteins were detected in all these animals and the degree of protection was shown to be correlated with the titre level of these antibodies (Hirsch et al. 1996; Langlois et al. 1992). Whether whole inactivated vaccines elicit a protective immune response or not, this approach has been neglected due to the high risk of disease development, the possible side effects of alloantigen immunisation, the resulting sporadic level of protection and the associated use of various adjuvant treatments that need to be further evaluated (Arthur et al. 1998; Girard et al. 2006; Rossio et al. 1998).

However, a recent study using a genetically modified and killed whole-HIV1 vaccine, SAV001, describes a current ongoing Phase I human clinical trial, the first of its kind. The study uses an inactivated and formulated killed whole-HIV (gmHIV-1NL4-3). The virus was propagated in the A3.01 human T cell line followed by virus purification and inactivation with aldrithiol-2 and γ-irradiation. The primary results suggested that the
vaccine had no potential toxicity and it triggered anti-HIV immune responses. However, it is still to be determined whether this immune responses can prevent HIV infection (Choi et al. 2016).

1.3.3.3. Virus-like particles (VLP)

Virus-like particles (VLP) are multi-protein structures, which mimic the organisation and the conformation of the original native viruses. VLPs lack the viral genome, and hence yield potentially safer and cheaper vaccine candidates. It has been noticed that the Gag and Env proteins of HIV or SIV, when expressed in cells, spontaneously assemble to form VLPs that include only the virus envelope and the core proteins (Girard et al. 2006; Levy 2007).

In many studies, SIV VLPs have been tested as immunogens in non-human primate models. These VLPs elicited low but to some extent, significant titres of neutralising antibodies against both homologous and heterologous primary HIV-1 isolates. However, the use of VLPs in vaccine production has failed due to the difficulty in producing VLPs in sufficient quantities. This could be a result of inefficient particle assembly and release, faulty Gag protein processing, or faulty incorporation of the virus envelope. Moreover, studies show that it is almost impossible to produce VLPs without contaminant cellular proteins that may produce complex immunogenicity (Cho 2000; Levy 2007). Significant improvement in the HIV VLP vaccine approach using VLPs containing different target antigens has been reported (Kushnir et al. 2012; Levy 2007). Chimeric VLPs could be produced using homologous or heterologous retroviruses such as the SIV Gag protein or the HIV Env protein. The assembled chimeric VLP could be enveloped or non-enveloped. The preparation of chimeric VLPs is however, substantially more difficult (Kushnir et al. 2012; Zhang et al. 2004). Compared to full pathogen-based inactivated or live attenuated vaccines, immunogenicity of VLPs is generally low thus requiring higher doses, booster administrations, co-administration of adjuvants, or development of alternative approaches for enhancing target-specific immunity.
1.3.3.4. Subunit vaccines

Subunit vaccines are usually composed of a mixture of protein components from a virus with no genetic material. The benefit of using such an approach is that these subunits can elicit both humoral and cellular immune responses (Cho 2000). Large amounts of viral peptides, which are needed for vaccine production, can be produced either by using recombinant vehicles, such as bacteria, yeast, insect cells, or mammalian cells, or they can be chemically synthesised (Levy 2007; Cho 2000). A set of peptides used in the production of the subunit can be safely manipulated to contain epitopes specific for B and/or T cells. As these epitopes lack the virus genome and are not infectious, increased safety is one of the most important advantages of this approach (Kushnir et al. 2012). Generally, subunit vaccines do not elicit an efficient CTL response. However, studies show that if a strong immune response was elicited when using a subunit vaccine, the purity of the vaccine preparation make it preferable to more complex mixtures such as inactivated pathogens or recombinant vehicles that would express pathogenic antigens (Emini et al. 1990; Murphey-Corb et al. 1991; Berman et al. 1990).

1.3.3.5. Envelope-based subunit vaccines

Recombinant envelope glycoproteins gp120 (exterior glycoproteins) and gp41 (transmembrane proteins) bear neutralisation epitopes that can elicit humoral immune responses, which HIV-1-neutralising antibodies target (Mascola et al. 1996; Jeffs et al. 2004). According to Klein et al. 2001, at least 13 different envelope-based vaccines have been evaluated in humans. Several trials have aimed to develop envelope-based subunit vaccines by evaluating gp120 and gp140 (gp120 coupled with the ectodomain of gp41) (Klein 2001; Girard et al. 2006). As reviewed in Girard et al. 2011, gp120 monomers from T-cell-line-adapted clade B isolates and gp140/160 oligomers have the same immunogenicity (Girard et al. 2011).
The results of past studies indicate that all envelope proteins elicit neutralising antibodies for homologous HIV strains, but not for heterologous ones. Despite the use of adjuvant, CTL responses were not induced in these cases. In spite of the use of booster immunisations, no trials have shown a statistically significant reduction of the viral loads in vaccines. Other approaches that have aimed to elicit HIV-neutralising antibody vaccine have been underdeveloped. For example, some studies have aimed to overlap CD4-binding and expose neutralising sites by stabilising gb140 or manipulating gp120. Higher titres of neutralising antibodies with limited ability to cross-neutralise heterologous virus strains have been isolated (Girard et al. 2006; Cho 2000; Levy 2007). In another example, developed by Merck and the University of Maryland, the co-receptor binding site has been overlapped and neutralising epitopes have been exposed by covalently linking gp120 or gp140 to soluble CD4 or to synthetic mimics of the CD4 receptor. The results suggested that a broad neutralising antibody response may be elicited (Devico et al. 1996); this finding has not been replicated (Girard et al. 2006). Targeting conserved epitopes on the CD4-binding site by engineering hyperglycosylated derivatives of gp120 and immunisation with recombinant gp41 has been studied to induce fusion-blocking antibodies. However, the extreme genetic variability of HIV makes it difficult to use such approaches to develop a vaccine capable of eliciting broadly neutralising antibodies that can challenge different primary isolates and different clades of the virus (Girard et al. 2006; Girard et al. 2011; Di Nunzio et al. 2012).

### 1.3.3.6. Non-structural protein subunit vaccines

An example of the development of a non-structural protein subunit vaccine is vaccination with a viral transactivator protein (Tat). Tat is a significant HIV virulence factor helping in virus gene expression, replication, transmission and progression of disease. Different approaches using Tat antigens have been developed (Levy 2007). Studies indicate that both humoral and cellular immune responses are elicited when cynomolgus monkeys are immunised using biologically active Tat antigens, while
immunised rhesus macaques are protected, to a certain level, from pathogenic SHIV (Gallo 1999; Ferrantelli et al. 2004; Rezza et al. 2005; Maggiorella et al. 2004). On the other hand, according to Liang et al., results concerning rhesus monkeys immunised with adenovirus type 5 (Ad5)-HIV tat recombinant vaccine did not indicate any efficacy against SHIV challenges, despite the elicitation of humoral and immune cellular responses (Liang et al. 2005). However, different studies suggest that in intra-rectally challenged rhesus macaques, inactivated Tat decreases the presence of the disease, as well as reducing the viral load (Silvera et al. 2002).

Combined vaccines using Tat antigen have also been developed, where Tat-adenyl cyclase fusion protein or a Tat–Nef fusion protein is combined with a recombinant gp120 subunit vaccine in AS02A adjuvant. This combined vaccine has been able to protect rhesus monkeys from the development of AIDS when they are challenged with pathogenic SHIV. Although further evaluation is still needed, several clinical trials using subunit Tat vaccine have indicated that the vaccine could be well-tolerated and would produce a significant immune response (Girard et al. 2006; Girard et al. 2011). Another approach is that of the AIDS Vaccine Integrated Project (AVIP), which is trying to develop a novel vaccine approach based on the use of a trimeric gp140 molecule deleted at the V2 loop (gp140 ∆V2 SF162) and mixing it with either Tat or Nef antigens. It seems likely that a multicomponent vaccine will be more efficacious because more epitopes will be presented. The anticipated diverse immune response should be less susceptible to viral mutation and immune escape (Levy 2007; Girard et al. 2006).

Anti-Tat antibodies are rarely found in natural infection and when found correlate with asymptomatic state and reduced disease progression, recent studies are focusing on using Tat as an optimal candidate for therapeutic immunisation and cART intensification more than a vaccination approach. Tat is produced very early upon infection and continues to be expressed under cART. It is found that Tat expressed and released extracellularly by infected cells, accumulates in tissues, induces immune
dysregulation, and promotes virus reactivation, entry and spreading (Ensoli et al. 2015; Ensoli et al. 2010).

The introduction of anti-Tat antibodies results in pathogenesis regulation and inhibition of disease progression thus helping in concentrating cART effectivity. Randomised phase II clinical trial (ISS T-002, ClinicalTrials.gov NCT00751595) conducted in 168 cART-treated volunteers in Italy, found that Tat-based vaccination is safe, immunogenic and capable of eliciting durable immune responses. Candidates used were HIV-infected anti-Tat Abs negative, virologically suppressed cART-treated (mean of 6 years) adult subjects. The vaccine induced anti-Tat Abs in 79% of patients. The vaccine contributed in HIV containment by promoting a durable and significant restoration of T, B, and natural killer (NK) cell numbers, increased CD4+ and CD8+ central memory subsets, and upregulating the expression of human leukocyte antigen-D related (HLA-DR+) on CD8+ killer T cells. A 70% reduction of blood proviral DNA was detected, which is significantly related to neutralization of Tat-mediated entry of Env in dendritic cells (DCs). DCs resulted from the expression of anti-Tat in IgG and IgM format (Ensoli et al. 2015; Ensoli et al. 2010; Longo et al. 2009).

Based on these data and to assess whether clade B Tat immunisation would be effective also in patients with different genetic background and mainly infected with a C clade virus, a phase II trial was conducted in South Africa (ISS T-003, ClinicalTrials.gov NCT01513135). Results indicated that immunisation with B clade Tat induced functionally effective cross-clade anti-Tat Abs and CD4+ T-cell responses. This finding reinforces that clade B Tat is a suitable candidate for therapeutic immunisation against different HIV clades in different geographical areas. As a result, phase III studies in South Africa were established (Ensoli et al. 2016).

1.3.3.7. Naked DNA vaccines

Evidence suggests that HIV-specific CD4+ and CD8+ T-cell immune responses are associated with reduced viral load, caused by better controlled viral replication. Thus,
the cellular immune response is a key factor in controlling HIV. Although live attenuated vaccines can elicit a strong cellular immune response, they can also evolve into virulent viruses over time. An emphasis on the development of new strategies that could produce a significant immune response against HIV without producing disease over time is a primary goal of HIV immunisation (Levy 2007; Cho 2000; Girard et al. 2006). Scientists have developed an immunisation strategy that is performed via the direct injection of naked plasmid DNA that encodes viral genes of interest. This injection can be made intramuscularly, intradermally, or via an epidermis gene gun.

It has been proven that using plasmid-encoding HIV envelope DNA vaccines in nonhuman primates, along with a prime-boost immunisation regimen, can elicit both humoral and cellular immune responses. This can be enhanced by cytokine administration (Levy 2007; Robinson & Pertmer 2000; Girard et al. 2011). The protective efficacy of DNA vaccines encoding envelope genes has been highly variable. Researchers have argued that macaques and chimpanzees immunised with plasmids encoding a viral genome can elicit cellular and relatively weak humoral immune responses (Boyer et al. 1997; Fuller, Corb, et al. 1997; Fuller, Simpson, et al. 1997; Haigwood et al. 1999). As reviewed by Girard et al., DNA vaccines demonstrated a higher rate of protection when immunised macaques were challenged by SHIV or HIV-1. Immunised macaques with both DNA and recombinant gp160 showed envelope-specific lymphoproliferative response and CTL activity (Girard et al. 2011; Levy 2007). However, DNA vaccines produce significantly lower level of immunogenicity in humans compared to animal models (Goyvaerts et al. 2013).

Wyeth Pharmaceuticals developed a naked DNA vaccine using an expressed HIV-1 gag gene with either IL-12 or IL-15 or both. The vaccine was tested on humans in Phase I trials in the USA, Brazil and Thailand (Clinicaltrials.gov NCT00115960 NCT00111605). The Phase I clinical trial with HIV-1-infected human patients clearly demonstrated the safety of DNA-based vaccines. IL-12 and IL-15 adjuvant were safe to
give, although offered limited ability to boost cellular immune responses (Kalams et al. 2012; Girard et al. 2006).

### Table 1: Comparison of VAX004 and VAX003 studies

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<th>VAX004 Study</th>
<th>VAX003 Study</th>
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<tr>
<td>Vaccine design</td>
<td>Bivalent rgp120 with alum</td>
<td>Bivalent rgp120 with alum</td>
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<tr>
<td>Vaccine antigens</td>
<td>MNrgp120/HIV-1 plus GNE8 rgp120/HIV-1 (subtype B and B')</td>
<td>MNrgp120/HIV-1 plus A244 rgp120/HIV-1 (subtype B and E)</td>
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<td>rgp120 dose</td>
<td>300 µg (MN)/ 300 µg (GNE8)</td>
<td>300 µg (MN)/ 300 µg (A244)</td>
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<td>Placebo</td>
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<td>Vaccine administration</td>
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Between 1998 and 2003, the world’s first Phase III clinical trials of a gp 120 HIV/AIDS vaccine (AIDSVAX) were conducted by VaxGen. Data from both VAX004 and VAX003 indicated no protective efficacy against acquisition of HIV infection (Table 1). AIDSVAX B/E alone conferred no protection among Thai people who inject drugs (PWID) (VAX003, Clinical-Trials.gov NCT00006327). Similarly, no overall significant protection was seen with a similar regimen of bivalent gp120 (AIDSVAX B/B) alone (VAX004, Clinical-Trials.gov NCT00002441) among mostly men who had sex with men (MSM) in North America and The Netherlands. Despite intriguing findings in subgroup analyses of VAX004, which suggested vaccine efficacy for acquisition of HIV infection in Blacks or non-whites, women and high-risk volunteers, vaccine efficacy in the subgroups could not be explained because of the small number of infections in each subgroup (Flynn et al. 2005; Pitisuttithum et al. 2006).

### 1.3.3.8. Live recombinant vaccines

Studies have suggested that the use of a vector can enhance vaccine protection level. Recombinant viruses are the current vectors that are used to deliver selected genes into the chosen host. Vectors are not usually derived directly from lentiviruses; poxviruses, adenoviruses and retroviruses are commonly used (Levy 2007; Girard et al. 2006). However, recently lentiviruses have been introduced as vaccine vectors.
Lentiviruses have successfully been used in gene therapy techniques, and their subsequent use in vaccination has evolved from the demonstration of their capacity to efficiently transduce dendritic cells.

In recombinant vaccination against HIV, envelope DNA is usually inserted into the genome of the vector virus that has been selected to produce a recombinant vaccine. The resulting virus will present envelope antigens to the host immune system, thus eliciting cellular immune responses as well as humoral responses (Girard et al. 2006; Morrow et al. 2012). DNA vaccines have been found useful when used within a prime-boost regimen. Although DNA vaccination itself does not elicit a strong immune response, it appears to prime the immune system quite effectively because a strong anamnestic response is observed when boosting with recombinant protein or vaccinia virus (Fuller, Simpson, et al. 1997; Fuller, Corb, et al. 1997; Haigwood et al. 1999). The strength of the immune response is strongly influenced by the choice of the vector system used, as well as the vaccination schedule (Fuller, Corb, et al. 1997; Fuller, Simpson, et al. 1997; Girard et al. 2011). The list of virus vectors used to construct live recombinant-vector-based HIV vaccines is long including lentiviruses, measles virus, vesicular stomatitis virus (VSV), Sendai virus and Venezuelan equine encephalitis virus (Sauter et al. 2005). Here I only highlight Pox viruses and Adenoviruses in detail, in favour to the fact that they have been used in several vaccination trials.

1.3.3.8.1. Pox viruses

Pox viruses are one of the most important vectors used in recombinant vaccination (Mercer et al. 2007). A variety of HIV antigens, such as Gag, Env, Pol and Nef, may be expressed using such vectors. Recombinant canarypox virus was used to express various HIV-2 antigens; high levels of antibody titres, together with specific HIV-2 CTLs, were detected. However, there was no correlation between immunologic response and protection, despite the immunisation boost (Levy 2007). Vaccinia viruses were the first to be used in vector based vaccines. In some early studies, a vaccinia virus vector vaccine expressing HIV gp120 was shown to protect chimpanzees from
low-dose HIV challenge. In humans, a vaccinia-virus-based vaccine has been shown to reduce the observed immune responses in subjects who had been previously vaccinated for smallpox (Levy 2007). However, the use of a prime-boost regimen with repeated boosting with gp160 has been shown to overcome this problem in the SIV model (Pitisuttithum et al. 2006). Due to safety concerns, highly attenuated pox viruses have been developed, such as modified vaccinia Ankara (MVA). Currently, several vaccination studies have been developed involving macaques and using MVA as a vector; MVA expresses multiple SIV antigens, most of which are provided within a prime-boost regimen. Generally, the results indicate reduced viral load, as well as a delay in the appearance of the disease among vaccinated macaques (Morrow et al. 2012; Girard et al. 2011). Promising studies in rhesus monkeys found that immunisation with recombinant MVA vector expressing SIV mac 239 Gag-Pol and HIV1 Env elicited strong Gag-specific CTL responses but did not create envelope-neutralising antibodies.

The first evidence to confirm that HIV-1 vaccine could confer protective efficacy against HIV-1 acquisition is provided by the Thai Phase III trial, RV144 (ClinicalTrials.gov NCT00223080). This prime-boost vaccine regimen consisted of a non-replicating recombinant canarypox vector (ALVAC; a Gag Pol Env prime and AIDSVAX gp120 B/E boost). The trial demonstrated a 31.2% reduction in the frequency of acquisition of HIV infection among vaccinated heterosexual men and women (refer to section 1.3.4).

1.3.3.8.2. Adenoviruses

An adenovirus-based vaccine system is of a major interest because it has effectively protected chimpanzees from HIV-1 infection. Replication-deficient adenovirus type 5 (Ad5) represents one of the most promising live viral vectors in HIV vaccination. It was first used by Merck Pharmaceuticals as a pilot monovalent Ad5 gag vaccine; it was tested on mice, nonhuman primates, and human volunteers. Strong, long-lasting, HIV-1-specific CD8+ T-cell responses were indicated in half of the volunteers (Casimiro et al. 2003; Shiver et al. 2002; Shiver & Emini 2004).
It has been argued that vaccination with recombinant Ad5, either delivered alone or as a boost after immunisation with a plasmid DNA vaccine, elicit significant immune responses to HIV-1 Gag, in which high numbers of long-lasting T-cells were found in human volunteer vaccine recipients (Levy 2007; Girard et al. 2006). However, frequent pre-existing anti-vector immunity in humans limits the efficiency of the use of Ad5. To overcome this problem, recombinant adenoviruses that are representative of less human-prevalent strains are under investigation; for example, Ad6, Ad35, Ad11 and Ad24. Non-replicative chimpanzee adenoviruses (AdC68, AdC6 and AdC7) could be used or chimeric adenoviruses developed to evade the pre-existing anti-Ad5 immunity, by replacing the fibre gene of an Ad5 vector with that of a rare Ad subtype. Adenovirus chimeras such as Ad5/Ad11 or Ad5/Ad35 have been constructed and successfully tested in animal models. Many other strategies using adenovirus vectors have been developed to increase the protective efficiency of the use of adenoviruses as recombinant vectors (Girard et al. 2006; Levy 2007; Girard et al. 2011).

Merck Pharmaceuticals, in collaboration with NIAID, engineered trivalent, recombinant Ad5-expressing Gag, Pol and Nef antigens and used these in the STEP trial (HVTN 502/ Merck V520-023). This was the first completed efficacy evaluation of a CMI-based HIV vaccine. It was conducted at 34 sites in North America, the Caribbean, South America and Australia, where the predominant circulating HIV-1 subtype is B, and enrolled 3,000 individuals. This Phase IIb trial started on Dec 2004 and terminated on Sep 2007. The findings indicated that the MRKAd5 gag/pol/nef HIV-1 vaccine neither prevented HIV-1 infection nor lowered the viral load (Buchbinder et al. 2008). The Step study revealed an increased risk of HIV-1 acquisition in vaccinees who were uncircumcised and/or Ad5 seropositive at baseline. However, later studies indicated that pre-existing seropositivity to multiple Ad serotypes is not intrinsically associated with increased risk of HIV-1 acquisition (Stephenson et al. 2012). The second Phase IIb trail of the MRK rAd5 vaccine, Phambili (HVTN 503), was undertaken in South Africa, where HIV-1 subtype c is predominant. Enrolment and vaccination was
terminated in Sep 2007 as a result of the STEP finding. Participants were followed-up for up to 3.5 years. Long-term follow-up analysis indicated no significant efficacy. Unlike the STEP study, statistical trends towards enhanced acquisition of HIV infection in vaccinees who were Ad5-seropositive at baseline or men who were uncircumcised were not observed (Gray et al. 2014) (refer to section 1.3.4).

1.3.3.9. Prime-boost immunisation regimens

Most of the vaccine strategies used to elicit immune responses against HIV have been shown to induce short–lived immune responses. In this regard, a prime-boost vaccination regimen has been developed. There are many types of boosting regimens (Girard et al. 2011). The initial use of a DNA vaccine candidate as a prime vaccine, followed by a recombinant live virus vector as a booster vaccine, is one example. Multiple studies concerned with HIV vaccination have used this approach, and it has been proven that the cellular immune response is improved by using such a method (Levy 2007). Synergy between DNA and live recombinant vectors has been observed, regardless of the type of recombinant. For example, compared to the Ad5 recombinant vaccine, the level of HIV-1 Env-specific T-cell responses is 7-fold higher in a DNA-Ad5 prime-boost regimen, which should dramatically increase the efficiency of the vaccine (De Rosa et al. 2011).

A prime-boost vaccine regime can be achieved using two live recombinant vectors that express the same antigens. The two live recombinants could be an Ad5 vector followed by a recombinant poxvirus (Casimiro et al. 2003), two successive recombinant poxviruses, such as MVA and FPV, or two successive recombinant adenoviruses, such as Ad26 followed by Ad5 or Ad35. It has been argued that the use of a heterologous prime-boost regimen, such as Ad5 followed by MVA or ALVAC, will elicit better cellular immune responses than those elicited via the use of a homologous Ad5 only regimen (Girard et al. 2011).
The final type of prime-boost immunisation regimen combined a DNA vaccine as a prime and a protein subunit as a booster an immunisation schedule which should elicit both cellular and humoral immune responses. It allows the use of a mixture of Env DNA plasmids for priming and boosting with a mixture of gp120 proteins from various HIV-1 clades in the hope of generating neutralising antibodies against primary heterogeneous HIV-1 isolates. It has been found that when a DNA-gp120 prime booster regimen was administered with the addition of an adjuvant, diverse antibody response was elicited with high levels of antibody avidity and cross-clade neutralising activity, as well as stronger cross-subtype T-cell responses (Girard et al. 2006; Levy 2007; Girard et al. 2011).

1.3.3.10. Other vaccine approaches

Many other vaccine approaches are at an early stage of clinical development. Many forms of vaccination use some combination of multi-epitopic peptides, fusion proteins and long lipopeptides (lipid connected to a peptide). Also, the use of adjuvants can enhance vaccine efficacy by stimulating the innate immune response thereby acting as immune stimulatory components (Levy 2007). These are undergoing evaluation either singly or in prime-boost recombinant virus regimens. It is argued that broad cellular immune responses can be elicited when patients are immunised with a multi-epitope DNA vaccine. Strong CD+8 T cell responses against HIV were detected in mice, higher primates and humans when immunised with synthetic lipopeptides containing MHC class I restricted T-cell epitopes without the addition of adjuvant (Girard et al. 2006).

A quite different method of vaccination is under development; it attempts to prevent the depletion of CD+4 T-cells. This approach has been focused on in therapeutic HIV vaccination. It is believed that using such a strategy would delay or prevent the onset of immunodeficiency and disease in HIV-infected patients (Girard et al. 2006; Levy 2007; Girard et al. 2011).
1.3.4. Current status of HIV vaccines

The six clinical efficacy trials (Figure 4) that have been completed to date and their results are summarised in Table 2. Each study was performed as a result of promising early pre-clinical and clinical data. The most promising of these efficacy studies was the RV144 study, also known as the Thai Prime-Boost trial, which was the first AIDS vaccine trial to show protection against HIV in humans. The prime-boost combination reduced HIV risk by an estimated 31.2% over three years of follow-up. To date, none of these studies have supported the licensing of an HIV/AIDS vaccine regimen.

![Figure 4: Timeline of HIV vaccine efficacy trials](image)

**Figure 4: Timeline of HIV vaccine efficacy trials.** Vaxgen: HIV gp120 protein, Merck/NIAID Step Trial: Adenovirus type 5, Sanofi/MHRP/NIAID/Thai Ministry of Health: RV-144 Trial : Canarypox + gp120, HVTN 505: NIAID-VRC DNA + Adenovirus type 5. Adapted from (Corey et al. 2011).

| Table 2: Phase 2b or Phase 3 efficacy studies |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | VAX004          | VAX003          | STEP            | Phambili        | RV144           | HVTN 505        |
| Clinical Trial Sponsor | VaxGen           | VaxGen          | Phase 3 trial   | Phase 2b        | Phase 3 trial   | Phase 2b        |
|                  |                 | and the Ministry | test-of-concept | test-of-concept | of vaccination  |                |
|                  |                 | of Public Health | study          | study companion | study to the   |                |
|                  |                 | of Thailand      |                | study          | STEP study      |                |
|                  |                 | Merck and NIAID  |                |                |                |                |
|                  |                 | Merck and NIAID  |                |                |                |                |
|                  |                 | USMHRP, the      |                |                |                |                |
|                  |                 | Ministry of      |                |                |                |                |
|                  |                 | Public Health of |                |                |                |                |
|                  |                 | Thailand         |                |                |                |                |
|                  |                 | Sanofi and NIAID |                |                |                |                |
|                  |                 | NIAID            |                |                |                |                |
|                  |                 | VRC              |                |                |                |                |
|                  |                 |                  |                |                |                |                |
| Clinical Trial Sponsor | VaxGen           | VaxGen          | Phase 3 trial   | Phase 2b        | Phase 3 trial   | Phase 2b        |
|                  |                 | and the Ministry | test-of-concept | test-of-concept | of vaccination  |                |
|                  |                 | of Public Health | study          | study companion | study to the   |                |
|                  |                 | of Thailand      |                | study          | STEP study      |                |
|                  |                 | Merck and NIAID  |                |                |                |                |
|                  |                 | Merck and NIAID  |                |                |                |                |
|                  |                 | USMHRP, the      |                |                |                |                |
|                  |                 | Ministry of      |                |                |                |                |
|                  |                 | Public Health of |                |                |                |                |
|                  |                 | Thailand         |                |                |                |                |
|                  |                 | Sanofi and NIAID |                |                |                |                |
|                  |                 | NIAID            |                |                |                |                |
|                  |                 | VRC              |                |                |                |                |
|                  |                 |                  |                |                |                |                |
| Place of study    | North America   | Thailand         | Australia, the  | South Africa    | Thailand        | U.S.           |
|                  | and the         |                 | Caribbean and   |                |                |                |
|                  | Netherland      |                 | North and South |                |                |                |
|                  | s               |                 | America         |                |                |                |

To date, none of these studies have supported the licensing of an HIV/AIDS vaccine regimen.
<table>
<thead>
<tr>
<th>Eligibility</th>
<th>Sample size</th>
<th>Vaccine</th>
<th>Candidate vaccine</th>
<th>Vaccine component</th>
<th>Placebo</th>
<th>Randomization: Vaccine to Placebo</th>
<th>Vaccine administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-uninfected MSM and high-risk heterosexual women</td>
<td>5400</td>
<td>AIDSVAX® B/B'</td>
<td>Bivalent rgp120 with alum</td>
<td>MNrgp120/HIV-1 plus A244 rgp120/HIV-1 (subtype B and E)</td>
<td>alum</td>
<td>2:1</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>Intravenous drug users who were not infected with HIV-aged 20–60</td>
<td>2500</td>
<td>AIDSVAX® B/E</td>
<td>Bivalent rgp120 with alum</td>
<td>MNrgp120/HIV-1 plus GNE8 rgp120/HIV-1 (subtype B and B')</td>
<td>Alum</td>
<td>1:1</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>Both Ad5-seronegative and seropositive men and women aged 18–45 with different risk criteria</td>
<td>3000</td>
<td>MRKAd5 HIV-1 gag/pol/nef</td>
<td>Trivalent replication-incompetent Adenovirus type 5-vector expressing clade B HIV-1 gag,pol, and nef genes</td>
<td>The bivalent B/E gp120 vaccine used is the same as the VAX003 study with an ALVAC vector prime expressing Gag and Pro from clade B (LAI strain) and gp120 from clade CRF_01 (A/E) strain 92Th023</td>
<td>801</td>
<td>Un-blinded</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>Sexually active men and women aged 18–35</td>
<td>16402</td>
<td>ALVAC and AIDSVAX</td>
<td>ALVAC-HIV (vCP1521) boosted by bivalent AIDSVAX gp 120 B/E</td>
<td>A prime mixture of six DNA plasmids expressing Gag, Pol, Nef, and Env from clade B, and two additional Env proteins from clade A and C. A single boost dose given consists of a mixture of four Ad5-vectors expressing Env from clades A, B, and C and a Gag-Pol fusion protein from clade B</td>
<td>2504</td>
<td>1:1</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>Healthy adults aged 18–30 at primarily heterosexual risk and MSM, commercial sex workers (CSW) and IDU/PWID based on self-reported risk behaviors</td>
<td></td>
<td>VRC DNA/rAd5</td>
<td>Retrivial replication-incompetent Adenovirus type 5-vector expressing clade B HIV-1 gag,pol, and nef genes</td>
<td>A single boost dose given consists of a mixture of four Ad5-vectors expressing Env from clades A, B, and C and a Gag-Pol fusion protein from clade B</td>
<td></td>
<td>Un-blinded</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>Circumcised men who were Ad5-seronegative MSM or transgender (male-to-female) aged 18–50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:1</td>
<td>Intramuscular</td>
</tr>
</tbody>
</table>

**Dose**

<table>
<thead>
<tr>
<th>HIV-uninfected MSM and high-risk heterosexual women</th>
<th>Intravenous drug users who were not infected with HIV-aged 20–60</th>
<th>Both Ad5-seronegative and seropositive men and women aged 18–45 with different risk criteria</th>
<th>Sexually active men and women aged 18–35</th>
<th>Healthy adults aged 18–30 at primarily heterosexual risk and MSM, commercial sex workers (CSW) and IDU/PWID based on self-reported risk behaviors</th>
<th>Circumcised men who were Ad5-seronegative MSM or transgender (male-to-female) aged 18–50</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 µg (MN)/ 300 µg (GNE8)</td>
<td>300 µg (MN)/ 300 µg (A244)</td>
<td>A dose of 1.5 × 10^10 vector genomes in 1 mL</td>
<td>ALVAC at all four doses and gp120 at the final two doses</td>
<td>Priming dose of 4 mg of plasmid mixture given on days 28 and 56. Single boost dose given on day 168 of Ad5 vector is 1 × 10^10 particle units (PU) total</td>
<td></td>
</tr>
</tbody>
</table>

**Placebo**

|  | alum | Alum |

| Randomization: Vaccine to Placebo | 2:1 | 1:1 | Un-blinded | 1:1 | Un-blinded |

| Vaccine administration | Intramuscular | Intramuscular | Intramuscular | Intramuscular | Intramuscular |
Description

Vaccinations were given at months 0, 1, 6, 12, 18, 24, and 30, with the final study visit at month 36.

Vaccinations were given at 0, 1, 6, 12, 18, 24, and 36 months.

Vaccinations were given at 0, 1, 6 month schedule.

Vaccinations were given at 0, 4, 12, and 24 weeks.

A single boost dose given at month six, after a four-month rest from the priming series.

Efficacy

No significant efficacy was observed.

No significant efficacy was observed.

No significant efficacy was observed. Statistical trends towards enhanced acquisition of HIV infection in vaccinees who were Ad5-seropositive at baseline or men who were uncircumcised were not observed.

No significant efficacy was observed. Unlike the STEP study, statistical trends towards enhanced acquisition of HIV infection in vaccinees who were Ad5-seropositive at baseline or men who were uncircumcised were not observed.

Estimated at 31.2% (95% CI of 1.1–51.2%, p = 0.04) achieving statistical significance.

Vaccine efficacy was estimated at −25% (95% CI, −121.2 to 29.3, p = 0.44). Overall, there were 41 vaccinees and 31 placebo-recipients who had become HIV infected, thus the study had to be halted.

1.4. Humoral immune responses to HIV infection

1.4.1. Overview

Evidence from HIV-related animal models and other virus studies have suggested a need for effective humoral-based protection, in addition to cellular responses, in order to produce effective vaccination. The classical approach of mimicry, including attenuated viruses, killed viruses and subunit viruses, failed because of the instability of HIV glycoproteins and the low immunogenicity of the conserved regions of the glycoproteins, especially compared to immune-dominant variable regions. The discovery of neutralising antibodies in HIV infected patients during the 1980s raised hopes that humoral immunity could be readily employed for passive immunisation. Chronically HIV-1-infected serum were found to have moderate to broadly neutralising antibodies as analysed by serum mapping, suggesting that the human immune system is capable of generating broadly neutralising antibodies responses against HIV. However, this proved far more challenging to achieve than imagined. The antibody
response to HIV is generally vigorous, and it is predominantly directed to the structural proteins of the virus (Klein et al. 2013; Walker & Burton 2010; Ferrantelli & Ruprecht 2002).

Studies have confirmed that shortly after HIV infection, antibodies are detectable. The evolution of antibodies begins with their early activity against the Gag proteins, followed by Nef, Rev and Env (Levy 2007). The response to these different viral proteins can elicit antibodies with different isotypes. Circulating antibodies that react against HIV and HIV-infected cells can possess one of four functions: neutralisation, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cytotoxicity and complement-binding (Figure 5) (Klein et al. 2013; Walker & Burton 2010; Ferrantelli & Ruprecht 2002; Huber & Trkola 2007). In infected individuals, the antiviral response to Gag can elicit several subclasses of antibodies, unlike anti-Env, where only one specific type is usually found. Env is the only known target for neutralising antibodies.

Figure 5: Function of antiviral antibodies. (a) Neutralisation of free virus by antibodies, (b) complement-mediated lysis of free virus and infected cells triggered by antibodies, (c) opsonization of virus particles by antibodies and phagocytosis of virus particles via Fc- or complement-receptors, (d) antibody-dependent cellular cytotoxicity (ADCC) against infected cells. Neutralising antibodies (red), non-neutralising (blue), Fc-receptors (violet), complement components (light-blue), complement-receptors (black). Reproduced from (Huber & Trkola 2007).
However, among the various isotypes, only IgG1 was found to play a dominant role at all stages of infection (Walker & Burton 2010; Levy 2007; Kwong et al. 2013).

1.4.2. Neutralising antibodies in HIV infection

Studies have suggested that neutralisation is often considered to be the most efficient mode of antibody-mediated defence against viral infections, and is therefore considered the most significant humoral response elicited by vaccines. Although antibodies can be detected during the first months of HIV infection, natural infection predominantly induces non-neutralising or strain-specific antibodies. Recent studies have argued that around 20% of HIV-1 chronically infected individuals elicit neutralising antibodies (Klein et al. 2013; Yang & Wang 2014). Studies have shown that a small fraction of these antibodies (2–4%) elicit a broadly neutralising response capable of neutralising most tested HIV strains (Simek et al. 2009). It is argued that neutralising antibodies could alter viral replication during the early asymptomatic stage of infection. However, very low titres of these antibodies can be detected; this may favour the selection of escape mutations. The mechanism of neutralisation lies either in blocking the virus from its interaction with the specific receptors, preventing fusion or entry into the target cell, or less commonly inhibiting transport to cytoplasm by preventing viral budding or uncoating (Klein et al. 2013; Ferrantelli & Ruprecht 2002; Huber & Trkola 2007).

Recent studies have indicated that known HIV-1 neutralising antibodies bind to HIV envelope glycoproteins—more specifically, functional viral envelope glycoprotein spikes. Structural analyses of the HIV envelope proteins have indicated that many viral neutralising epitopes are hidden from the immune system due to their spatial arrangement. In addition, it has been found that the viral spikes can be protected from neutralisation by evolving a shield of glycans, variable immunodominant loops and conformational masking of important viral epitopes. However, studies have confirmed that complete prevention can be achieved through passive delivery of neutralising antibodies in animal models of HIV-1 transmission (Shibata et al. 1999; Mascola et al.}
2000; Hessell et al. 2009; Mascola 2003). IgG has a higher general neutralising ability than IgA (Kozlowski et al. 1994).

Passive transfer studies have suggested that relatively high concentrations of neutralising antibodies are required for protection in the SHIV/macaque model (Mascola et al. 2000; Burton & Parren 2000; Lu et al. 2003). However, there is some indication that passive antibody protection requires antibody activity against infected cells as well as free viruses (Hessell et al. 2007). In general, the level of antibodies needed to provide benefit remains unclear as different factors can determine the sensitivity of various HIV strains to antibody neutralisation. However, the ability to neutralise HIV appears to require binding to most if not all of the surface envelope glycoprotein molecules (spikes), which usually ranges from 7 to 14 per virion (Levy 2007; Klein et al. 2013).

Functional glycoprotein spikes on HIV particles are composed of trimeric heterodimer of noncovalently attached surface subunit gp120, which is involved in CD4 receptor recognition, and transmembrane subunit gp 41, which is responsible for the viral fusion/entry and target cell membrane (Levy 2007; Kwong et al. 2013). Only antibodies that interact with the intact trimer are considered to be neutralising. Following the interaction of the virus spikes with the cell receptors and the co-receptors, conformational changes occur to these spikes, exposing hidden neutralising epitopes (Kwong et al. 2013).

Thus far, most studies have indicated that at least five epitopes of the viral envelope could be involved in neutralisation (Figure 6 and Figure 7). Within gp120, there are four regions involved in antibody neutralisation—the V3 loop, the V1/V2 loop, the CD4-binding site (CD4BS) and the CD4-induced (CD4i) binding domain—which are involved in the interaction with the co-receptors. On the other hand, only a few antibodies directed to a specific region of the gp41—the membrane proximal external region (MPER)- have been identified including the monoclonal antibodies 2F5, 4E10 and Z13. Although MPER was found to have highly conserved structures, antibodies to this
region are difficult to detect in sera. Most recently, additional neutralisation epitopes within gp41, signified by antibodies to the CBD1-binding domain and fusion intermediates, have been described. Further analysis is expected to confirm their efficacy. According to Binley et al., anti-gp120 showed selective reactivity against clade B viruses, while anti-gp41 antibodies had broader cross-clade efficacy. Moreover, envelope carbohydrate moieties and cell surface proteins, including LFA-1, ICAM and HLA on the envelope, act as regions of HIV sensitive to antibody neutralisation (Binley et al. 2004; Levy 2007; Kwong et al. 2013; Klein et al. 2013; Ferrantelli & Ruprecht 2002).

Figure 6: Sites of HIV-1 vulnerability to neutralising antibodies. The HIV-1 envelope spike is the target of known virus-directed neutralising antibodies. An image of the viral spike obtained by cryoelectron microscopy (light grey) is shown fitted with atomic-level ribbon models for three portions of the HIV-1 envelope glycoprotein (Env; red): the membrane-proximal external region (MPER) of gp41 is shown towards the top of the image, the structure of the core gp120 with intact amino- and carboxyl termini96 is shown in the middle of the image, and the V1/V2 domain55 is shown towards the bottom of the image. Antibodies that effectively neutralise HIV-1 primarily target four specific regions in Env: the MPER, which is bound by antibody 10E8 (cyan), the CD4-binding site on gp120, which is bound by antibody VRC01 (fuchsia), and two sites of N-linked glycosylation, one of which is in the V1/ V2 region at residue Asn160 and is bound by antibody PG9 (green), and the other which is a glycan V3 epitope that generally includes residue Asn332 and is bound by antibody PGT128 (blue). Reproduced from (Kwong et al. 2013).
1.4.3. HIV envelope protein

Prior to the isolation of human HIV neutralising monoclonal antibodies, research on vaccine development concentrated on particular regions of the HIV envelope that were immunogenic to elicit neutralising antibodies (Klein 2001).

1.4.3.1. V3 loop

The V3 loop is found in the central portion or crown of the third variable region, which is located within the mid portion of gp120. The V3 loop is also called the principal neutralising domain (PND); it is a critical immune-dominant neutralisation domain of gp120. The V3 loop is involved in a post-CD4-binding step with a co-receptor that HIV requires to enter cells. Although V3 is a variable region, the PNDs of numerous strains...
differ only slightly in amino acid structure. It has been suggested that immunisation within this region can elicit antibodies that neutralise a large number of HIV strains that share this envelope domain (Levy 2007; Kwong et al. 2013).

Investigations found that homologous HIV-1 strains are not always neutralised by sera with high titre antibodies to V3 peptides, suggesting that a V3 loop can have both neutralising and non-neutralising epitopes. Neutralisation-escape studies have indicated that the V3 loop rapidly mutates, leading to a reduction in its ability to be neutralised. The V3 region can present both linear and conformational determinants recognisable by antibodies. The neutralisation that resulted from using the V3 regions might have resulted from inhibiting the V3 envelope cleavage, thus preventing viral attachment. Changes in other envelope regions might also alter the neutralisation of the V3 loop (Levy 2007; Kwong et al. 2013; Kwong et al. 2011).

1.4.3.2. CD4 binding site

The CD4 binding domain, which is a large complex conformational region of the envelope, is the second principal neutralising region in gp120. Polyclonal antibodies that cross-neutralise a large number of primary- and laboratory-adapted strains, including those with different V3 regions, are directed against this domain. Conversely, sera from HIV infected individuals' have neutralising monoclonal antibodies that recognise discontinuous epitopes in the gp120 that is involved in CD4 receptor interaction. The antibodies are thought to prevent the attachment of HIV strains to CD4. However, broad antiviral activity by one type of monoclonal antibody might not be achieved because numerous regions of the HIV envelope are involved and, thus, numerous monoclonal antibodies might be needed to attain virus neutralisation. Several studies indicate that conformational changes in gp120 result from point mutations in gp41, as well as in the V3 loop, and affect the neutralisation ability in this domain (Levy 2007; Kwong et al. 2013; Kwong et al. 2011; Zhou et al. 2007).
1.4.3.3. Other V regions

The third and fourth principal neutralisation sites on gp120 are located in the V1 and V2 regions, involving both linear and conformational determinants. Antibodies that are directed by both glycosylated and non-glycosylated epitopes have been found to recognise such regions. Removal of the V2 loop from gp120 exposes epitopes on the envelope that are conserved among various HIV-1 clades; this increases the virus’s susceptibility to neutralisation. In contrast, neutralisation escape was observed in HIV strains with a V1 deletion. It is still unclear whether V4 and V5 regions in HIV envelope proteins contain domains that allow neutralisation (Levy 2007; Kwong et al. 2013; Kwong et al. 2011).

1.4.3.4. Gp41

Gp41 is a common fusion domain where only one specific region, which is named the membrane proximal external region (MPER), is involved in neutralising antibody binding. Immunisation studies of laboratory animals found that the amino terminal portion of this region elicits antibodies against homologous and heterologous strains. Certain broadly neutralising monoclonal antibodies target the MPER and interact with the ectodomain of the HIV protein. Although the MPER displays highly conserved structures, it poses the problem of auto-antigen mimicry. Antibodies to these structures tend to be autoantibodies and react with normal cellular proteins (Levy 2007; Kwong et al. 2013; Kwong et al. 2011).

1.4.3.5. CD4 Induced neutralising antibodies

CD4-induced or CD4i antibodies are the antibodies that bind to HIV-1 gp120 after it interacts with CD4. Studies have indicated that these epitopes compete with the co-receptor binding sites and that CD4i antibodies may broadly neutralise different HIV-1 strains, as well as HIV-2. The need for further analysis of these epitopes remains, although the cross-reacting neutralisation that was observed is encouraging and might
indicate a high degree of antigenic conservation that is linked to co-receptor binding (Levy 2007; Kwong et al. 2013; Kwong et al. 2011).

1.4.4. **HIV neutralising monoclonal antibodies**

The definition and characterisation of monoclonal antibodies that mediate a broad neutralisation range, as well as the structural basis for its interaction with the HIV envelope, have provided opportunities for designing HIV vaccines that stimulate the production of antibodies that are directed against specifically conserved regions of the virus (Yang & Wang 2014; Kwong et al. 2011).

1.4.4.1. **First-Generation broadly neutralising Abs against HIV**

Initially only a few broadly neutralising monoclonal antibodies (mAbs) were isolated and confirmed to neutralise primary isolates of HIV-1. These antibodies displayed limitations in the overall potency or range of reactivity (Yang & Wang 2014; Kwong et al. 2011).

The first broadly neutralising human Ab against HIV to be found was the antibody IgG1b12 or IgGb12, which recognises a unique epitope on the gp120 surface of the HIV Env and overlaps the CD4-binding site (Burton et al. 1991). IgG1b12 was isolated from a clade B-infected patient by applying a novel method of phage screening of libraries of Fab’s heavy and light chains. Because of the manner of their discovery, the presence of IgG1b12 in nature has been questioned (Klein et al. 2013). Moreover, the source material for the library was also unique as HIV-1- from an infected individual's bone marrow RNA was used (Kessler et al. 1997). IgG1b12 can neutralise almost 50% of clade B viral strains and approximately 30% of non-clade B strains (Yang & Wang 2014). In another study, the IgG1b12 antibody was found to neutralise approximately 35% of circulating isolates (Kwong et al. 2011).
Structural studies indicated that IgG1b12 differed from the naturally elicited Abs, because it used the heavy chain only as a means of recognition. This dose did not affect the site recognition ability of IgG1b12 (Zhou et al. 2007). However, antigenic variation was found because of the extended recognition ability of IgG1b12, which could recognise outside targets, especially around the CD4-binding loop, particularly in the case of non-B clade isolates (Wu et al. 2009). Burton et al., indicated that the human monoclonal antibody IgG1 b12 can prevent SHIV SF162 infection in rhesus macaques. However, the dose of antibodies that is required to achieve complete neutralisation was suggested to be extremely high, which is unlikely to be achieved through current immunisation techniques (Burton et al. 1994; Davis et al. 2013; Kessler et al. 1997). Few studies have been conducted so far that could effectively neutralise HIV using IgG1b12. Hessell et al., found that the neutralising effect of IgG1b12 could be decreased if the Fc region of the IgG was altered to elicit complement binding and ADCC activity (Hessell et al. 2007).

Another broadly neutralising antibody that was isolated is IgG 2G12. IgG 2G12 recognises a unique cluster of high mannose carbohydrates of N-linked glycosylated amino acid residues on the immunologically silent face of gp120 (Trkola et al. 1996; Wyatt et al. 1998; Sanders et al. 2002). It was also found that 2G12 has broad neutralising activity in vitro, where it binds to a glycan epitope, and that it can neutralise numerous clade B-derived viral strains and, to a lesser extent, other HIV subtypes (Burton et al. 1994; Trkola et al. 2005; Binley et al. 2004). IgG 2G12 has a unique structure, where heavy and light chains lie vertically and adjacent to one another, and, thus, are not associated in a traditional Y-like manner. It was suggested that this unique configuration provides the antibody with flexibility and thus enables it to undergo multivalent interactions with the gp120 oligomannose cluster (Calarese et al. 2003). Phase I and I/II clinical trial studies have been conducted using this antibody (Armbruster et al. 2002; Stiegler et al. 2002; Trkola et al. 2005).
The antibodies 2F5 (Muster et al. 1993; Purtscher et al. 1994; Trkola et al. 1996) and 4E10 (Stiegler et al. 2001; Zwick et al. 2001) were found to bind to both the gp41 MPER and its nearby lipids. Zhu et al. 2006, confirmed that the MPER constituted a suitable target for HIV neutralising antibodies. The antibodies 2F5 and 4E10 are the two most studied neutralising antibodies in this region (Zhu et al. 2006; Binley et al. 2004; Stiegler et al. 2001; Zwick et al. 2001; Muster et al. 1993). Although 4E10’s potency is modest, it displayed a substantial range of neutralisation across several clades. Both antibodies were successfully tested in Phase I studies (Armbruster et al. 2004; Armbruster et al. 2002).

An in vivo, passive transfer of IgG b12, IgG 2G12, IgG 2F5, or IgG 4E10 protected against SHIV infection in macaques (Mascola et al. 2000; Hessell et al. 2009; Mascola et al. 1999; Baba et al. 2000; Parren et al. 2002). A clinical study demonstrated that a combination of IgG 2G12, IgG 2F5, and IgG 4E10 could delay a viral rebound in numerous patients after cessation of a successful cART. An escape mutant analysis suggested that the activity of IgG 2G12 was crucial for the in vivo effect of the neutralising antibody cocktail in this trial (Trkola et al. 2005).

1.4.4.2. Second-Generation broadly neutralising Abs against HIV

Since 2009, following the application of new B cell technologies, a large panel of new HIV-1 mAbs of substantial breadth were identified. It has been argued that numerous additional neutralising mAbs might exist and will soon be detected. These new broadly neutralising Abs (bNAbs) have greater neutralisation potency and breadth than the first generation of bNAbs. They could provide an excellent resource for antibody gene transfers to target HIV. However, to improve the knowledge of them, further structural and escape mutant analytical studies are necessary (Klein et al. 2013; Yang & Wang 2014; Kwong et al. 2011).
Nussenzweig and colleagues combined the cell sorting of gp140-binding B cells and single-cell antibody cloning to reveal that multiple antibodies targeting a range of Env epitopes with broad neutralising activity could be detected in patients who were infected with HIV (Mouquet et al. 2011; Walker et al. 2009). Burton and colleagues, who screened over 30,000 activated memory B cells from clade A-infected African donors by utilising clonal B cell culture, succeeded in identifying two broad neutralising Abs, PG9, and PG16 that target the variable loops V2 and V3 of gp120 (Burton et al. 1991; Walker et al. 2009). The structure of this portion of the Env has yet to be determined, and its functional importance remains unclear. Beside PG9 and PG16, numerous antibodies have been identified that recognise this region, including human monoclonal antibody (Klein et al. 2013) and a number of rhesus antibodies (Walker et al. 2009). Studies have indicated that these antibodies vary dramatically in their neutralisation breadth. Human monoclonal antibody 2909 was found to be extremely strain-specific, whereas PG9/PG16 could neutralise 70%–80% of circulating isolates (Honnen et al. 2007; Walker et al. 2009).

Antibody HJ16 is a natural human antibody that was isolated by direct assessment of neutralisation coupled with single B cell antibody sequencing. Further structural analysis is yet to be conducted to determine its characteristics and structure. However, studies suggested that HJ16 targets the “loop D” region, which is outside the known neutralisation sites. HJ16 neutralises 30%–40% of tested HIV strains (Klein et al. 2013; Corti et al. 2010).

Antibodies VRC01, VRC02, and VRC03 targeted the site of vulnerability more precisely. Studies suggested that they bind to a functionally conserved region of gp120 that interacts with the host cell receptor CD4 (Zhou et al. 2010; Wu et al. 2010; Zolla-Pazner 2004; Pantophlet & Burton 2006; Mascola & Montefiori 2010). It is suggested that these antibodies could neutralise over 90% of circulating isolates (Wu et al. 2010). Structural studies reveal that the VRC01 heavy chain, in complex with gp120, mimics the CD4 receptor (Zhou et al. 2010).
Recently, the 10E8 broad neutralising antibody has been identified. It targets the MPER region and can neutralise more than 90% of tested HIV strains (Klein et al. 2013; Huang et al. 2012). The first monoclonal antibody against HIV applied in passive immunisation \textit{in vivo} was MAb F105, which binds to the CD4-binding site (Posner et al. 1991). The monoclonal antibodies 2G12, 2F5, 4E10, and F105 and the immunoglobulin fusion protein PRO 542 have all been probed for efficacy in established HIV infection (Trkola et al. 2005; Armbruster et al. 2004; Wolfe et al. 1996; Cavacini et al. 1998; Jacobson et al. 2004; Jacobson et al. 2000). Recent studies isolated substantially more broadly neutralising antibodies. The list of these new second-generation broadly neutralising antibodies against HIV is lengthy; because most of them are yet to be understood, further research is necessary (Klein et al. 2013).

1.4.5. \textit{Passive immunisation using neutralising antibodies}

Passive immunisation, less commonly known as antibody mediated prevention (AMP), is the transfer of pre-made antibodies to patients. Protection with transferred antibodies can occur naturally, such as when maternal antibodies are transferred to the fetus through the placenta, as well as artificially.

Several studies have shown that neutralising antibodies with broad specificity can protect from the onset of HIV-1 infection if present at threshold concentration at the time of the viral inoculum. Passive administration of broadly neutralising monoclonal HIV-1 envelope-specific antibodies to macaques shortly before infection with SHIVs carrying an HIV-1 envelope was found to prevent infection (Parren et al. 2001). Similar results have been reported in macaque trials where vaginal administration of neutralising antibodies before the viral challenge resulted in full protection from systemic infection (Mascola et al. 2000; Pegu et al. 2017). Further evidence came from mice with severe combined immunodeficiency (SCID) that were reconstituted with human lymphoid tissue followed by infection with HIV-1. This experiment indicated that
the antibodies have to be inoculated and elicited before the virus encounter, as virus escape mutation are quickly selected (Veazey et al. 2003). Levels of antibodies declined over time, thus repeated doses are required to maintain a protection level. Passive immunization clinical trials are under planning and additional research is ongoing to find an approach to enhance the potency and half-life of existing bNAb.

Two multinational clinical trials of an intravenously delivered investigational antibody for preventing HIV infection have been launched, known as the AMP Studies, for antibody-mediated prevention. The trials will evaluate the safety and efficacy of the human monoclonal antibody (mAb) VRC-HIVMAB060-00-AB (VRC01) in preventing HIV-1 infection. The study will also examine safety of these antibody infusions and determine the time the antibody is in the blood of study participants receiving different amounts or doses of the antibody. The AMP Studies are being conducted jointly by the NIAID-funded HIV Vaccine Trials Network (HVTN) and HIV Prevention Trials Network (HPTN). Both are Phase Ib clinical trials. The first AMP Study, HVTN 704/HPTN 085 (ClinicalTrials.gov:NCT02716675), is taking place in Brazil, Peru and the United States. Enrol 2,700 men and transgender people who have sex with men. The study started on March 2016. The second of the two AMP Studies, HVTN 703/HPTN 081 (ClinicalTrials.gov:NCT02568215), started on April 2016, aiming to enrol 1,500 high risk HIV-uninfected women. This study is being conducted in Botswana, Kenya, Malawi, Mozambique, South Africa, Tanzania and Zimbabwe. The volunteers in both studies are adults aged 18 to 50 years, at high risk for HIV infection, but HIV-negative when they enter the study. In each trial, volunteers will be assigned at random to receive an intravenous infusion of either VRC01 at a dose of 30 milligrams per kilogram (mg/kg), VRC01 at a dose of 10 mg/kg, or a saline solution (a placebo). It is a double-blind study where neither the volunteers nor the study investigators will know who receives which type of infusion until the end of the study. Volunteers will receive a total of 10 infusions, once every 8 weeks, and then will be followed for 20 more weeks. The results of both trials are expected in 2022. If the current AMP Studies provides proof of
concept, it could lead to additional passive immunization trials, as well as research on
vaccines to help the body make these protective antibodies on its own
(https://ampstudy.org/ acceded on 07/05/18).

1.5. Antibody gene cloning

1.5.1. Antibody structure

Immunoglobulins are Y-shaped molecules whose basic units are composed of 4 polypeptide chains arranged in 2 identical pairs of heavy (H) and light (L) chains (Figure 8). Each antibody molecule has 2 Fab fragments that contain identical antigen binding sites at their tips. IgG antibodies have one Fc (fragment crystallisable) fragment joined to the Fabs by a flexible hinge (Fc has a propensity to self-associate and crystallise into a lattice). The Fab region consists of the entire light chain combined with part of the heavy chain. At the protein level, each of the polypeptide chains is folded into discrete domains. The N-terminal variable regions (V-domains), VH and VL in the heavy and light chains respectively, are variable in sequence. Within each V-domain are 3 hypervariable regions (complementarity determining regions or CDRs) that indicate the antibody specificity. The combination of the pairs of variable heavy and light chains is referred to as the Fv of the antibody and is responsible for the binding of antigens. In contrast, the C-terminal domains of both heavy and light chains are more conservative and are called the constant regions (the C regions of the heavy chains mediated effector functions).

There is only one constant region in the light chain’s CL, while the IgG heavy chains have 3 constant regions: CH1, CH2 and CH3. The constant regions of the immunoglobulin are responsible for a variety of effector functions, including complement fixation and clearing by cytotoxic mechanisms. Almost all antibodies share the same structural characteristics but display remarkable variability in the regions that bind the antigens (Davies & Chacko 1993; Travers et al. 1999).
1.5.2. Antibodies and molecular immunology

Because of modern advances in molecular technologies, antibodies (including human antibodies) are prepared using different methods than in the past. For example, transgenic mice strains can be genetically manipulated to produce much larger amounts of fully human antibodies, whereby the mouse immunoglobulin heavy- and light-chain loci are deleted in embryonic stem cells and replaced by human artificial chromosomes containing the entire human heavy chain and gamma light-chain loci. Mammalian cells have also been genetically manipulated to express fully human antibodies through viral recombinant transgenic antibodies (Klein et al. 2013; Bennett & Akkina 2013; Joseph et al. 2010). Since the discovery of the mAb in the 1980s, the use of gene therapy and vaccine production has been studied extensively. By the middle of 2008, the FDA had approved the use of 21 mAb as therapeutic agents, only one of which was directed against infectious disease (Palivizumab – used for prophylaxis against respiratory syncytial virus in infants). Among different types of antibodies, IgG is the preferred isotype. The high plasma half-life, long-time stability, suitability for efficient affinity purification and immunological effector functions all indicate that IgG is a better choice (Giacca 2010; Jostock 2011; Marasco 2005).

Hybridoma technology was the first technique used to manufacture therapeutic monoclonal antibodies. However, while this method can be applied to mouse
antibodies, the method is not effective for less immunogenic chimeric and humanised antibodies and is difficult to apply for fully human antibodies (Giacca 2010; Jostock 2011).

1.5.3. Antibody gene transfer for HIV immunoprophylaxis

Antibody gene transfer is a novel protective strategy that allows the transfer of genes that are encoding antibodies into host cells, bypassing the natural immune response. To date, few studies have focused on this approach in attempts to develop a vaccine against HIV (Figure 9) (Giacca 2010; Walker & Burton 2010; Balazs & West 2013). The usefulness of this approach has been demonstrated in two studies. One was directed against simian immunodeficiency virus (SIV) in macaques (Johnson et al. 2009), while the other was directed against the HIV in humanised mice (Balazs et al. 2012).

**Figure 9: Examples of HIV prophylaxis approaches.** Traditional vaccines work by engaging the adaptive immune system to produce a response that recognises the administered antigen. Conventional vaccines use a viral vector to deliver genes encoding a given antibody where the production of antibodies is directed to nonhematopoietic tissues, such as muscle that then express the desired antibody and secrete it into the circulation without using the immune system. Reproduced from (Balazs & West 2013).
Johnson et al. used adeno-associated virus (AAV) serotype 1 as a vector to deliver genes encoding anti-SIV immunoadhesins, which are chimeric antibody-based molecules that combine the functional domain of a binding protein with immunoglobulin crystallizable fragment (Fc) domains (Figure 10), to protect macaques. This study showed consistent results wherein macaques expressing the immunoadhesin 4L6 had sustained circulating concentrations of approximately 20 mg/mL of 4L6 for 5 years with no adverse health effects after 4 intramuscular injections given at the initiation of the study. The macaques also demonstrated a stable erythropoietin expression for over 6 years (Johnson et al. 2009).

Figure 10: Schematic representation of immunoadhesin constructs. For molecules derived from macaque Fabs (4L6, 8S, 5L7 and 3V), VH and VL domains were joined by a synthetic linker. Rhesus CD4 (domains 1 and 2 (D1 and D2)) was cloned as described in the Methods online. Antigen-binding domains were attached to the Fc fragment of a rhesus IgG2 molecule. Reproduced from (Johnson et al. 2009).

Burton presented early results of experiments where a remarkable protection in macaques was demonstrated by giving different doses of PGT121. After intravaginal challenge, all monkeys that received a dose of 5 mg/kg or 1 mg/kg remained uninfected, whereas 3 of 5 monkeys that received a dose of 0.2 mg/kg were protected from challenge. The antibody was not detected at the vaginal surface of any challenged monkeys (Burton et al. 1994; Balazs & West 2013).
Baltimore also reported a study wherein humanised mice were given vector immunoprophylaxis to express antibody b12 or VRC01, both challenged with the REJO.c transmitted founder strain. In his experiment, substantial protection against infection with REJO.c was noted in mice expressing VRC01 but not in those expressing b12; this was consistent with results obtained in vitro for these antibody strain combinations (Balazs & West 2013; Balazs et al. 2012).

Balazs et al. conducted a study based on vector-mediated gene transfer to engineer secretion of broadly neutralising antibodies into the circulation and to describe the practical implementation of vectored immunoprophylaxis (VIP) (Balazs et al. 2012). In this study, humanised mice were found to induce long lasting expression of monoclonal antibodies at high concentrations from a single intramuscular injection. Adeno-associated viruses were used as optimised vectors to produce full-length antibody from muscle tissue. Results from this study indicated that humanised mice receiving VIP were fully protected from HIV infection. Intravenous challenge with very high doses of replication-competent virus was successful. Based on this experiment, it was suggested that effective prophylaxis against HIV could be achieved, as the feasibility of direct translation of broadly neutralising antibodies (bNAbs) into functional immunoprophylaxis using VIP in vivo was demonstrated.

The first phase 1 clinical trial involving the use of adeno-associated viruses (AAV) for the expression of antibody from muscle tissues (Clinical Trials.gov: NCT01937455) started on 2014 and estimated to be completed by 2017. It is a randomised (3:1) blinded, dose-escalation study to evaluate the safety and tolerability of rAAV1-PG9DP when administered intramuscularly at $4 \times 10^{12}$ vg, $4 \times 10^{13}$ vg, $8 \times 10^{13}$ vg and $1.2 \times 10^{14}$ vg in healthy male adults aged from 18 to 45 years. A total number of 24 volunteers were screened up to 42 days before injection and followed for 12 months after the single administration. The trial was held in the United Kingdom in the Surrey Clinical Research Centre and was sponsored by IAVI, NIAID, and the Children’s Hospital of Philadelphia. Findings from the current PG9 study will provide important information
about vector safety, antibody concentration, duration of expression, and neutralization capacity. On the other hand, Baltimore and the Vaccine Research Centre at the US National Institutes of Health are planning a separate trial of AAV serotype 8 that expresses an immunoadhesin to the CD4-binding site from muscle in infected patients receiving treatment with antiretroviral drugs (Balazs & West 2013; Johnson et al. 2009).

1.5.4. Expression vectors

In gene therapy, a cloned gene is inserted into a cell using one of many delivery methods; viral vectors are often used. Viruses are highly efficient at infecting cells, inserting their genomes and promoting gene expression they attach to suitable host cells by recognising and binding specific receptor proteins on host cells. The transfer of DNA into human mammalian cells using viruses is known as transduction. The viral vector genome usually integrates into the cell genome, thus providing a long lasting transgenic expression. However, integrating vectors raises safety risks, thus viral vectors are usually designed to be replication deficient and modified to have a reduced or defective immunogenicity. Gene therapy protocols can be performed in vivo as well as ex vivo to lower these risks (Giacca 2010; Jostock 2011).

Different viral vector systems have been used in gene therapy. Depending on the targeted gene, the type of cells used for gene expression, the viral vector chosen, various transfection methods can provide different advantages. Generally, high transfection efficiencies are required for high-producing transient expression systems. It has been observed that small vectors reach higher gene transfer rates, while large vectors tend to be less stable (Geisse 2009; Jostock 2011). Viruses with large genomes can be favourable in accepting large DNA inserts. In transient expression, it is advantageous to use minimal vector backbones with only the necessary elements. Nonessential components of the viral genome must be eliminated to allow correct insertion and packaging into the viral protein coats (Giacca 2010; Jostock 2011).
1.5.5. Expression cells

The tetrameric nature of an IgG molecule and the fact that glycosylation is essential for many antibody functions make it a challenge to efficiently express antibodies from microbial cells. Typically, host cells are well transfectable and capable of episomal replication if suitable expression vectors are used. Among those, derivatives of the HEK293 (human embryonic kidney) cell line are particularly popular (Giacca 2010). Two variants of episomal replicating derivatives, HEK293-T and HEK293-EBNA, are well established. Moreover, HKB-11 (HEK293 fusion with lymphoma cell line) and 293 Freestyle (293-F) cells are commonly used for transient expression of antibodies and other proteins (Geisse 2009). Chinese hamster ovary cells (CHO), which have typically lower yields, have been more widely used as host cells for stable antibody expression and manufacture in recent studies (Suen et al. 2010; Li, Menzel, et al. 2007; Geisse 2009). To a lesser extent, African green monkey kidney (COS) cells (Johnson et al. 1999; Jostock et al. 2001) and baby hamster kidney (BHK) cells (Bi et al. 2003) have also been utilised for transient antibody expression.

1.5.6. Methods of co-expression of antibody heavy and light chains

Different strategies can be followed to transfect the host cell with the antibody heavy- and light-chain sequences to express both chains of the selected antibody (Jostock 2011). Light and heavy chains can be encoded on separate plasmids in which co-transfection has the advantage of small vector sizes and the ability to vary the ratio of heavy- to light-chain encoding plasmids (Schlatter et al. 2005). Alternatively, both chains can be combined in a single vector and expressed from one plasmid with a monocistronic ‘tandem’ or ‘sandwich’ setup and an internal ribosomal entry site (IRES)-based bicistronic setup or with a monocistronic single open reading frame (ORF) setup (Fang et al. 2005; Jostock et al. 2010; Li, Menzel, et al. 2007).
A classical way to produce co-transfected cells with antibody sequences uses 2 plasmids, where one encodes the heavy chain and the other encodes the light chain of the selected antibody (Schlatter et al. 2005; Kaloff & Haas 1995; Montaño & Morrison 2002). Heavy and light sequences of this type can be cloned in one of two forms, either in genome-like intron-exon structure (Kalwy et al. 2006) or cDNA structure (Li, Menzel, et al. 2007). This strategy allows the use of small viral vectors. Moreover, systems using this strategy provide the option to vary the ratio of heavy-chain to light-chain encoding in the plasmids. However, lack of control over the ratio of heavy to light chains inserted into the host genome, as well as the risk of multiple insertion sites in the different plasmids, raise safety concerns (Figure 11) (Jostock 2011).

![Figure 11: Mono-cistronic: Light and heavy chain encoded on separate plasmids.](image)
P, promoter; LC, light chain; HC, heavy chain; pA, Polyadenylation.

A commonly used method where cassettes for heavy and light chains are combined on a single plasmid has been proven to be suitable for generating high-producing cell lines (Schlatter et al. 2005; Kalwy et al. 2006). Tandem vectors have light and heavy cassettes positioned sequentially in one plasmid with the same orientation. On the other hand, sandwich vectors contain both heavy and light cassettes, but they are in opposed orientation (Figure 12) (Jostock 2011).

![Figure 12: Single plasmid cassettes for heavy and light chains expression.](image)
(A) Tandem vector (B) Sandwich vector. P, promoter; LC, light chain; HC, heavy chain; pA, Polyadenylation.
In these types of monocistronic double gene vectors, the probability of generating equal copy numbers of both cassettes after integration is high, even with gene amplification. However, such vectors can be limited in size, which can make handling and production of the plasmid difficult; this may affect the efficiency of gene transfer during transfection. Combining the heavy and light chains in a single expression cassette can be done using a bicistronic setup, where an internal ribosomal entry site (IRES) is included (Figure 13) (Jostock et al. 2004; Li, Zhang, et al. 2007; Li, Menzel, et al. 2007).

![Figure 13: Bi-cistronic containing an internal ribosomal entry site (IRES). P, promoter; LC, light chain; HC, heavy chain; pA, Polyadenylation.](image)

IRES sequences are viral in origin and can drive cap-independent translation initiation of cistrons located downstream from the element (Borman et al. 1994). In bicistronic vectors, both chains are encoded on a single mRNA in the expressing cell, thus balanced expression of both polypeptides is found. Using different translation initiation efficiencies, studies show that mutant IRES elements create strong overall expression levels (Li, Zhang, et al. 2007).

A different technology has been described recently, consisting of co-expression of the heavy and light chains from a single open reading frame (ORF) (Fang et al. 2005; Jostock et al. 2010), where heavy and light chains are encoded as a single polypeptide with a self-processing 2A sequence motive of viral origin and a furin cleavage site in-between both antibody chains (Figure 14).

![Figure 14: Single Open Reading Frame (ORF). P, promoter; LC, light chain; HC, heavy chain; pA, Polyadenylation.](image)
Forced equimolar expression of the heavy- and the light-chain genes is one of the possible advantages of the single ORF approach.

1.5.7. PCR-based antibody gene cloning

The cloning of human immunoglobulin genes is typically conducted using PCR-based cloning. The isolation of the immunoglobulin heavy- and light-chain variable region genes that encode the binding domains of an antibody is the first step in any antibody-engineering project. This is conducted by PCR. However, reverse transcription of mRNA with constant region primers that anneal in the CH gene (Cγ 1, Cγ 2, Cγ 3, Cγ 4, Cμ, Cε, Cα and/or Cδ) and/or the CL gene (Cκ and/or Cλ) must be conducted first to obtain cDNA for PCR amplification. Primers are usually designed so that Fab, Fv or single-chain Fv (scFv) antibody fragments can be created. For the creation of Fabs, heavy chain VH-CH1 (fd) and light chain (VL-CL) are amplified.

Primer design is usually straightforward if the sequence of the constant domain exons is known. In case of Fv or scFv, only the rearranged VH and VL are amplified. Primers for the 3’ end of rearranged murine or human VH and VL genes and cDNA can be based on the (J) gene segments, whose sequencing is well known. This may facilitate the design of primers. Universal V-gene primers based on the extensive published V-regions are available and contain internal restriction sites that are suitable for amplification of murine, human, chicken and rabbit V-genes. In the transient expression of monoclonal antibodies, antibody heavy- and light-chain cDNAs are usually cloned separately into expression vectors such as pCEP4 or pTT5 (Marks & Bradbury 2004; Marks et al. 1991; Clackson et al. 1991).

1.5.8. HIV and human monoclonal antibody expression

HIV has been the target of huge research efforts, including a great amount of work attempting to develop genetically engineered vaccines. Joseph et al. conducted a proof-of-concept study to test the capacity of a single lentiviral vector to express the heavy and light chains of the 2G12 antibody (Joseph et al. 2010). In their study, cloning
of the 2G12 heavy and light chains into the lentiviral vector was done by combining the mRNA sequences and encoding the light- and heavy-chain genes that combine into the secreted 2G12 antibody into a single transcript linked by a “self-cleaving” 2A peptide (light chain-2A-heavy chain) using a PCR-based cloning strategy (Figure 15). The 2G12-lentiviral vector construct used in the study included deletion of the membrane region of the IgG heavy-chain gene to maximise secretion of the vector-encoded 2G12 antibody (Joseph et al. 2010; Giacca 2010).

Another approach to serum antibody generation-sustained HIV-1 neutralising activity through antibody gene transfer was demonstrated by Lewis and co-workers. In their study, rAAV was used as a vector to transfer the human monoclonal antibody IgG1b12 into mouse muscle cells. After a single vector administration, neutralising activity was maintained in the serum for the 6-month duration of the study. Lewis et al., constructed a novel rAAV expression vector to achieve efficient antibody expression within target muscle cells. A newly developed dual-promoter rAAV vector (pCMV/HC/EF1a/LC) has demonstrated optimal co-expression of heavy- and light-chain proteins within the same cell (Figure 16). The rAAV vector contains 2 constitutive promoters that are active in skeletal muscle in the context of a rAAV vector (hCMV promoter-enhancer and the human EF1-alpha promoter). The IgG1b12 heavy-chain introns were removed by RT-PCR to reduce vector size and remained within the packaging limit of wild-type AAV (Lewis et al., 2002).

Figure 15: PCR cloning of the vector expressing the 2G12 light chain-2A-2G12 heavy chain. The product was cloned into the BamH1/Xbal restriction site of a lentiviral transfer vector regulated by the human phosphoglycerate kinase (hPGK) promoter upstream of an internal ribosome entry site (IRES)-regulated enhanced green fluorescent protein (eGFP) reporter gene. Reproduced from (Joseph et al. 2010).
Figure 16: Dual-promoter rAAV antibody vector. Unique restriction sites are labelled at the top of the schematic, restriction enzyme sites were incorporated into the vector to allow for the rapid replacement of promoter elements or heavy and light chain coding sequences. Vector components are labelled as follows: HC and LC, the heavy- and light-chain antibody genes, respectively; CMVp, human immediate-early promoter-enhancer; and I, SV40 small T-antigen intron. Antibody leader sequences are labelled “L,” and “pA” denotes the bovine growth hormone polyadenylation site. The second transcriptional unit contains the human elongation factor 1α (EF1-a) promoter and has been modified to enhance stability of DNA and RNA by using the R segment and part of the U5 sequence (R-U5') of the HTLV-1 long terminal repeat. This promoter also contains the I117 intron, which is derived from plasmid pGT62LacZ (InVivoGen, Inc.). The light-chain polyadenylation site is from SV40. Reproduced from (Lewis et al. 2002).

1.5.9. Regulatory and Enhancement Elements

To drive antibody expression in mammalian cells, strong viral promoter combinations are used such as cytomegalovirus (CMV) immediate early (IE) gene region promoters. Alternatively, strong constitutively active cellular promoters, such as those of translation elongation factors (EFs), are applied (Li, Menzel, et al. 2007; Cacciatore et al. 2010). The promoters should have high transcription levels and 5′ un-translated regions (UTR) with high translation initiation activity. Promoters should also be susceptible to gene silencing of the transgene expression by epigenetic gene methylation (Mutskov & Felsenfeld 2004). It is believed that transgene expression and RNA stabilization may benefit from inclusion of an intron in the expressed RNA. A suitable polyadenylation signal is needed to complete the expression cassettes on the 3′-end (Jostock 2011).

The expression cassettes of the antibody chains could be flanked with chromatin modulating motifs such as S/MARs (scaffold/matrix attachment regions), UCOEs (ubiquitous chromatin opening elements) or STAR® (stabilising and anti-repressor) elements (Figure 17), which have been shown to increase the ratio of high-producing clones after random integration of the vector. These have also been shown to support long-term stability of transgene expression (Cacciatore et al. 2010; Benton et al. 2002; Kwaks et al. 2003). Using such vector elements can have the same upper limit of
productivities to vectors without chromatin opening motifs. However, chromatin opening motifs vectors need less clone screening to identify high producers. Such vectors can therefore be useful in cases where clone-screening efforts are limited. Moreover, high production stability could be obtained due to anti-repressor activities of such elements (Kwaks et al. 2003).

![Diagram of special vector setups for stable cell line generation](image)

**Figure 17:** Examples of special vector setups for stable cell line generation. (A) Chromatin modulating elements: Flanking the antibody expression cassettes with chromatin modulating elements such as S/MAR, UCOE, STAR®. (B) STAR® select: Selection marker and antibody coding regions are positioned in single expression cassettes with the selection marker upfront. (C) Tri-cistronic vectors: IRES elements are used to combine antibody chains and selection marker a reporter gene like GFP in a single expression cassette and to drive translation initiation of the downstream cistrons. Reproduced from (Jostock 2011).

1.6. Lentiviral vectors and gene therapy

1.6.1. Overview

Gene therapy was initially developed as a tool to deliver corrective genetic DNA into cells in the hope of compensating for specific genetic mutations that cause cellular dysfunction and the consequent development of systemic disease (Verma & Somia 1997). However, the principle of gene therapy can also be used to introduce protective factors into the cells of healthy individuals and generate intracellular immunisation (Baltimore 1988). This immunotherapy option aims to use genetically engineered viruses (recombinant viruses) as vectors for delivery to the relevant genetic targets and is concerned with eliciting cellular and humoral immune responses needed for
protection against a multitude of different pathogens. In genetic immunotherapy, recombinant viruses have been investigated in the past as a potential advanced option for prevention and treatment of chronic infectious diseases and cancer (Morrow et al. 2012; Sutter et al. 1994). Recombinant DNA methods are a promising technique in the development of a protective vaccine, as they enable the cloning of appropriate viral genes into other non-pathogenic vectors to produce immunogenic proteins under high standards of purification and quality control (Morrow et al. 2012; Sutter et al. 1994).

1.6.2. Lentiviruses as gene therapy vector

Lentiviral vectors (LV) are an efficient vehicle for gene transfer in mammalian cells due to their ability to stably deliver and express a gene of interest in non-dividing as well as dividing cells. Several other characteristics make LV favourable tools for gene therapy, including sustained gene delivery through vector integration, applicability to different target cell types, absence of expression of viral proteins after transduction, delivery of complex genetic elements, low genotoxicity and the relative ease of vector manipulation and production (Escors & Breckpot 2010). This leads to a promising list of applications such as transgene overexpression, persistent gene silencing, immunisation, generation of transgenic animals, as well as many applications targeting cancer cells (Goyvaerts et al. 2013). The use of LV has exponentially grown both in research and in clinical gene therapy protocols. LVs can be derived from primate as well as non-primate lentiviruses, such as HIV-1 and simian immunodeficiency virus (SIV). The generation of recombinant LVs has been accompanied by safety concerns such as the generation of replication-competent lentiviruses (RCLs) and induction of insertional mutagenesis (Manilla et al. 2005).

Basically, LVs are produced by transiently transfecting HEK 293 or 293T cells with plasmids encoding structural and functional sequences, imperative for proper LV particle generation. However, recent studies indicated that in order to develop recombinant LV with minimum risk, only critical viral structural and functional sequences are used. These sequences should be divided over a certain number of
individual plasmids in either *cis* or *trans* acting manner, with as minimal overlap as possible between viral sequences.

![Figure 18: Schematic representation of the four generations of lentiviral packaging constructs. A) First generation packaging vector. B) Second generation packaging vector. C) Third generation packaging vector. D) Fourth generation packaging vector. Reproduced from (Helio et al. 2013).](image)

A system of three expression cassettes was developed in 1996 by Naldini et al where LV production is accomplished using three different plasmids. First, to generate functional particles a packaging plasmid encoding all viral structural and enzymatic sequences in *trans* is developed. Then, a transfer plasmid providing the expression cassette of gene of interest in *cis* is cloned into the non-coding regions of the original lentiviral genome including a packaging signal and the two long terminal repeats (LTRs) of which the promoter activity has been deleted from the 3’ LTR. Finally, an envelope plasmid encoding an envelope glycoprotein (gp) consisting of a transmembrane domain (TM) and a receptor binding domain (SU) is developed in which the LVs’ tropism can be determined (Naldini et al. 1996). This system of three expression cassettes is considered to be the first generation of LVs. Four generations of LVs have now been described (Figure 18). In the first generation LV packaging plasmids the entire gag and pol genes were encoded together with all accessory
regulatory and virulence genes. This system provided good antibody production, but its level of safety was not very high as the chances of production of RCL were frequent.

To overcome safety concerns and to decrease the cytotoxicity of LVs, all accessory genes that are not essential for viral replication were removed without any negative effects on vector yield or infectivity, giving rise to the second generation vectors. In this generation, multiplication was attenuated by removal of four virulence genes, but not the regulatory ones (i.e. tat and rev) (Zufferey et al. 1997). However, this did not affect the number of homologous events to generate RCL. A third generation was developed in order to produce a more effective and safer version of the lentiviral gene delivery vehicle. In this approach the rev gene is expressed from a separate plasmid and the tat gene is removed by insertion of a strong constitutive promoter replacing the U3 region in the 5’ LTR of the transfer plasmid (Dull et al. 1998). The U3 region of the 3’ LTR of the transfer plasmid cancels the production of full-length vector RNA in transduced cells. Vector of the third generation presents both high titres as well as a high level of biosafety, thus they are now the most commonly used LVs. A further major improvement was achieved with the development of self-inactivating LVs (SIN). That minimised the production of RCLs and reduced the chance of viral LTR enhancer’s interference with the expression cassette, which lowers detectable expression of adjacent cellular coding regions. In contrast, in the fourth generation the homology between constructs is severely reduced, but the titres of antibody produced have also been affected (Romano et al. 2003).

1.6.3. Pseudotyping of lentiviruses

In addition to packaging and transfer plasmid optimisation, the envelope plasmid can be modified by replacing the natural lentivirus envelope gp with an alternative gp gene, most often the gp of vesicular stomatitis virus (VSV.G). This concept is called pseudotyping. Pseudotyping of lentiviruses is mainly used to expand the host range of retroviral vectors. The G glycoprotein of the VSV-G is almost always used to replace the Env proteins of the lentiviruses. VSV-G pseudotyped lentiviral vectors have
expanded cell tropism and spurred applications for gene therapy that can infect most types of cells. Studies have indicated that pseudotyped lentiviruses are particularly stable (Burns et al. 1993).

Joseph et al. generated a pseudotyped HIV-based third-generation lentivirus carrying the light-chain-2A-heavy-chain construct of the 2G12 antibody. In their study, co-transfection of 293T cells with 4 plasmids was made under calcium phosphate-mediation. A cytomegalovirus promoter was used to drive the packaging construct, expressing the gag and pol genes. A Rous sarcoma virus (RSV) promoter was used to drive construct expression of the rev gene. A cytomegalovirus promoter was also used to drive expression of the VSV-G envelope gene. Finally, a self-inactivating transfer construct was used, driven by the hPGK promoter containing HIV cis-acting sequences and an expression cassette for the 2G12 light-chain-2A-heavy-chain coding sequence inserted upstream of an IRES-regulated eGFP (2G12-lentivector) or an empty expression plasmid (Joseph et al. 2010).
2. Chapter 2

HIV neutralising antibody delivered by gene therapy with a stable retroviral vector encoded in vaccinia expression systems

2.1. Abstract

Production of an effective vaccine against human immunodeficiency virus (HIV) is elusive. This project used a novel replication-restricted, poxvirus split-vector system to insert HIV-neutralising monoclonal antibody sequences into a simian retroviral gene therapy agent pseudo-typed with vesicular stomatitis virus glycoprotein, all encoded within a poxvirus. Recombination between split-vector recombinants does not produce viable, fully replication-competent retroviruses. To circumvent this, a recombinant modified vaccinia Ankara (MVA) was constructed for functional retrovirus production. Production of these gene therapy retroviral particles delivered *in vivo* by a safe, stable poxvirus promises to produce long-lasting, high titres of neutralising anti-HIV monoclonal antibody. A hybrid dual-vector system using the large packaging capacity of MVA was used. The expression cassette of the gene therapy agent was split into two independent MVA vectors. The ultimate aim is to insert IgG1 b12 antibody genes into the human genome. The first rMVA (rMVA/IgG1b12/T7) carried the HIV-neutralising monoclonal antibody (mAb) sequence as well as a T7 polymerase for delivery into the cytoplasm of mammalian cells. A second recombinant MVA (rMVA/gag/pol/VSV-G) encoded a simian retroviral particle pseudo-typed with vesicular stomatitis virus glycoprotein to facilitate the delivery of the mAb genes into the human genome inside the nucleus. The second rMVA expressed heterologous polypeptides capable of assembling into defective non-self-propagating viral particles mimicking the packaging structure of SIV. The new non-infectious artificial lentivirus that results from
the hybrid dual-vector system infects neighbouring cells, penetrating the nucleus and inserting its genetic material into the mammalian genome to function as a vehicle for gene therapy. The insert comprised the expression cassette carrying a human CMV promoter, allowing constant expression of the neutralising monoclonal antibody over a prolonged period. This proof of principle will potentially allow subsequent development of long-acting vaccines for prevention of HIV infection and long-lasting immunotherapy of existing HIV infection.
2.2. Objectives and aims

- Generate a genomic rMVA/IgG1b12/T7 by homologous recombination between wt MVA and a plasmid-based transfer vector encoding monoclonal antibody IgG1b12, CMV promoter, T7 polymerase and a selective marker.

- Generate a packaging rMVA by homologous recombination between rMVA/gag/pol and a plasmid-based transfer vector encoding vesicular stomatitis virus glycoprotein (VSV-G) and Rev.

- Construct and validate recombinant MVA for functional gene therapy and pseudotyped retrovirus production.

- Produce gene therapy retroviral particles that will be delivered *in vitro* as a proof of principle to demonstrate production of a long-acting immunotherapy vectors for prevention of HIV infection and treatment of existing HIV infection.
2.3. Introduction

2.3.1. Recombinant vaccinia viruses and vaccination

2.3.1.1. Poxviruses in vaccination

Poxviruses, in particular Vaccinia viruses, are excellent candidates for the development of gene vectors. Vaccinia viruses are members of the Orthopoxvirus genus of the poxvirus family (Figure 19). They can be used in recombinant virus production to express and deliver foreign genes for use in novel vaccines. Several characteristics ensure the success of poxviruses in recombinant vaccination. The viruses have large double-stranded DNA genomes with packaging capacity to accommodate a large amount of foreign DNA (Sutter & Staib 2003; Amara et al. 2001).

Figure 19: Electron micrographs of negatively stained, naturally released virions. (A) Vaccinia virus. (B) Parapoxvirus. (bar = 100 nm). Reproduced from (Baxby 1996).

Poxviruses replicate in the cytoplasm of mammalian cells and do not integrate into the host genome while their gene expression is regulated by a strong poxvirus promoter. Poxviruses are also known to have high immunogenicity, and are well suited to large scale vaccine production (Blanchard et al. 1998; Mercer et al. 2007; Collier, L., Kellam, Paul, Oxford, J. S. 2011; Collier et al. 2011). There are safety concerns around the use of non-host range restricted vaccinia virus as human vaccines. These concerned are exemplified by the observation of complications when chorioallantoic vaccine Ankara (CVA) was administered as vaccine (Herrlich & Mayr 1957). It has been proven that poxviruses, in particular vaccinia viruses (VV), can replicate in human cells and cause
significant harm; thus, they have to be handled under biosafety level 2 laboratory conditions (Sutter & Staib 2003; Blanchard et al. 1998).

2.3.1.2. Smallpox vaccination

Smallpox has been successfully eradicated by a global effort in the 1960s and 1970s using a panel of live vaccinia virus vaccines. The last naturally occurring case was reported in 1977 in Somalia and in May 1980 the World Health Organisation (WHO) declared that smallpox had been eradicated (Breman & Arita 1980).

First generation smallpox vaccines where produced and successfully used during most of the intensified eradication program. Various vaccinia virus strains were demonstrated globally (e.g. Lister, NYCBH, Tiantan, EM63), manufactured on skin or lymph of animals (e.g. calf, sheep). However, towards the end of the eradication phase a second generation vaccine was developed using culturing techniques and the same smallpox vaccine strains employed for the manufacture of first generation vaccines or clonal virus variants plaque purified from traditional vaccine stocks. Using cell culture approach not only reduced the risk of contamination with adventitious agents but also improved the manufacturing processes primarily in providing consistency between lots.

At the end of the eradication phase, replication deficient third generation smallpox vaccines were developed which represent more attenuated vaccine strains, MVA based vaccines, specifically developed as safer vaccines by further passaging in cell culture or animals. Second and third generation vaccines are produced using modern cell culture techniques and current standards of Good Manufacturing Practices (GMP). Most adverse reactions to vaccinia virus/MVA administration involve the skin and central nervous system (CNS). Expected vaccine-related common local and systemic reactions with mild to moderate severity were detected. Local reactions included pain, intense erythema and inflammation at the vaccination site. Systemic reactions included fever, malaise, myalgia, headache, chills, nausea, fatigue, and lymphadenopathy. These reactions were usually self-limited and resolved within 2-3 weeks. Rare but
serious postvaccinial complications were documented during the eradication phase dependent on the vaccine strain used and age of the vaccinee. Reports described generalised vaccinia, eczema vaccinatum, progressive vaccinia (vaccinia necrosum), postvaccinial encephalitis (pvE) and death (Fulginiti et al. 2003; Breman & Henderson 2002).

Kretzschmar et al analysed and reviewed vaccination data on the frequency of pvE and death after primary and revaccination with smallpox vaccine with respect to age and vaccinia strain used, observations are summarised in Table 3 (Kretzschmar et al. 2006). Aragón et al reviewed data on the experience of adverse events after smallpox vaccination in the USA with the NYCBH strain that were published by Neff, Lane, Melin and Ratner (Aragón et al. 2003). The data were gathered between 1963 and 1968 and represent approximately 13 million primary vaccinations and 18 million re-vaccinations (Table 4). In regard to age and following primary vaccination, the risk of pvE (Risk Ratio 2.8) and generalised vaccinia (Risk Ratio 3.14) was highest among infants under 1 year of age, while risk of progressive vaccinia was highest in adults 20 years and older (Risk Ratio 7.27). However, almost all adult cases occurred in persons with a previously diagnosed hematopoietic malignancy or immunodeficiency condition (Aragón et al. 2003).

### Table 3: Estimated frequency of postvaccinial encephalitis (pvE) and death after primary vaccination with different vaccinia virus strains

<table>
<thead>
<tr>
<th>Vaccinia Virus Strain</th>
<th>Expected cases per million vaccinations by vaccinia strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bern</td>
<td>Copenhagen</td>
</tr>
<tr>
<td>PVE</td>
<td>44.9</td>
</tr>
<tr>
<td>Death</td>
<td>55</td>
</tr>
</tbody>
</table>

### Table 4: Estimated frequency of specific postvaccinial complications after primary and re-vaccination with NYCBH strain

<table>
<thead>
<tr>
<th>Risk of postvaccinial complication per million vaccinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVE</td>
</tr>
<tr>
<td>Primary vaccinee</td>
</tr>
<tr>
<td>Re-vaccinee</td>
</tr>
</tbody>
</table>
2.3.1.3. Modified Vaccinia virus Ankara (MVA) and vaccination

Highly attenuated poxviruses have been generated, including the MVA and NYVAC (both derived from Vaccinia virus strains), as well as TROVAC (derived from a Fowlpox strain) and ALVAC (derived from a Canarypox strain) (Verheust et al. 2012). To generate MVA, the CVA strain was attenuated through repeated passage in primary chicken embryo fibroblasts (CEF) (Meyer et al. 1991). The attenuated strain MVA was obtained after the 516th passage. Genomic studies revealed that after more than 570 passages, the CVA strain lost approximately 15% of its genome. Depending on the passage number, different MVA strains or isolates have been generated. The genome of the MVA is 178 kb in length, and a high number of deletions and disruptions of the viral genome have resulted from the extensive passage. Genomic studies show that six large genomic deletions have been identified in the MVA genome compared to the parental CVA (Figure 20). In addition, many shorter deletions, insertions, and point mutations occur resulting in gene fragmentation, truncation, or deletions of open reading frames (ORFs). These deletions and disruptions resulted in the loss of functional ability to encode for many poxviral immune evasion and virulence factors, including host range genes and some structural genes, making MVA defective in replication in human cells and a virulent in test animals (Morrow et al. 2012; Sutter & Staib 2003; Drexler et al. 2004).

Figure 20: Arrangement of HindIII genome DNA fragments of vaccinia virus CVA wild-type, CVA 382 and MVA. Fragments are labelled alphabetically according to size. Location and size of six major deletions (I to VI) are indicated by arrowheads. Reproduced from (Meyer et al. 1991).
MVA provides the advantages of genetic stability and high-level gene expression as well as deficient replication in human cells. Thus, depending on the nature of the inserted target gene, constructs can be handled under biosafety level 1 laboratory conditions. MVA has an excellent clinical safety record and induces significant humoral and cellular immune response. It has limited replication in some human cells and is clearly safe in immunosuppressed patients (Sutter & Staib 2003; Amara et al. 2001; Blanchard et al. 1998). Studies suggest that the high immunogenicity of MVA vectored vaccines may result from apoptosis of human DCs upon MVA infection (Verheust et al. 2012).

MVA is widely considered as the vaccinia virus strain of choice in preclinical and clinical research, in particular in the field of recombinant vectors for both vaccinations against various pathogens or as a delivery vector for gene therapy. A study showed that MVA-572 and MVA-I721 strains demonstrate ability to replicate in some human cell lines (human keratinocyte cell line HaCat, human embryo kidney cell line 293, human bone osteosarcoma cell line 143B, and human cervix adenocarcinoma cell line HeLa) and in immunodeficient mice (Suter et al. 2009). However, many MVA-based vector studies have not indicated any major side effects in preclinical animals or in human clinical trials undertaken so far. However, the use of MVA and recombinant MVA vectors must include an assessment of potential risks for human health and the environment (Verheust et al. 2012).

MVA has the adaptability to express a high level of genes as well as to produce heterologous proteins. Further, although MVA is characterised by severe growth deficiency in human cells, it shows good efficacy of recombinant gene expression (Morrow et al. 2012; Verheust et al. 2012). In 2003, Moorthy et al. reported the first safety data profile of recombinant MVA immunisation in humans, testing the efficacy of MVA vaccine against *Plasmodium falciparum* malaria. Since then, other studies testing MVA vaccines for prophylaxis or immunotherapy against AIDS and other chronic infections and cancers have been completed and published (Verheust et al. 2012;
Moorthy et al. 2003). As the development of an effective vaccine against HIV is an urgent need, investigations into the development of an adequate recombinant MVA vector vaccine against HIV increased. Thus far, many studies have been dedicated to developing recombinant MVA, producing immunodeficiency virus antigens to elicit humoral and/or cellular immune responses. Many MVA-based vaccine studies have successfully reported that both antibody and high-level cellular responses can be elicited when different SIV or SHIV antigens expressed in model hosts are expressed by rMVA vaccine candidates. However, further investigation is still needed into the efficacy of elicitation of the potent immunity and induction of broadly virus-neutralising antibody responses required to achieve a successful HIV vaccine (Verheust et al. 2012; Schnierle et al. 2007).

### 2.3.1.4. Vaccinia Retroviral Hybrid Vector

Retroviral vectors have become important tools in gene therapy as a result of a number of highly desirable properties, mainly their stable genomic integration. However, retroviral vectors cannot be grown to high titers and they tend to be unstable and are sensitive to inactivation by complement when transfused into patients. The search for more robust retroviral delivery systems has led to the development of hybrid viral vectors with advantageous properties of another viral vector system.

A hybrid vector can be generated by integrating the retroviral unit into the genome of a VV. Hence, instead of being delivered by transfection of a proviral plasmid DNA, the retroviral vector is backpacked onto a carrier virus (MVA) which actively infects cells with high efficiency and produces the genomic RNA in the cytoplasm. Both replicating and replication-defective VVs are appropriate as carriers. The approach is depicted in Figure 48 on page 136.

MVA transcribes its genes, replicates its DNA, and assembles its virions in the cytoplasm. It provides its own specific transcription apparatus, whereas retrovirus genomes are transcribed from nuclear proviruses by cellular factors. To serve as an
efficient MVA early transcription unit, the proviral retrovirus vector has to be substantially modified, and is then inserted into the vaccinia DNA genome because of the cytosolic life cycle of MVA. Cellular promoters and other eukaryotic transcriptional signals like polyA sites and splicing signals, are not recognised. Gene expression is controlled solely by virus-specific elements.

Early transcripts are capped mRNAs of discrete length terminating about 50 bp downstream of a specific viral termination signal (TTTTTNT). Thus, early genes usually carry no termination signal within the coding region, but in the 3’ untranslated region (UTR) of a gene. In order to convert the proviral DNA, existing in a proviral plasmid, into a functioning vaccinia early transcription unit, internal TTTTTNT signals have to be removed. The vector is cloned downstream of a strong vaccinia early promoter precisely fused to the retroviral R region, thus replacing the retroviral 5’-U3. A new transcriptional stop is introduced downstream of the 3’-R region to obtain properly processed RNA genomes from the vaccinia system (Konetschny et al. 2003).

### 2.3.1.5. Generation of recombinant MVA

Currently, the most frequently used method of generating recombinant poxvirus is allowing homologous DNA recombination in infected cells between a plasmid-based transfer vector and the virus. Homologous recombination occurs relatively frequently during poxviral replication. Recombination into specific sites of the MVA genome is typically directed by a plasmid-based transfer vector encoding homologous sequences flanking the transgene (Figure 21 and Figure 22).

An expression cassette including the gene of interest and a vaccinia-specific promoter is inserted forming a recombinant virus. Multiple cloning sites are encoded, facilitating the insertion of the foreign gene of choice (Verheust et al. 2012; Schnierle et al. 2007). A selection marker system is usually inserted to aid the clonal isolation of the
recombinant virus by plaque purification. This is usually carried out by the additional insertion of selection marker gene expression cassettes.

In brief, homologous recombination occurs between genomic and transfer plasmid DNA during MVA replication. Thus, when genomic DNA is packed to yield MVA progeny, a proportion of the population is recombinant at a frequency of approximately 1:1000. Homologous sequences flanking the expression cassette direct the recombination to a desired locus in a non-essential region of the MVA (Morrow et al. 2012; Schnierle et al. 2007).

Figure 21: Generation of recombinant MVA virus for expression of foreign gene. The insertion of the gene of interest into the MVA genome carried on by the presence of the homologous sequences (HS). MCS: Multiple cloning site.
Figure 22: Conventional recombinant MVA production through spontaneous homologous recombination. (a) rMVA formed when an initial single recombination action occurs in either the left or the right homology sequence (HS-L or HS-R) between the wild-type MVA and the transfer plasmid vector, where the entire transfer plasmid encodes the gene of interest. (b) In (A), a secondary crossover event occurs owing to spontaneous intra genomic recombination between the right homology sequences in rMVA which results in the formation of a marker-gene-free rMVA, a much more stable form of rMVA that lacks the marker gene as well as the plasmid genome. In (B), a secondary crossover event occurs due to spontaneous intra genomic recombination between the left homology sequences in rMVA results in back reversion of the rMVA to wild-type MVA where the whole insert is lost.
2.3.1.5.1. **Insertion sites**

Several regions have been targeted for exogenous DNA insertion. Initially, classical gene loci encoding for the vaccinia virus proteins thymidine kinase (TK) and the haemagglutinin gene (HA) were used. However, sites of naturally occurring deletions formed during the course of MVA attenuation (Del) are now more commonly used. Moreover, intergenic regions (IGR) have also been used (Morrow et al. 2012; Schnierle et al. 2007).

Deletion (Del) sites represent non-essential regions in the MVA genome whose deletion minimally impacts MVA viability in chicken embryo fibroblasts (CEFs). These regions formed during the process of 570 serial passages on CEFs that transformed the pathogenic vaccinia virus Ankara strain into MVA (Meyer et al. 1991). Deletion sites have been routinely used for insertion of recombinant genes to form MVA derived recombinant vaccines (Drexler et al. 2004). However, the stability of certain transgenes in Del sites is variable depending on the toxicity of the expressed product during cellular and viral replication (Wyatt et al. 2008). In some cases, mutations or deletions that occur in transgenes after several passages of rMVA in culture may result in reduced or complete loss of expression (Wyatt et al. 2009).

2.3.1.5.2. **Marker selection**

There are several well-established methods for clonal isolation of rMVA involving the co-expression of a marker gene. Early techniques relied on insertion of the foreign gene into the TK locus; then bromodeoxyuridine (BrdU) enrichment selection based on the TK phenotype was carried out. However, this technique showed some non-specific isolation. TK mutation from the cytotoxic influence of the BrdU could be detected, and, as a result, false-positive isolates could be selected. Later, this method was combined with the simultaneous insertion of the *E. coli* LacZ gene (Morrow et al. 2012).

The *E.coli* enzyme β-galactosidase coded in the LacZ gene is one of the most frequently used marker genes. The LacZ gene has the ability to turn plaques of rMVA
from non-stained into blue-stained plaques when located with the synthetic chromogenic substrate X-gal. The infected cell line containing the active LacZ gene will code for β-galactosidase, which cleaves the β-glycosidic bond in D-lactose found in X-gal. When X-gal is cleaved by β-galactosidase, galactose and 5-bromo-4-chloro-3-hydroxyindole is formed, producing a characteristic blue-coloured plaque (Kremer et al. 2012; Serebriiskii & Golemis 2000). To improve versatility in recombinant isolation, other genes can be added to the transfer gene vector. Studies have shown that antibiotic resistance markers such as the E.coli neomycin gene and the xanthine-guanine phosphoribosyl transferase (gpt) gene, and additional markers can be inserted such as green fluorescent protein (GFP) or detection of the product of the E.coli β-glucuronidase A gene (gus). Frequently used for recombinant virus purification is the E.coli gpt gene encoding the enzyme xanthine guanine phosphoribosyl transferase. This method allows dominant positive selection for resistance against mycophenolic acid (MPA). As long as marker genes are not cross-reactive, it is possible to combine more than one marker gene into one recombinant, to improve the selection. This can occur under the control of a single vaccinia promoter, as in the case of gpt-gus fusion (Morrow et al. 2012; Schnierle et al. 2007).

### 2.3.1.5.3. Promoters

Since the discovery of the first vaccinia virus promoter, the 7.5 kb early/late promoter, in 1982, significant improvements have been made in designing and synthesising vaccine promoters. Studies showed that using rational optimisation of promoters results in enhanced immune responses. Using an optimised promoter in rMVA vaccine development leads to higher and more efficient expression of desired antigens. Resulting in a significant enhancement in immune responses as well as potential lower doses required for vaccination. Vaccinia promoters are active in one of the three transcriptional stages – early, intermediate or late – and some, such as the 7.5 kb and H5 promoters, contain both early and late elements in tandem (Morrow et al. 2012).
In designing rMVA-based vaccines it is important to achieve high levels of antigen expression while avoiding conflicting effects. To help reach this goal, assessment of optimal promoter activity must be undertaken in vitro. It is important to ensure that the transgene is not located close to the vaccinia virus early transcription termination sites TTTTTNT. Impairment of gene expression is prevented by excision of non-essential DNA between the promoter and transcription start site (Morrow et al. 2012). Ideally all these modifications should be employed simultaneously.

2.3.1.5.4. **T7 RNA polymerase hybrid system**

In this study all genes of interest were under the control of a T7 promoter. Hence, the transient cytoplasmic expression system used in this study relies on synthesising the bacteriophage T7 RNA polymerase in the cytoplasm of the mammalian cells. Mammalian cells are co-infected with two recombinant vaccinia viruses, one contains the genes of interest while the other encodes bacteriophage T7 RNA polymerase (driven by a poxviral promoter, p7.5).

During incubation, the genes of interest are efficiently transcribed by T7 RNA polymerase and translated in the cytoplasm of the infected cells. The decision to utilise this system was based on several factors. Such a system will provide a control mechanism for the expression of transgenes (no expression without co-infection), thus enhancing the safety profile of the proposed gene therapy candidate. As transgenes are not expressed during the manufacturing stage, more stable recombinant viruses are achieved. T7-RNA polymerase known to be extremely promoter specific and only transcribes genes downstream of a T7-promoter with a very low error rate, this serves as a desirable characteristic for a viral vector vaccine. The DNA-dependent T7 RNA polymerase is a very active enzyme that less frequently terminates transcription and is found to be highly selective for initiation of its own promoter sequences (Tabor 2001; Elroy-Stein & Moss 2001).
2.3.1.5.5. **Genes of interest**

The final result in this project will be the production of a pseudotype virus like particle (VLP) encoding IgG1b12 sequence in its core. The VLP is of SIVmac239 origin. Expression cassettes used contained two common genes (gag, pol) and five additional accessory genes (tat, rev, vpx, vpr and vif).

Genes encoding the structural proteins of VLPs were organised in three major regions of the genome: - gag (group specific antigen), internal structural proteins; pol (polymerase) enzymes and envelope genes were pseudotyped with VSV-G envelope proteins. The gag gene codes for the Gag polyprotein (matrix, capsid, p2, nucleocapsid, p1 and p6), which is then processed by the viral protease to generate proteins associated with the viral mRNA and some may form a part of the viral capsid. The pol gene (polymerase), translated into a Gag-Pol polypeptide via a ribosomal frame-shift, codes for the viral enzymes reverse transcriptase RT (p55/p51), integrase IN (p32), RNase H, and protease PR (p11).

The accessory proteins are essential for viral replication and virion infectivity. Tat (transactivator of transcription) is a powerful activator of viral gene expression. The protein binds a structured region present at the 5’ end of the transcribed viral RNAs and recruits different cellular factors to the viral LTR promoter. Rev (regulator of expression of virion proteins) binds a highly structured RNA sequence (Rev-responsive element, RRE), present in correspondence to the env gene and is thus contained as a potential intron in the fully spliced mRNAs. The role of Rev is to mediate transport of these RRE-containing mRNAs outside the nucleus.

Vpr (viral protein R) blocks progression of the cell cycle in the infected cells by accumulating them in the G2-M phase, is incorporated into virions, and contributes to nuclear transport of the reverse transcribed viral cDNA in the infected cells. Vif (virion infectivity factor) is essential for proper virion infectivity since it blocks the function of a cellular enzyme, the RNA deaminase APOBEC, which would otherwise inactivate the
viral genome by introducing mutations. Vpx (virion-associated protein) targets a host restriction factor for proteasomal degradation and is involved in the import of the viral pre-integration complex into the host nucleus (Collier et al. 2011; Carter & Saunders 2013).

Pseudotyping of lentiviruses is mainly used to expand the host range of retroviral vectors. The G glycoprotein of the VSV-G is almost always used to replace the Env proteins of the lentiviruses. VSV-G pseudotyped lentiviral vectors have expanded tropism that spur applications for gene therapy and can infect most types of cells. Studies have indicated that pseudotyped lentiviruses are particularly stable (Burns et al. 1993).

The expression cassette of the gene therapy agent is split into two independent MVA vectors. It is speculated that the final result should be the insertion of IgG1 b12 antibody genes into the mammalian genome. The first genomic rMVA (rMVA/IgG1b12/T7) will carry the HIV-neutralising monoclonal antibody (mAb) sequences as well as the T7 polymerase and will deliver them into the cytoplasm of mammalian cells. A second packaging rMVA (rMVA/gag/pol/VSV-G: encodes a simian retroviral particle pseudo-typed with vesicular stomatitis virus glycoprotein) will be used to facilitate the delivery of the mAb genes into the human genome inside the nucleus. The second rMVA is a viral vector that expresses heterologous polypeptides capable of assembling into defective non-self propagating viral particles mimicking the packaging structure of an SIV. The new non-infectious artificial lentivirus that results from the hybrid dual-vector system will infect neighbouring cells, penetrate the nucleus and will insert its genetic materials into the mammalian genome, functioning as a vehicle in this gene therapy project.


2.4. **Materials and Methods**

Genetically Modified Organisms approval for this study was obtained for the laboratory and the project. The lab permissions and the GMO permission were granted by the University of Manchester safety committee, the GMO Establishment license# 541 (p5603-4).

**2.4.1. Molecular Cloning**

All the plasmids were designed using Lasergene Genomics Suite (DNASTAR, Madison, USA) software, as well as NCBI GenBank database. The transfer plasmid was designed (refer to section 2.5.1) and sent for manufacture to Blue Heron Biotechnology, Bothell, WA, USA.

For multiplication of plasmid DNA the following *E.coli* strain was used:

SCS1 Supercompetent Cells were obtained from Stratagene/Agilent Technologies UK Ltd., (Wokingham, UK). SCS1 are *E.coli* cells hation 5.1.1.ve a recA1 endA1 gyrA96 thi-1 hsdR17 (RK-, MK+) supE44 relA1 genotype and are characterised by endA (endonuclease) and recA (recombination) deficiency which improves insert stability and the quality of miniprep DNA.

**2.4.1.1. Plasmid designing**

**2.4.1.1.1. pLF-IgG1b12-mTK**

The transfer plasmid was prepared from a pBR322 backbone. The cloning vector pBR322 complete sequence was obtained from GenBank: J01749.1. MVA sequences from the classical insertion site, the thymidine kinase gene locus (TK), were selected after slight modification to the flank the gene of interest to form the functional cassette. The gene of interest (IgG1b12) is to be inserted into the TK locus of the MVA strain by *in vitro* recombination in primary chicken embryo fibroblasts (pCEFs). Modified TK sequence was obtained from vaccinia virus strain MVATGN33.1 complete genome, GenBank: EF675191.1. The CMV promoter and its enhancer were obtained from
Promega Corporation. The *E.coli* xanthine guanine phosphoribosyl transferase (*gpt*) gene and the *E.coli* β-galactosidase lacZ gene sequences were encoded in this plasmid as a dual marker system. A LacZ sequence was added to the TK gene flanking regions by traditional cloning. β-galactosidase sequence was obtained from GenBank:AAA24053.1, while *gpt* sequences were obtained from *E.coli* KLY, complete genome, GenBank: CP008801.1. To drive *gpt* expression, synthetic E/L promoter derived from Psc6 was used after reverse complementation of its sequence. The promoter sequence from Chakrabarti et al, 1997 was used and direct repeats of repetitive MVA-DNA fragments homologous to the right end of flank1 were added, allowing for the deletion of the marker expression cassette (*gpt* and lacZ marker system) by homologous recombination. The last 50 bp from the right end of flank1 encoding the right TK locus fragment was copied and added before the Reverse complement pE/L promoter (Figure 28).

The heavy and light sequences of IgG1b12 were retrieved from the crystal structure of the intact human IgG1b12 (Figure 23). A slight modification was done via codon de-optimisation and/or nucleotide mutation to breakup any C repetitive sequences, to disrupt potential kozak consensus and/or to introduce a silent stopping codon. Also, few amino acids were replaced to fit consensus for IgG1 heavy chain (Figure 24).

*Figure 23: Crystal structure of the intact human IgG1b12.* (Adapted from: http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=1HZH accessed 18/10/17).
Figure 24: Heavy and light chain sequences after codon modification.

2.4.1.1.2. pLF-VSV-G

The plasmid was designed using Lasergene Genomics Suite (DNASTAR, Madison, USA) software. The sequence of VSV-G was obtained from Vesicular stomatitis Indiana virus, complete genome; Sequence ID: J02428.1 (NCBI GenBank database). The plasmid was constructed by Blue Heron. VSV-G sequence was flanked by BglIII
and PacI. This plasmid was used to replace the Env sequence in already existing plasmid pCoEnvRev.D4 that was provided by Dr Tom Blanchard.

### 2.4.1.2. DNA extraction and purification from bacterial cells

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN #27104). Following the manufacturer instructions, an overnight *E.coli* culture was pelleted by centrifugation. Bacterial cells were then lysed by alkaline lysis followed by adsorption of DNA onto silica membranes in the presence of high salt concentrations. Impurities were washed with the supplied washing buffer and pure plasmid DNA eluted in a small volume of elution buffer. The concentration of the plasmid DNA was determined using the ND1000 NanoDrop Spectrophotometer (Labtech, Uckfield, UK).

### 2.4.1.3. Preparation of vector and insert: Restrictions digest

In a total volume of 50μl: 1μg of DNA, 5μl of 10xNEBuffer and 1μl (10unit) enzyme were mix. Both incubation times and temperature are enzyme dependent.

### 2.4.1.4. DNA End Modification: Dephosphorylation

1 μL (1 unit/μL) of Recombinant Shrimp Alkaline Phosphatase (rSAP) (NEB #M0371) was added to each 1 pmol of DNA (about 1 μg of a 3 kb plasmid). After incubation at 37°C for 30 minutes, the reaction was stopped by heat inactivation at 65°C for 5 minutes.

### 2.4.1.5. Agarose gel electrophoresis and gel extraction

#### 2.4.1.5.1. Agarose gel electrophoresis

PCR products and DNA digests were analysed by gel electrophoresis on the basis of their molecular weight. Fragment sizes were determined in relation to the bands of a mixture of standard molecules (marker ladder) on the gel. The DNA fragments in agarose gels were visualised with UV light using SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, USA) or Gel Red (Biotium Inc., Hayward, USA) following the
manufacturer’s guidelines. 6x Gel Loading Dye (New England Biolabs, Herts, UK) was mixed with each DNA sample. Either 1kb DNA ladder or 100 bp DNA ladder molecular weight standards (Figure 25) (New England Biolabs, Herts, UK) were used for DNA band size approximation.

The percentage of agarose (Table 5) in preparing the gel was determined according to the expected band sizes. UltraPure agarose (ThermoFisher Scientific #16500500) was used in preparing the agarose gels.

![Image of DNA Ladders](image)

**Figure 25: DNA Ladders.** Ladders visualised by ethidium bromide staining on a 0.8% TAE agarose gel. Mass values are for 0.5 µg/gel lane.

**Table 5: Recommended agarose gels for electrophoretic separation of DNA fragments**

<table>
<thead>
<tr>
<th>Agarose gel, %</th>
<th>Range of effective separation, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>800-10000</td>
</tr>
<tr>
<td>0.9</td>
<td>600-10000</td>
</tr>
<tr>
<td>1.0</td>
<td>400-8000</td>
</tr>
<tr>
<td>1.2</td>
<td>300-7000</td>
</tr>
<tr>
<td>1.5</td>
<td>200-3000</td>
</tr>
<tr>
<td>2.0</td>
<td>100-2000</td>
</tr>
</tbody>
</table>
2.4.1.5.2. Purification of vector and insert by DNA extraction from agarose gels

Following gel electrophoresis of DNA from both vector and insert, bands of interests were excised and purified using the Monarch® DNA Gel Extraction Kit (New England Biolabs, Herts, UK). Following the manufacturer's protocol, DNA fragments were excised using a scalpel (Disposable scalpels number 21, Ref # 0507. Swann-Morton, Sheffield, UK) visualised under UV light transillumination (Syngene, Cambridge, UK). Dissolving buffer was added to the gel slice; DNA was then adsorbed on a silica membrane, and purified DNA eluted in the provided Elution Buffer.

2.4.1.6. Ligation of vector and insert

Ligation of DNA fragments was performed using Instant Sticky-end Ligase Master Mix (New England BioLabs, Herts, UK) containing a T4 DNA ligase. The ligation mix was prepared by combining 20–100 ng of vector with a 3 to 5 fold molar excess of insert, the volume was adjusted to 5μl with nuclease free water. 5μl of Instant Sticky-end Ligase Master Mix was added to the ligation mix, the mix is ready to be used for transformation. In case of blunt ends or single-base overhangs, an incubation time of 15-30 minutes at room temperature is required to ensure high efficiency ligation. The amount of vector and insert in the ligation mix was calculated using NEBBioCalculator (http://nebiocaculator.neb.com/#/main accessed 09/05/2018).

2.4.1.7. Transformation

SCS1 Supercompetent Cells (Stratagene/Agilent Technologies UK Ltd., Wokingham, UK) were transformed with plasmid DNA according to the manufacturer’s instructions. Briefly, 1-2.5 μl of plasmid DNA or ligation mix (50-100ng DNA) were suspended in 50μl competent cells and incubated on ice for 30 minutes. After a heat shock at 42°C for 45 seconds the cells were incubated on ice for 2 minutes. The competent cells were mixed with 450μl SOC medium (Super optimal broth medium; Hanahan, 1983) and incubated at 37°C for one hour at 300rpm. Following the one-hour recovery step, the
cells were plated (100μl and 400μl) on selective LB-agar plates with the respective antibiotic (ampicillin [100mg/ml] or kanamycin [25mg/ml]).

2.4.1.8. Sequencing

Sanger nucleotide sequencing was used for confirmation of each cloning experiment. Sequencing was performed using plasmid and gene specific primers, spanning the region of plasmid and gene insertion (Table 6). Big Dye Terminator v1.1 cycle sequencing reagents (Applied Biosystems) were used for the sequencing reactions, cleaned up using ethanol precipitation and placed on the ABI 3130 genetic analyser for sequence reading. Post sequencing analysis was performed using ReCALL (beta v3.03) software (http://pssm.cfenet.ubc.ca/account/login). Consensus sequences were aligned in SnapGene software (http://www.snapgene.com) to ensure gene insertion in the correct reading frame. The sequencing primers were designed manually following common primer designing guidelines and subsequently ordered from Eurofins/MWG Operon (Ebersberg, Germany).

Table 6: Primers used for sequencing

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Target</th>
<th>Tm</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' AATGGGCTGGATAGCGGTTTG 3'</td>
<td>Light Chain</td>
<td>62.1</td>
<td>Forward</td>
</tr>
<tr>
<td>5' CAGGCTGGAGACAGGGAGTAGGT 3'</td>
<td>Light Chain</td>
<td>67.8</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' CCGCCTCCGCTGTTGTTG 3'</td>
<td>IRES and eGFP</td>
<td>67.5</td>
<td>Forward</td>
</tr>
<tr>
<td>5' CTTCGCGCATTGCGGACTTTGA 3'</td>
<td>IRES and eGFP</td>
<td>63.7</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' GACCAAGCCACGCGAGGAGCAGTA 3'</td>
<td>Heavy Chain</td>
<td>67.8</td>
<td>Forward</td>
</tr>
<tr>
<td>5' TAGACGGCAGCCAGGATTCAGAG 3'</td>
<td>Heavy Chain</td>
<td>67.8</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' ACTCTTGGCCATTTTTGACTTTGAA 3'</td>
<td>VSV-G</td>
<td>57.6</td>
<td>Forward</td>
</tr>
<tr>
<td>5' CGGGGCCGGTGGGTTAG 3'</td>
<td>VSV-G</td>
<td>64.8</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

2.4.2. Generation and amplification of rMVA

2.4.2.1. Processing of primary Chicken Embryo Fibroblasts

Primary Chicken Embryo Fibroblasts (pCEFs) were prepared from specific pathogen free embryonated eggs obtained from the Institute of Animal Health, Compton-UK. pCEF cells were maintained in liquid nitrogen in serum free Dulbecco modified Eagle
medium (DMEM). Each frozen vial containing 1mL of pCEFs (3.5 × 10⁸ cells) was resuscitated using cell growth medium prepared from DMEM (Lot no. RNBC6720) containing 10% heat-inactivated foetal calf serum (FCS) and 10 mM L-glutamine into a tissue culture flask (either T175 or T75). The tissue culture flasks upon seeding the cells (one frozen vial/flask) were incubated at 37°C in a tissue culture incubator supplied with 5% CO₂ for 2–3 days or until 100% confluency was achieved. For a 175 cm² flask, cells would usually be ready for cell passage on the third day at a ratio of 1:3, whereas for a 75 cm² flask, cells would usually be ready for splitting on the second day at the same ratio. Adherent cells were removed from the culture flask surface by treatment with TrypZean® Solution, 1x (SIGMA-ALDRICH #T3449). Cells were incubated with TrypZean for 5 minutes. Trypsinization reaction was stopped by adding FCS.

2.4.2.2. Virus stock

The modified vaccinia Ankara (MVA) used in this project was a gift from Professor Anton Mayr (Ludwigs Maximilian University, Munich, Germany). He provided sealed vials of wild type MVA (wtMVA) used in the smallpox eradication campaign. The wtMVA had been passaged twice on pCEFs since then, but not plaque-purified. The virus then amplified and the titre was determined to be 8.5 x 10⁸ pfu/µL by plaque assay (refer to section 2.4.2.3).

2.4.2.2.1. Virus titration

2% Low melting point agarose

- 5 g Low melting point agarose (Sigma-Aldrich; cat #A9045)
- 250 mL water (Sigma Aldrich; cat #W3500-500ML
- The suspension was autoclaved for 15 minutes at 121°C (Astell, Kent, UK; Model AMB420BT)

The stock concentration of virus samples was determined using plaque assay in pCEFs. Tenfold dilutions of the virus-stock sample in DMEM medium were adsorbed on pCEFs in duplicates using 6-well tissue culture plates. After 3 hours incubation at
37°C, the cells were overlaid with 2ml/well of pre-warmed (42°C) 2% low melting point agarose (Sigma-Aldrich; cat #A9045) with 2X MEM (Life Technologies Ltd.; cat #11935-046) medium and 10% FCS in a 1:1 ratio. The plates were then incubated at 37°C with 5% CO₂ and 95% humidity until plaques were visible. To determine the virus titre only wells with isolated plaques were counted and where the number of plaques exceeded 20.

2.4.2.2. Generation of recombinant MVA

In order to generate recombinant MVA, the transfer plasmid of choice that was obtained by molecular cloning was transfected into pCEFs, which were then infected with either wtMVA or rMVA. Transfer vector plasmids were transfected into MVA-infected cells, and homologous recombination between MVA and plasmid DNA generated a recombinant virus.

2.4.2.3. Transfection of primary chicken embryo fibroblasts

pCEFs were transfected with endotoxin free plasmid DNA using SuperFect Transfection Reagent (QIAGEN; cat #301305) following the manufacturer’s guidelines. On the day of transfection the density of pCEFs was 8x10⁵ cells/well (6-well dish). For one well 5-8μg of plasmid DNA were mixed with DMEM to make up a final volume of 100μL. Then 10μL of SuperFect transfection reagent was added to the mix and vortexed for 10 seconds. To allow for transfection-complex formation the solution was incubated for 10 minutes at room temperature while growth medium was aspirated from pCEFs. Then, 600μL of growth medium (DMEM + FCS + L-glutamine) was added to the transfection mix and pipetted onto the pCEF cell layer. The plates were incubated at 37°C with 5% CO₂ and 95% humidity for 3 hours with a rocking step every 15 minutes.
2.4.2.4. **Infection of pCEFs with wildtype MVA or recombinant MVA**

Pre-titrated wtMVA or rMVA stock was thawed and diluted to a multiplicity of infection (MOI) of 0.5 in 1mL DMEM. Following a three-hour infection step, the transfection mix was aspirated from the pCEFs cell layer and replaced with 1mL infection mix per well. The plates were incubated (37°C with 5% CO₂ and 95% humidity) for 3 hours and rocked every 15 minutes. Subsequently the infection mix was aspirated and the cell layer was covered with 2 mL DMEM+ 10% FCS + 2 mM L-glutamine. The plates were placed in an incubator for 48 hours. The pCEFs and virus were then harvested using cell scraper (Falcon, Corning GmbH; Cat # 353086). The cell lysate underwent three freeze-thaw cycles and was stored at -80°C until further usage.

2.4.2.3. **Plaque assay and rMVA isolation**

Primary overlay

- 2% Low melting point agarose (melted in a microwave)
- 2X Minimum essential medium eagle (MEM, Life Technologies Ltd.; cat #11935-046) 10% Fetal Calf Serum (FCS, Biosera, Gentaur Ltd.; cat #FB-13770/500).

Secondary overlay

- 2% Low melting point agarose (melted in a microwave)
- 2X Minimum essential medium eagle (MEM, Life Technologies Ltd.; cat #11935-046 )
- Supplement with 1/1000 volume of 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Fisher Scientific: cat # BP1615-1)

Virus lysate was homogenised for 10 seconds in a sonicator (Misonix, sonicator 3000 cabinet) at 150 W. 80% confluent pCEFs monolayer (in 6-well dishes) was infected with variable volumes ranging from 50μL – 200μL of lysate, dilutions were prepared in DMEM, and incubated for 3 hours at 37°C with 5% CO₂ and 95% humidity. The inoculum was aspirated and the cells were covered with 2 mL/well pre-warmed (42°C)
primary overlay. Plates were left at room temperature until solidification. The assay plates were then incubated at 37°C until the plaques are clearly visible. A second agarose overlay containing X-gal (2 mL/well) was added and the plates were re-incubated at 37°C for 8-24 hours until blue plaques appeared. The plaques were picked with a Pasteur pipette and diluted in 1.5 mL DMEM, and subjected to three freeze-thaw cycles and before storage at -80°C until further usage. After each round of plaque purification the picked plaques were screened for the insert using PCR (refer to section 2.4.2.3.4). White plaques were also screened for the loss of the LacZ marker gene.

2.4.2.3.1. **Antibiotic resistance system gpt selection**

Confluent cells were incubated in the gpt selection medium (DMEM, 10% FCS, 25µg of MPA per mL, 250µg of xanthine per mL, 15µg of hypoxanthine per mL) for 14 to 42 hours prior infection. Cells were infected with the virus for 3 hours, incubated at 37°C with rocking every 15 minutes. Following infection, cells were overlaid with the gpt-selective medium containing 1% of low-melting-point agarose. After plaque formation cells were overlaid with a second agarose overlay containing X-gal (2 mL/well) and were incubated for 8-24 hours until blue plaques appeared.

2.4.2.3.2. **Screening for transient β-galactosidase expression and plaque purification**

Recombinant MVA expressing the recombinant antigen and transiently co-expression β-galactosidase were subjected to consecutive rounds of plaque purification in pCEF cell monolayers stained with X-gal, selecting blue foci. The first 4 rounds of plaque purification were made by selecting isolated blue plaques. To remove the reporter gene from rMVA, a further round of plaque purification was carried out, screening for nonstained viral foci in the presence of X-gal.
2.4.2.3.3. Isolation of virus DNA from cells

To isolate virus DNA from pCEFs, PureLink Genomic DNA Mini-Kit was used (Life Technologies Ltd; Paisley, UK cat #K1820-01). 70% confluent pCEFs in 6-well dish were infected with 200μL of re-suspended blue or non-stained plaque and incubated for 3-4 days at 37°C with 5% CO₂ and 95% humidity. Cells in each well were harvested by washing the cell layer by forced pipetting. The suspension was centrifuged at 10000rpm for 10 minutes to collect the cell-virus pellet. Cells were lysed with lysis buffer at 55°C for 10 minutes. Residual RNA and proteins were digested with Rnase A and Proteinase K respectively (20 μL each). The released DNA was then applied to silica-based membranes (spin tube, supplied with the kit) in the presence of chaotropic salts. After two washing steps the DNA was eluted with a low-salt elution buffer and used for PCR analysis.

2.4.2.3.4. Characterisation of recombinant MVA genomes by PCR

To monitor the retention of the inserted gene, plaques were analysed by PCR using oligonucleotide primer sets (Table 9) designed to amplify DNA fragments at the specific insertion site used within the MVA genome. After each round of plaque purification, harvested rMVA was screened for the presence of the target genes and/or for the absence of marker gene. For amplification Taq 5x Master Mix (New England Biolabs; Cat #M0285L) was used. The conditions for a routine PCR was as following:

Table 7: PCR reaction setup

<table>
<thead>
<tr>
<th>Component</th>
<th>25 μL Reaction</th>
<th>50 μL Reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM Forward Primer</td>
<td>0.5 μL</td>
<td>1 μL</td>
<td>0.2 μM (0.05–1 μM)</td>
</tr>
<tr>
<td>10 μM Reverse Primer</td>
<td>0.5 μL</td>
<td>1 μL</td>
<td>0.2 μM (0.05–1 μM)</td>
</tr>
<tr>
<td>Template DNA</td>
<td>Variable</td>
<td>Variable</td>
<td>&lt;1,000 ng</td>
</tr>
<tr>
<td>Taq 5X Master Mix</td>
<td>5 μL</td>
<td>10 μL</td>
<td>1X</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>to 25 μL</td>
<td>to 50 μL</td>
<td></td>
</tr>
</tbody>
</table>
Table 8: Thermocycling conditions for a routine PCR

<table>
<thead>
<tr>
<th>STEP</th>
<th>TEMP</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>30 Cycles</td>
<td>95°C</td>
<td>15-30 seconds</td>
</tr>
<tr>
<td></td>
<td>45-68°C</td>
<td>15-60 seconds</td>
</tr>
<tr>
<td></td>
<td>68°C</td>
<td>1 minute/kb</td>
</tr>
<tr>
<td>Final Extension</td>
<td>68°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4-10°C</td>
<td></td>
</tr>
</tbody>
</table>

All PCR reactions were conducted in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Thermo Fisher Scientific, UK). For all PCR reactions nuclease-free water (Ambion, Thermo Fisher Scientific; cat # AM9930) was used.

Table 9: Primers used for PCR screening

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Target</th>
<th>Tm</th>
<th>Direction</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ TGAGACCCAACGGCAAGAAGAAGTA 3’</td>
<td>MVA-Gag</td>
<td>62.5</td>
<td>Forward</td>
<td>577 bp</td>
</tr>
<tr>
<td>5’ TCGGCCGCCTCCTCGTGATGATGT 3’</td>
<td>MVA-Gag</td>
<td>62.5</td>
<td>Reverse</td>
<td>577 bp</td>
</tr>
<tr>
<td>5’ TGTCCGGTCAGGGCATCAGGAAGG 3’</td>
<td>MVA-Rint</td>
<td>63.8</td>
<td>Forward</td>
<td>568 bp</td>
</tr>
<tr>
<td>5’ GCCGAGGCGAGTAGGGAAGAACA 3’</td>
<td>MVA-Rint</td>
<td>62.6</td>
<td>Reverse</td>
<td>568 bp</td>
</tr>
<tr>
<td>5’ GTTTTGCTGGCGCTGGTGGTGG 3’</td>
<td>MVA-Tat</td>
<td>62.6</td>
<td>Forward</td>
<td>568 bp</td>
</tr>
<tr>
<td>5’ CTAAGACGGGCGCAGTAGTGAGGAG 3’</td>
<td>MVA-Tat</td>
<td>62.6</td>
<td>Reverse</td>
<td>568 bp</td>
</tr>
<tr>
<td>5’ ATGAGGGCTGGATAGGCGAGTCGTTGG 3’</td>
<td>MVA-LC</td>
<td>62.3</td>
<td>Forward</td>
<td>823 bp</td>
</tr>
<tr>
<td>5’ CAGGGGTAGGACAGGAGTAGTGT 3’</td>
<td>MVA-LC</td>
<td>62.3</td>
<td>Reverse</td>
<td>823 bp</td>
</tr>
<tr>
<td>5’ CCGCCTCCGCCTGCTGGTG 3’</td>
<td>MVA-eGFP</td>
<td>63.1</td>
<td>Forward</td>
<td>473 bp</td>
</tr>
<tr>
<td>5’ CTTCGGGACTGGGCACTTGGA 3’</td>
<td>MVA-eGFP</td>
<td>63.1</td>
<td>Reverse</td>
<td>473 bp</td>
</tr>
<tr>
<td>5’ GACCAAGCCACGCGAGGAGCACTAGA 3’</td>
<td>MVA-HC</td>
<td>64.3</td>
<td>Forward</td>
<td>606 bp</td>
</tr>
<tr>
<td>5’ TAGACGGCGGACAGCTAGCTAGGAG 3’</td>
<td>MVA-HC</td>
<td>64.3</td>
<td>Reverse</td>
<td>606 bp</td>
</tr>
<tr>
<td>5’ CGTTTACAGGGCGCGCTGCTCTGT 3’</td>
<td>MVA-LacZ</td>
<td>60.7</td>
<td>Forward</td>
<td>473 bp</td>
</tr>
<tr>
<td>5’ AGGGCGCTGTGATGGCGCTTAGCTGGA 3’</td>
<td>MVA-LacZ</td>
<td>60.7</td>
<td>Reverse</td>
<td>473 bp</td>
</tr>
<tr>
<td>5’ CCATCGGCAAGGAGGCTACTACT 3’</td>
<td>T7Pol</td>
<td>62.4</td>
<td>Forward</td>
<td>404 bp</td>
</tr>
<tr>
<td>5’ TGGCCCGCTGGAGATCGCTTGTAAT 3’</td>
<td>T7Pol</td>
<td>62.4</td>
<td>Reverse</td>
<td>404 bp</td>
</tr>
<tr>
<td>5’ AGCCCTCTAATCCGGGCTCAGTTCTGCTCT 3’</td>
<td>MVA-Vif</td>
<td>62</td>
<td>Forward</td>
<td>452 bp</td>
</tr>
<tr>
<td>5’ GCCTGGGCTAGGAGGGAAGGAGTA 3’</td>
<td>MVA-Vif</td>
<td>62</td>
<td>Reverse</td>
<td>452 bp</td>
</tr>
<tr>
<td>5’ ACTCGTGAGTCATTTTGACTTGTA 3’</td>
<td>MVA-VSV-G</td>
<td>55.6</td>
<td>Forward</td>
<td>443 bp</td>
</tr>
<tr>
<td>5’ CGGGGCGGTGGGCTAGG 3’</td>
<td>MVA-VSV-G</td>
<td>55.6</td>
<td>Reverse</td>
<td>443 bp</td>
</tr>
<tr>
<td>5’ CGGGGCAAACATCGAAGTGGCAG 3’</td>
<td>MVA-LacZ</td>
<td>62.2</td>
<td>Forward</td>
<td>534 bp</td>
</tr>
<tr>
<td>5’ CAGCGTTGACCAGGCGGTTAGG 3’</td>
<td>MVA-LacZ</td>
<td>62.2</td>
<td>Reverse</td>
<td>534 bp</td>
</tr>
</tbody>
</table>
2.4.3. Transfection using Lipfectamine 2000

In a 24-well plate, 70-90% confluent cells were transfected with rMVA encoding eGFP. Four different amounts of Lipofectamine reagent (2, 3, 4 and 5µL) each was diluted in Opti-MEM medium 50 µL. 5 µg of plasmid DNA was diluted in opti-MEM medium to give a final volume of 250µL. Diluted DNA was added to diluted Lipofectamine 2000 reagent at 1:1 ratio (50µL:50µL). DNA-lipid complex was incubated for 5 minutes at room temperature. The complex was then added to the cells and incubated for 6 hours at 37°C. The mixture was then replaced with fresh 10% FCS/DMEM media. Cells were incubated up to 5 days at 37. Then transfected cells were analysed for fluorescence.
2.5. Results

2.5.1. Design and construction of MVA transfer plasmid vectors

In all vector design pBR322 was the plasmid of choice. It is a relatively small plasmid encoding the ampR gene (source plasmid RSF2124) that produces the ampicillin resistance protein and the tetR gene, which forms the tetracycline resistance protein.

In case of traditional molecular cloning, if a vector is linearised by a single restriction enzyme or has been cut using two enzymes with compatible ends, a dephosphorylation step must be carried out to ensure that the vector does not recircularise during ligation.

2.5.1.1. pLF-IgG1b12-mTK

Several promoters were used in this design. A T7 promoter was placed upstream in the R region (U5 region + 5'LTR) and the tRNA primer-binding site. The T7 promoter sequence was obtained from bacteriophage T7 complete genome NCBI Reference Sequence: NC_001604.1. Human CMV IE gene promoter was added to drive expression of the monoclonal antibody sequence and the eGFP. The CMV promoter is one of the strongest mammalian promoters known. It has been frequently used in several studies for a high level mammalian expression and it is found to be optimal in driving LC-HC expression. The lacZ sequence wasn’t included in our plasmid design, as it is already has been designed in the lab.

In this project, combining the heavy and light chains in a single expression cassette was done using a tri-cistronic setup, where two different IRES elements were included (Figure 26). IRES elements are used to combine antibody heavy and light chains together with the marker a reporter gene GFP in a single expression cassette. Additionally, IRES allows the driving translation initiation of the downstream cistrons (Jostock 2011).
Tri-cistronic vector utilised two different IRES elements (EMCV, HTLV-1) to combine antibody light and heavy chains together with a reporter gene (eGFP) in a single expression cassette and to drive translation initiation of the downstream cistrons.

The internal ribosome entry site (IRES) form of the encephalomyocarditis virus (EMCV) is a noncoding RNA fragment noted for its ability to initiate high levels of cap-independent protein synthesis in mammalian cells and cell-free extracts. When compared head-to-head, an EMCV IRES generally directs higher translation levels than any other IRES or capped mRNA. The preferred IRES sequence was obtained from (Figure 27) (NCBI Reference, GenBank: M81861.1). To minimise any sequence duplication from using the same IRES, different IRES was used to separate between the heavy chain sequences and the eGFP. HTLV-1 IRES is commonly used. The complete sequence was retrieved from GenBank: KF797887.1. Linking the product gene to a fluorescent reporter by IRES will ease the screening process. Thus, cells with high green fluorescent level will hopefully be co-expressing high levels of the product gene. Green fluorescent protein (GFP) is a protein that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. Sequence obtained from GenBank: AAB02572.1.

The optimal Kozak sequence 5’-GCCACCATG-3’ was arranged as the cap-dependent translation initiation, in the polycistronic vectors EMCV IRES and HTLV-1 IRES were used to mediate internal protein translation initiation. In this project, the Kozak consensus sequence (ccAcCATG) was added. Sequence derived from Marilyn Kozak’s original paper (Kozak 1987). Polyadenylation [poly(A)] signals are a defining feature of eukaryotic protein-coding genes. The central sequence motif AAUAAA was identified in
the mid-1970s and subsequently shown to require flanking, auxiliary elements for both 3′-end cleavage and polyadenylation of premessenger RNA (pre-mRNA) as well as to promote downstream transcriptional termination (Beauvoir & Gautheret 2001). Several unique restriction enzymes were used in the design.

![Encephalomyocarditis virus (EMCV) IRES](image)

**Figure 27: Encephalomyocarditis virus (EMCV) IRES.** Reproduced from (Bochkov & Palmenberg 2006).

Long terminal repeat (LTR) is the control centre for gene expression in lentiviruses. LTRs are identical sequences that are repeated in the virus genome when integrated as a provirus. The most important function of LTRs is to insert the virus genomic material into the host. In our design, the gene of interest is flanked by SIVmac 239 long terminal repeats (LTRs) subdivided in U3, R and U5 regions; which are responsible for regulation and expression of the viral genome, including polyadenylation, replication and integration of the provirus into the host DNA. The R regions are essential cis-acting elements that have to be maintained by retroviral vectors. The 5′ and 3′ R regions are located at the start and the end of the genomic retroviral RNA, respectively, and their homology is imported for strand transfer during reverse transcription. U3 is the SIV promoter. The R region marks the starting point of transcription, and U5 region is
critical for reverse transcription. The other key elements are the packaging signal (Ψ) and the polypurine tract (PPT). The packaging signal, as in many other virus species, allows RNA genome encapsidation during virion assembly in the cytoplasm. The PPT element is a key element for reverse transcription. The R region is an important element in many steps of the lentiviral life cycle, such as transactivation of transcription, polyadenylation and first-strand transfer during reverse transcription. SIV mac 239 LTR sequences were retrieved from GenBank: M62835.1. The synthesised plasmid is shown in diagrammatic form in figure below.

**Figure 28:** Schematic representation of the transfer plasmid pLF-IgG1b12-mTK. The thymidine kinase (tk) locus used as an intergenic insertion site for the IgG1b12 expression cassette.

Restriction digestion was used either to screen for positive clones or to prepare DNA samples for cloning. Both vector and insert from a plasmid source were digested with appropriate restriction enzymes. Restriction digests were carried out as recommended by New England BioLabs. Double digests were only performed when both enzymes showed 75-100% activity in the same buffer. Depending on an enzyme's activity rating in a non-optimal NEBuffer, numbers of units and/or incubation time were adjusted to compensate lower rate of cleavage. If there was no buffer in which both restriction enzymes exhibited >50% activity, a sequential digest was performed. First digestion
with the enzyme required lowest salt concentration. Then, a sufficient amount of NaCl is added to bring the salt concentration to that of higher salt concentration buffer.

pLF-IgG1b12-mTK plasmid DNA was received as a bacterial stab from Blue Heron, single colonies were obtained after culturing the stab. The DNA from these colonies was isolated and prepared for digestion with restriction enzymes. As shown in Figure 29, DNA digests were analysed by gel electrophoresis on the basis of their molecular weight. Fragment sizes were determined in relation to the bands of a mixture of standard molecules on the gel. Expected band sizes are indicated in the Figure. In the single digest using restriction enzyme Ascl two fragments of sizes 6328bp and 5650bp resulted, while fragments of sizes 8486bp and 3492bp were created when XhoI was used. Double digestion using NotI and SpeI resulted in the formation of fragments of sizes 7636bp and 4342bp.

![Figure 29: 1% agarose gels of DNA restriction digests product of pLF-IgG1b12-mTK. Ascl (fragments 6336 bp and 5642 bp) and XhoI (fragments 8517 bp and 3461 bp) were used for single digest, while double digest was performed using NotI and SpeI (fragments 7636 bp and 4340 bp). M is a 1kb DNA ladder.]

### 2.5.1.2. pLF-IgG1b12-LacZ-mTK

This plasmid was constructed by cloning LacZ from a LacZ containing plasmid into pLF-IgG1b12-mTK. Fragment (size 3135bp) encoding LacZ was removed from the LacZ plasmid and inserted into pLF-IgG1b12-mTK between the FseI and Pmel restriction enzyme sites resulting in the recombinant transfer vector pLF-IgG1b12-
LacZ-mTK. A summary of the steps in cloning is shown in Figure 30. Plasmid DNA was extracted from randomly selected colonies and was subjected to restriction digestion with XbaI and SacI to confirm the cloning, results are illustrated in Figure 31.

**Figure 30**: Schematic representation of the construction of transfer plasmid pLF-IgG1b12-LacZ-mTK. A fragment (size 3135bp) encoding LacZ expression cassette was removed from a donor LacZ plasmid and inserted into pLF-IgG1b12-mTK between the FseI and Pmel restriction enzyme sites resulting in the recombinant transfer vector pLF-IgG1b12-LacZ-mTK.

**Figure 31**: 1% agarose gel of DNA restriction digests product of pLF-IgG1b12-LacZ-mTK. M is a 1kb DNA ladder. Restriction enzymes used are Xbal and SacI. Fragments sizes 9635bp, 3172bp and 2300bp.
2.5.1.3. pLF-VSV-G

The plasmid was constructed by Blue Heron using the complete genomic sequence of Vesicular stomatitis virus (VSV) strain Indiana (complete genome; Sequence ID: J02428.1). The plasmid was provided in kanamycin resistant pUC vector (Figure 32). Results of plasmid DNA restriction digestion are illustrated in Figure 33 confirming the cloning.

![Schematic representation of the transfer plasmid pLF-VGV-g.](image)

Figure 32: Schematic representation of the transfer plasmid pLF-VGV-g.

![DNA restriction digest product of pLF-VGV-g.](image)

**Figure 33: 1% agarose gels of DNA restriction digests product of pLF-VGV-g.** Restriction enzymes used in (A) are Stul and SacI. Fragments sizes 922bp and 3391bp. Restriction enzymes used in (B) are Stul and AflIII. Fragments sizes 3016bp and 1297bp.
2.5.1.4. pLF-VSV-G-Rev-D4

Transfer vector pLF-VSV-G-Rev-D4 was constructed by replacing a fragment between the PacI and BglII sites in an already existing pCoEnvRev.D4 plasmid with a fragment (size 1629 bp) containing VSV-G was excised from pVSV-G. A summary of the cloning is illustrated in Figure 34. To confirm the cloning, plasmid DNA was extracted from randomly selected colonies and was subjected to restriction digestion, results are illustrated in Figure 35.

Figure 34: Schematic representation of the construction of transfer plasmid pLF-VSV-G-Rev-D4. A fragment (size 1629bp) encoding the sequence of VSV-G was removed from a donor pLF-VGV-g plasmid and inserted into pCoEnvRev.D4 between the PacI and BglII restriction enzyme sites resulting in the recombinant transfer vector pLF-VSV-G-Rev-D4.

Figure 35: 1% agarose gel of DNA restriction digests product of pLF-VSV-G-Rev-D4. M is a 1kb DNA ladder. Restriction enzymes used are SacI and AgeI. Fragments resulted with sizes 9351 bp and 2384 bp.
2.5.1.5. pLF-IgL1b12-LacZ-D4

The modification to the TK locus region during the design of the transfer plasmid was lethal to the rMVA expressing IgG. Thus, shifting the insertion site of the IgG expression cassette from TK locus to a different insertion site was fundamental. A fragment size of 5650bp encoding IgG expression from the TK targeting plasmid pLF-IgL1b12-LacZ-mTK replaced the expression cassette of pLF-VSV-G-Rev-D4 at the Ascl site, resulting in the formation of pLF-IgL1b12-LacZ-D4 which target Del4. A summary of the cloning is illustrated in Figure 36. Plasmid DNA was extracted from randomly selected colonies to confirm the cloning by restriction digest; results are illustrated in Figure 37.

**Figure 36:** Schematic representation of the construction of transfer plasmid pLF-IgL1b12-LacZ-D4. A fragment (size 5650bp) encoding IgG expression from pLF-IgL1b12-LacZ-mTK replaced the expression cassette of pLF-VSV-G-Rev-D4 at the Ascl site, resulting in the formation of pLF-IgL1b12-LacZ-D4.
2.5.2. Generation of recombinant MVA by homologous recombination

rMVA encoding human codon optimised sequences of SIVmac239 responsible of forming the packaging component of the VLP (reverse transcriptase, integrase, protease, vpr, vif, gag and tat) was provided by Dr Blanchard (University of Manchester). The recombinant packaging virus was generated by homologous recombination between transfer plasmids containing the codon optimised DNA sequences and wtMVA virus in pCEFs. These desired sequences were targeted for insertion into Del-I and Del-II of MVA. The insertion site was chosen randomly to accommodate different sequence fragments of SIV at different locations on the MVA genome (Figure 38).
rMVA encoding human codon optimised sequence of T7 RNA polymerase responsible for the expression of T7 RNA polymerase was provided by Dr T Blanchard (University of Manchester). Virus was generated by homologous recombination between transfer plasmids targeting Del-II contain human codon optimised T7 RNA polymerase and wtMVA virus in pCEF (Figure 39). The recombinant virus will control and ensure that an efficient transcription and translation of genes driven by T7 promoter.

**Figure 38:** Schematic representation of the recombinant packaging MVA. Within the rMVA genome, Del-I and Del-II used as insertion sites of expression cassettes encoding human codon optimised sequences of SIVmac239 responsible of forming the packaging component of the VLP (reverse transcriptase, integrase, protease, vpr, vif, gag and tat).

**Figure 39:** Schematic representation of the recombinant T7 MVA. Within the rMVA genome, Del-II used as insertion sites of expression cassettes encoding T7 RNA polymerase and Vpx.
To expedite clinical use, the generation and amplification of rMVA is performed in compliance with certain regulatory requirements. Most importantly, it is considered a safety advantage to exclusively processing of primary Chicken Embryo Fibroblasts (pCEFs).

The uptake of the foreign DNA material into MVA is monitored using unstained/blue stained plaque screening. Plaques are discrete foci of infection originated from a single infectious virion. A plaque denotes a spot of cell lysis or cytopathic effect (CPE), in other words a hole, within the cell monolayer (Figure 40). Plaque formation is only observed in pCEFs. rMVA plaques were picked from well-separated viral foci from wells infected with highest dilutions, which drastically reduced the number of plaque passages needed to isolate clonally pure rMVA.

![Figure 40: Microscopic observation of established CPE where individual virus plaques differentiated.](image)

(a) X20 and (b) X40 shows a blue-stained plaques, while (c) X20 and (d) X40 are non-stained plaques. A plaque denotes a spot of cell lysis or cytopathic effect (CPE), in other words a hole, within the cell monolayer.
Homologous recombination between the transfer plasmid vector and the virus was visualised in infected pCEF monolayers by the formation of blue plaques in the presence of X-gal (Figure 41). The primary overlay medium prevents the development of secondary plaques by forcing the released virus particles from the infected cell to infect only neighbouring cells. rMVA incorporating and expressing gpt was first subjected for an initial round of selection in the presence of mycophenolic acid (MPA). This selection system was carried out only for the first round of plaque purification. It is based on plaque isolation in the presence of MPA, an inhibitor of purine metabolism. Recombinant virus incorporating and expressing the E.coli xanthine-guanine phosphoribosyl transferase gene (gpt), unlike normal gpt(-) virus, can form plaques in medium containing MPA and the nucleotide precursors xanthine and hypoxanthine.

![Figure 41: Blue plaque formation on 6-well dish.](image)

(A) A well visualises infected pCEF monolayers with rMVA through the formation of blue plaques in the presence of X-gal. (B) A well shows uninfected pCEF monolayers.

Four consecutive rounds of blue plaque selection in the presence of X-gal was conducted, then the plates were screened for non-stained plaques for the loss of LacZ. A higher dilution of virus inoculum was related to better plaque separation and an intense blue colour formation. Starting from the fifth round of plaque selection, non-stained viral foci in the presence of X-gal were selected. The formation of non-stained plaques resulted from a second intragenomic recombination event where the reporter gene from rMVA is removed. In regard to a complex vaccine for human use, the construction of a marker-free variant is very desirable. Plaques were picked only after
microscopic observation of established CPE where individual virus plaques clearly differentiated. These white plaques were further purified until pure rMVA was obtained. rMVA lysate was defined as pure if ≥ 12 plaques picked from one well of a 6-well plate gave a positive PCR signal for the insert while negative for the LacZ.

The expression cassette of the gene therapy agent was split into two independent recombinant MVA vectors to achieve insertion of IgG1 b12 antibody genes into mammalian genome. The packaging rMVA (rMVA/gag/pol/VSV-G) vector encoded all genes required to form a pseudo-typed simian retroviral particle. A recombinant virus rMVA/gag/pol encoding Gag, Tat, Pol had already been prepared in the lab by Dr. Tom Balnchard. To this, VSV-G was added to form the final pseudo-typed particle (Figure 42). The titre of this virus was determined to be $3.7 \times 10^{12}$ pfu/µL.

![Figure 42: Schematic representation of the first rMVA (rMVA/gag/pol/VSV-G).](image)

To produce the genomic rMVA (rMVA/IgG1b12/T7), IgG1 b12 sequences were inserted into the TK-locus after altering the site of insertion (Figure 43). The modification that was made to the TK gene resulted into further attenuation of an already poorly replicating virus and possibly inactivation of the TK-gene, this clearly altered the growth properties of the rMVA. Throughout the plaque screening plaques were smaller than expected and after 11-plaque purification rounds the recombinant plaques were not
detectable. Suggesting that the TK deletion incorporated in the plasmid design terminated the viral replication.

Figure 43: Schematic representation of the second rMVA (rMVA/IgG1b12/T7). Del-II used as insertion sites of expression cassettes encoding T7 RNA polymerase and Vpx, while TK locus used to insert the IgG1b12 expression cassette.

Cloning the IgG1b12 sequences into a different insertion site was successfully performed using Del site IV (refer to section 2.5.1.5). This time rMVA encoding T7 polymerase was used to produce the recombinant virus (Figure 44). However, no CPE was observed when the virus was cultured. Several attempts were carried to form a successful recombination. However, each time there was no CPE detected which might indicate the failure of the formation of recombinant virus.

Figure 44: Schematic representation of the second rMVA (rMVA/IgG1b12/T7). Del-II used as insertion sites of expression cassettes encoding T7 RNA polymerase and Vpx, while Del-IV used to insert the IgG1b12 expression cassette.
2.5.3. **Characterization of recombinant MVA genome by PCR**

To confirm the retention of the expression cassettes in the different deletion sites of MVA, virus plaques were expanded and then screened with a specific primer set (Table 9). In later rounds (round 5-10) of plaque purification the desirable loss of the LacZ-marker was confirmed with a LacZ-specific primer set. Positive non-stained plaques underwent several rounds of plaque purification, until a pure rMVA carrying the inserted sequence could be confirmed.

The packaging recombinant virus rMVA/gag/pol/VSV-G was stable during the first couple rounds of plaque purification, where all genes of interest are detectable by PCR. Unfortunately, after plaque purification in round 3 the recombinant started to show instability. Pol expression cassette, inserted into site Del II, was completely lost after round 4. Also, VSV expression cassette couldn't be detected on the 7th round of plaque purification. However, Gag expression cassette was detected in all blue and white plaques. Results are illustrated in Figure 45.

The genomic recombinant virus rMVA/IgG1b12/T7 formed smaller CPE (plaques) than normally seen with any rMVA. Through the plaques purification, CPE was getting smaller with the increase number of the purification round. After the 10th round of plaque purification, plaque formation was not detectable and the virus disappeared from the cells. Suggesting that the TK alteration incorporated in the plasmid design is actually an impediment to viral replication. Also, LacZ gene was detectable though all the plaque purification rounds despite selection of white plaques (Figure 46). In the designed transfer plasmid, the marker genes were destabilised by a 50bp repetitive homologous sequence used to drive spontaneous intra genomic recombination. However, it appeared that such small sequence (50bp) is not enough to generate a marker-gene-free rMVA, as the LacZ gene was detectable.
Figure 45: Agarose gel electrophoresis after PCR screening for the insertion of packaging components into the packaging virus. (M) 100 bp DNA Ladder; (C1) negative control: nuclease free water; (C2) negative control: pCEF; (C3) negative control: pCEF+wtMVA; (C4) positive control: transfer plasmid. (P1-P3) blue plaques purification rounds 1 to 3, (P4-P10) white plaques purification rounds 4 to 10. (A and B) Agarose gel electrophoresis after PCR screening for insertion of Gag and Tat expression cassettes into deletion site II of MVA. Gag PCR product: 577bp and Tat PCR product: 568bp. (C) Agarose gel electrophoresis after PCR screening for insertion of Pol and Vif expression cassettes into deletion site I of MVA. Pol PCR product: 568bp. (D) Agarose gel electrophoresis after PCR screening for insertion of VSV-g and Rev expression cassettes into deletion site 4 of MVA. VSV-g PCR product: 443bp. (E) Agarose gel electrophoresis after PCR screening for the loss of lacZ MVA. Lac Z PCR product: 534bp.
2.5.4. Transfection

Vero cells were transfected with pLF-IgG1b12-mTK using Lipofectamine® 2000 reagent which delivers the plasmid DNA with excellent transfection performance. This step was made to test protein expression, as the IgG sequence together with eGFP incorporated in our plasmid is driven by CMV promoter. Thus, it is speculated that if the plasmid was delivered inside the cells proteins will be expressed and green fluorescent
will be detected. Figure 47 indicated that vero cells expressed weak fluorescence after the 5th day of incubation using inverted fluorescence microscopy. The cells were monitored daily for fluorescence expression.

![Fluorescent vero cells infected with pLF-IgG1b12-mTK](image)

**Figure 47:** Fluorescent vero cells infected with pLF-IgG1b12-mTK. Cells were visualised using UV light (40X).

Lipofectamine® 2000 Transfection Reagent is a versatile transfection reagent that has been shown to effectively transfect wide variety of adherent and suspension cell lines. Lipofectamine® 2000 Reagent delivers DNA or siRNA with excellent transfection performance for protein expression, gene silencing, and functional assays.
2.6. Discussion and Conclusion

This project used a replication-restricted poxvirus split-vector system developed utilising a Vaccinia Retroviral Hybrid Vector, where MVA is used to deliver SIV like particles into mammalian cells. The first hybrid dual-vector system was developed to double the packaging capacity of recombinant adeno-associated virus (rAAV) by splitting the expression cassette between two independent AAV vectors (Beaudoing & Gautheret 2001; McClements & MacLaren 2017). Studies indicated that this split vector system was safe in vivo and stable during vaccine production and probably enabled higher titres of vaccine.

In this study, MVA was employed as the viral vector to overcome the limited packaging capacity of AAV and other vectors. The research hypothesis is illustrated in Figure 48. The recombination between the split-vector recombinants with MVA origin will result in the formation and the production of simian like particles. The resulted VLPs are attenuated and thus not fully replication-competent retroviruses. The fact that SIV replicates and infects several species of African non-human primates, gives the system an excellent safety profile.

VLPs used encode HIV-neutralising monoclonal antibody sequences into its core to function as a simian retroviral gene therapy agent. Theses virus particles are pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G). Pseudotyping with VSV-G was performed to expand the retroviral tropism. The replication-restricted poxvirus split-vector system developed is composed of recombinant MVA, rMVA/gag/pol/VSV-G and rMVA/IgG1b12/T7 responsible for the production of the packaging component and the neutralising antibody respectively.
Figure 48: Schematic representation of the hybrid dual-vector system (poxviral/retroviral system) and generating HIV-neutralising monoclonal antibody. A retroviral vector transcription is integrated into the double-stranded DNA genome of the rMVA. (A) In a first step, dual infection of a packaging cell leads to cytoplasmic transcription of the retroviral expression cassette. The transcripts serve as genomic RNA and are packaged by the nascent protein components constitutively produced by the packaging cells. (B) In a second step, the resulting retroviral vector particles infect target cells. Transduction results in the expression of the gene of interest (mAbs) from the integrated provirus under control of the nuclear transcription signals.
Packaging rMVA (rMVA/gag/pol/VSV-G) is a viral vector that expresses heterologous polypeptides capable of assembling into defective non-self-propagating VLPs mimicking the packaging structure of an SIV. A plasmid containing the VSV-G gene was designed to allow final formation of rMVA/gag/pol/VSV-G vector. Following synthesis of the plasmid by Blue Heron, confirmation of the plasmid insert was performed by restriction digestion (refer to section 2.5.1.3). Several unique restriction enzymes were incorporated in the plasmid design that was used to clone the VSV-G fragment into a transfer vector to form pLF-VSV-G-Rev-D4 (refer to section 2.5.1.4). Both restriction digestion and sequencing were used to confirm the results of the cloning. VSV-G was added to rMVA/gag/pol by homologous recombination (refer to section 2.5.2).

Before homologous recombination, rMVA/gag/pol was first subjected to 11 rounds of plaque purification to ensure its purity and stability. Transfer vector pLF-VSV-G-Rev-D4 was constructed by replacing Env in pCoEnvRev.D4 with VSV-G from pVSV-G. Homologous recombination of rMVA/gag/pol and pLF-VSV-G-Rev-D4 encoding VSV-G and Rev driven by T7 promoter and a selective marker was performed successfully as confirmed by PCR results. The expression cassette was arranged back-to-back resulting in a bi-directional T7 promoter system. This system was followed for all plasmids used in the formation of the recombinant packaging virus. This allowed the insertion of two foreign genes per one deletion site. The insertion of genes in sense or anti-sense direction of the open reading frame of the poxvirus should not affect expression levels, as the expression of target genes is independent from poxviral promoters. The resulting recombinant packaging virus encodes all of Gag, Tat, Pol, Rev, Vif and VSV-G. Screening for the presence of inserts was limited to the detection of Gag, Tat, Pol and VSV-G sequences due to better primer sets. Despite the fact that they were the best of choice, primers designed to detect both Vif and Rev were insignificant to be used as they resulted in the formation of several nonspecific
fragments and thus cannot be reliable for screening. This is related to the small size of the sequence of interest, the template.

In the first round of plaque formation, 40 blue-stained plaques were picked for initial screening. Half of these blue-stained plaques were positive for Gag, Pol and VSV-G. Out of these, a random blue-stained plaque was selected for further purification. In the second round 36 blue-stained plaques were picked. 12 were screened but only 7 were positive. In the third round, 19 blue-stained plaques were selected. 10 plaques were screened and all were positive for all inserts. This suggests that the blue-stained plaque selected on round two formed from a stable recombinant virus. In the fourth round, 20 blue-stained plaques were selected and screened. All of which lost the pol construct completely, while still encoding Gag, Tat, Rev and VSV-G constructs. Following several re-attempts and around 60 blue-stained plaques screening, only one plaque was found to give positive signal for all inserts during the fourth round of screening. Unfortunately, by the fifth round the Pol construct was again lost. This indicates that Del site I used in inserting the Pol construct is dramatically unstable. Insertion of the expression cassette into a different deletion site was a necessary following step. The resulted recombinant virus (after the loss of Pol) rMVA/gag/VAS-G was further purified to re-insert the Pol cassette into it targeting different insertion site. However, during purification Del site IV started to show instability, as VSV-G could not be detected in all of the screened plaques during plaque purification round 7 (refer to section 2.5.3). Further work on the recombinant virus was suspended as due to shortage of pCEFs supply.

For the genomic (rMVA/IgG1b12/T7) recombinant virus, a plasmid-based transfer vector, encoding monoclonal antibody IgG1b12 expressed under the control of a CMV promoter flanked by an LTR of simian origin, was designed and the whole expression cassette was driven by a T7 promoter. Homologous recombination between wtMVA and the plasmid resulted in the formation of recombinant virus. Detailed mapping of the transfer plasmid is found in section 2.4.1.1.1. The backbone of all plasmids used was
pBR322 which is known to cause less metabolic stress to *E.coli* due to its low copy number. This can result in lower DNA yield but enhances the stability of large recombinant DNA inserts. The TK locus was selected to be the insertion site. Unlike deletion regions, the TK locus is well characterised to allow genetically stable integration of foreign genes in many vaccinia strains (Scheiflinger et al. 1996). This will provide much more stable recombinant virus especially where the insertion gene is of large size. The gpt and lacZ dual selection system was used to reduce rounds of purification of the recombinant MVA as well as to increase the specificity of the selection (Morrow et al. 2012).

Optimised promoters were used in rMVA vaccine development, these can significantly enhance the immune response and potentially lower the dose required for vectored immunoprophylaxis. IE promoter was inserted to drive the marker expression. A T7 promoter was placed upstream in the R region (U5 region + 5’LTR) and the tRNA primer-binding site to drive transcription of the immunoglobulin expression cassette in the presence of T7 RNA polymerase. CMV IE promoter was added to drive the expression of the monoclonal antibody sequence and the eGFP after integration in nuclear DNA. To avoid the resulting transcriptional interference by using multiple promoters in close proximity, a single strong promoter was used in this study. CMV IE is known to be optimal in driving LC-HC expression. Protein coding sequences must contain a translational start codon (ATG) and translational stop codon, plus a Kozak sequence upstream of the start codon. Thus, Kozak consensus sequences were added to ensure efficient translation.

In retroviruses, the long terminal repeat (LTR) is the control centre for gene expression. All of the requisite signals for gene expression are found in the LTRs: Enhancer, promoter, transcription initiation (capping), transcription terminator and polyadenylation signal. Expression directed by the viral LTR signals is carried out entirely by host cell enzymes (RNA pol II, poly A synthetase, guanyl transferase). The enhancer and other transcription regulatory signals are contained in the U3 region of the 5’ LTR, and the
The TATA box is located roughly 25 bp from the beginning of the R sequence. The integrated provirus has two LTRs. 5' LTR normally acts as an RNA pol II promoter and 3' LTR acts in transcription termination and polyadenylation. The transcript begins, by definition, at the beginning of R, it is capped, and proceeds through U5 and the rest of the provirus, and is usually terminated by the addition of a poly A tract just after the R sequence in the 3' LTR. In addition, the R region in this plasmid was introduced to serve as regions of homologous recombination. This would allow the integration of the IgG1b12 mAb gene sequences into the packaging virus in the presence of T7 polymerase.

To shorten the time required to generate and clone rMVAs and to increase the level of expression of the gene of interest, we employed a tri-cistronic cassette under the control of a single promoter. The strategy is based on the use of an internal ribosomal entry site (IRES) sequence that permits both the gene of interest and the selection marker to be translated from a single tricistronic mRNA (Borman et al. 1997; Gaines & Wojchowski 1999). When an IRES element is placed between two genes, the cap-independent translation of the second gene is mediated. This cap-independent translation is less efficient than a typical cap-dependent translation, resulting in lower peptide levels for the second gene. Using such a design enables strict control of the amounts of light chain (LC) to heavy chain (HC) peptide. It has been found that IRES-mediated tri-cistronic vector with LC as the first cistron controlled the LC:HC at a 4:1 ratio in all the clones. This design is preferred because excess LC is found to generate a greater proportion of high mAb-producing clones where the product has a low aggregation and consistent glycosylation. Green fluorescent protein (GFP) was added after the mAb sequence to ease the detection of the protein expression.

The screening system relies on the co-production of the reporter enzyme, E.coli β-galactosidase, which allows screening of the foci of the MVA-infected cells by colorimetric assays as well as the co-expression of the E.coli gpt gene encoding xanthine guanine-phosphoribosyl transferase, which allows the selection of resistance
against the antimicrobial agent, mycophenolic acid. The gpt and lacZ marking system is frequently used in recombinant virus purification. This method would allow dominant positive selection of the resulted rMVA for resistance against mycophenolic acid as well as blue plaque formation (Morrow et al. 2012; Schnierle et al. 2007; Scheiflinger et al. 1996).

Free plasmid DNA was ordered from Blue Heron to perform transfection. As GFP was incorporated in our plasmid and driven by CMV promoter, expression of green fluoresce could be detected if DNA plasmid were introduced to mammalian cells by transfection. Lipofectamine® 2000 was used as a transfection reagent to couple the expression plasmid to a cationic lipid or polymer producing a liposome that interacts with the cell membrane and results in endocytosis of the molecule. Vero cells were used to perform the transfection. After the fifth day of incubation, weak fluorescence was detected using an inverted fluorescence microscope. This confirmed that the CMV promoter expressed the gene of interest if the open reading frame was delivered to the cell nucleus.

Following receipt of the synthesised plasmid from Blue Heron restriction digestion was conducted and DNA digests were analysed by gel electrophoresis confirming the plasmid sequence. LacZ was cloned successfully in the plasmid which was confirmed by restriction digest and sequencing. Homologous recombination between the transfer plasmid and wt-MVA was performed to form the genomic rMVA. Following the formation of the recombinant virus, an initial round of plaque purification indicated that the resultant virus encoded the gene of interest. Blue-stained plaques were selected for screening up to round 4 then non-stained plaques were selected for virus purification. By the 11th round of plaque purification the recombinant virus disappeared from the cells. LacZ marker gene was detected in both blue and white plaques (refer to section 2.5.3). The modification that was made on the TK gene resulted in further attenuation of an already poorly replicating virus and probable inactivation of the TK-gene, which clearly altered the growth properties of the rMVA (Scheiflinger et al. 1996). Thus after
11-plaque purification rounds the recombinant plaques were dramatically smaller, suggesting that the TK deletion incorporated in the plasmid design is actually an impediment to viral replication. In addition, in the designed plasmid the marker genes were destabilised by a 50bp repetitive homologous sequence used to drive spontaneous intra-genomic recombination. However, after the 10th plaque purification round it appeared that such a small sequence is not enough to generate a marker-gene-free rMVA, as the LacZ gene was still detectable. Findings indicated that cloning of the expression cassette into a different insertion site was necessary, Del IV was selected. Cloning of IgG1b12 gene into a transfer plasmid targeting D4 was performed successfully, results are confirmed by restriction digest and sequencing. Unfortunately, plaques formation couldn’t be detected which suggest that the homologous recombination was not successful. In such case both T7 polymerase and T7 promoter are present at the same time. This could possibly lead to the expression of the IgG, where the mAb could mask and neutralise the virus. Also, the possible IgG expression might affect the efficiency of the homologous recombination process. Thus result in the failure of the homologous recombination. Homologous recombination between the transfer plasmid and wild type MVA may provide the ultimate solution. However, this means that we will use 3 rMVA to co-infect pCEFs instead of our initial plan of using dual vector. Unfortunately, this is challenging for the cells where an overdose of MVA is most likely to be harmful to the cells. However, further work with MVA was precluded due to unexpected delays in chicken embryo fibroblast supply.

In conclusion, due to the challenges in the above work the whole system had to be changed. Baculovirus work was suggested and planned.
3. Chapter 3

HIV neutralising antibody delivered by gene therapy with a hybrid baculovirus/SIV vector

3.1. Abstract

Baculovirus (BV) (*Autographa californica* multiple nucleopolyhedrovirus, AcMNPV) is an insect, enveloped, virus that is often genetically engineered to allow recombinant protein production in insect cell culture. Conventionally, baculovirus expression systems (BES) have provided versatile platforms used for production of a wide range of recombinant proteins including the expression of virus-like particles (VLPs). Self-assembly of BV produced recombinant viral capsid proteins has allowed the production of VLPs, which resemble an authentic virion and maintains its original antigenic composition. VLPs provide potent immunogenicity and safety when used as vaccines given the fact that they are replication-incompetent virus shells. Baculoviruses are capable of transducing various types of mammalian cells and thus can serve as a vehicle to efficiently deliver genes to mammalian cells. However, these transgenes cannot be expressed unless they are driven by an appropriate promoter such as the CMV IE promoter (Kataoka et al. 2012). The baculovirus vector “BacMam” has been successfully used as a transient expression vector for gene delivery in mammalian cells. The BacMam system combines the advantages of transient viral expression, ease of generation of protein, and a broad cell tropism; enabling rapid, efficient and flexible protein expression in various mammalian cells (Kost & Condreay 2002).

The aim of this study was to develop retrovirus like particles to serve as a new gene therapy carrier system, permitting the insertion of an IgG1 b12 sequence into the mammalian cell genome with the intention of *in vivo* production of high titres of anti-HIV neutralising monoclonal antibody. We used two different BacMam systems to allow the
production of VLPs by transducing mammalian cells with different recombinant baculoviruses (rBVs). VLPs were expressed either under the control of T7 RNA polymerase system or under the cytomegalovirus immediate early gene promoter.

This proof of principle study not only determines the feasibility of antibody gene transfer, but also investigates the efficiency of SIV like particle production in mammalian cells, to support their candidacy as potential vaccines and/or immunotherapy agents.
3.2. Objectives

This chapter includes two separate studies describing the construction and validation of two independent BacMam systems for functional gene therapy and pseudotyped retrovirus production. Generated retroviral particles delivered in vitro provide a proof of principle to demonstrate production of long-acting immunotherapy vectors for prevention of HIV infection and treatment of existing HIV infection.

The first BacMam system allowed expression of VLPs by transducing mammalian cells with five different recombinant baculoviruses, under the control of the T7 RNA polymerase system. BacMam-Gag/Tat encodes for both the group-specific antigen (Gag) that forms the main structural proteins, and transactivator (Tat) a positive regulator of transcription. BacMam-Pol/Vif/Vpr expresses polymerase (Pol), which codes for reverse transcriptase (Rt), protease (Pro), and integrase (Int); together with viral infectivity factor (Vif) and the virus protein R (Vpr), both required for virus replication. To extend the limited cell tropism inherent in SIV, the VLP was pseudotyped with vesiculat stomatitis virus glycoprotein (VSV-G). BacMam-VSV-G/Rev encodes both VSV-G and regulator of expression of virion (Rev) which is essential for viral replication. BacMam-IgG1 b12 was designed to encode for the antibody gene sequence to be packed as a core sequence of VLP. BacMam-T7 is responsible for the expression of T7 RNA polymerase at high levels.

The second system allowed the production of VLPs by transducing mammalian cells with four different recombinant baculoviruses, under the control of CMV IE promoter. BacMam-Gag/Tat encodes for both the group-specific antigen (Gag) that forms the main structural proteins, and transactivator (Tat) a positive regulator of transcription. BacMam-Pol/Vif/Vpr expresses polymerase (Pol), which codes for reverse transcriptase (Rt), protease (Pro) and integrase (Int); together with viral infectivity factor (Vif) and virus protein R (Vpr), both required for the virus replication. To extend the limited cell tropism inherent in SIV, the VLP was pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G). BacMam-VSV-G/Rev encodes both VSV-G and the
regulator of expression of virion (Rev) which is essential for viral replication. BacMam-IgG1 b12 was designed to encode for the antibody gene sequence to be packed as a core sequence of VLP.
3.3. Introduction

3.3.1. Baculovirus

Baculoviruses are a large group of rod-shaped, enveloped, double-stranded DNA viruses. Their nucleocapsid has two distinct ends: apical cap end with a small protuberance and one end blunt (Figure 49). The size of the nucleocapsid varies, ranging from 250 to 300 nm in length and by 30 to 60 nm in diameter. The nucleocapsid encloses a circular, super-coiled, double-stranded DNA genome of 80–180 kb which encodes between 90 and 180 genes. Baculoviruses infect insect larvae most prominently of the order Lepidoptera. Members of the Baculoviridae family have been isolated from more than 700 host species. The most extensively studied virus used in gene expression is Autographa californica multiple nucleopolyhedrovirus (AcMNPV) (Possee & King 2007; Jarvis 2009)

The BV genome replicates and transcribes in the nuclei of infected host cells. Baculoviruses have a bi-phasic life cycle producing two types of infectious viral particles: the budded virion (BV) and the occlusion-derived virion (ODV). The ODV is involved in virus transmission as it initiates the infection at the larvae midgut of feeding caterpillars. After this primary infection, the infectious form BV produces systemic infection in larvae as it is responsible for cell-to-cell transmission within the host and in cell culture. The two forms of virions are produced at different times and different subcellular places during the replication cycle (Figure 50). Baculoviruses are produced during the initial replication when nucleocapsids bud from infected cells. These virions contain a single nucleocapsid and an envelope derived from the plasma membrane of the infected cell and contains the viral fusion proteins (GP64 or F protein). The ODVs are produced during the very late phase of replication and are formed in the nucleus by envelopement of single or multiple nucleocapsids, which then become incorporated within the protein matrix forming occlusion bodies (OB) or polyhedra that are released into the environment upon death of the infected larva. The ODV infect midgut epithelial
cells up to 10,000 fold more efficiently than BV. However in insect cell culture, BV are up to 1,000 fold more efficient than ODV. Almost all studies involving BVs use the BV phenotype to mediate viral propagation. Thus, most of the knowledge regarding the BV infection cycle is based on studies performed in insect cells infected by BV. The BV is responsible for gene delivery into the host insect cell for expression (Au et al. 2013; Kelly et al. 2007).

Figure 49: Baculovirus. (A) Rod-shaped BV nucleocapsid. (B) Section through a polyhedron showing occlusion-derived virus particles embedded in a matrix of polyhedrin protein. (C) Infected cell in culture showing polyhedra in the enlarged nuclei. (D) Schematic diagrams of the structure of BV occlusion bodies (OB), occlusion-derived virion (ODV) and budded virion (BV). ODVs are embedded in a crystalline matrix of protein to form OBs. Shown here is the OB of nucleopolyhedroviruses (NPVs) (the vesicle containing three round structures is a cross section of an ODV). The ODV and BV envelope and their nucleocapsid(s) contain numerous proteins; only the proteins discussed in this review are outlined in the diagram. Adapted from (Au et al. 2013).

3.3.2. Baculovirus Replication

Baculovirus infection exhibits both lytic and occluded life cycles that develop independently throughout three phases of virus replication including early, late and very late phase. The infection initiates by vertical transmission when a host insect feeds on
plants contaminated with OB or polyhedra. Once eaten the crystalline polyhedrin matrix is degraded in the alkaline environment in the insect midgut, effectively in the stomach. Occlusion derived virus (ODV) is released and embedded virions fuse to microvillla of epithelial cells. Infected cells release virion from the basement membrane side of the mid-gut cell into the haemolymph system. Once the virion is inside the cells, the nucleocapsids are transported from the cytoplasm to the nucleus where they replicate in preparation to form budded virus (BV). This early phase is also known as the virus synthesis phase where the virus prepares the infected cell for viral DNA replication. Steps included in this phase are virus attachment, penetration, uncoating, early viral gene expression, and termination of host gene expression.

The initial viral synthesis occurs 0.5 to 6 hours after infection. This is followed by the late phase, also known as the viral structural phase, where late genes codes for replication of viral DNA are expressed. Between 6 and 12 hours after infection, the cell starts to produce BV. Peak release of BV occurs 18 to 36 hours after infection. Secondary infection is initiated when BV spreads systematically through the cells. The very late phase or the viral occlusion protein phase, will then be started. During this phase the polyhedrin and p10 genes are expressed forming large numbers of occlusion bodies (OB). Between 24 and 96 hours after infection, the cell starts to produce OB, which contains nuclear membrane envelopes and the viral polypeptides gp41 and gp74. Multiple virions are produced and are surrounded by a crystalline polyhedral matrix. The virus particles produced in the nucleus are embedded within the polyhedrin gene product and a carbohydrate- and protein-rich calyx. Polyhedra are mainly made from polyhedrin a protein that is produced by the very powerful transcriptional activity of the polyhedrin gene (polh) promoter. The OB produced, protects the virus and allows survival between hosts. Within 10 viral generations, the insect dies due to extensive cell lysis and the OB is released into the environment (Au et al. 2013; Roy & Noad 2012).
Figure 50: Key stages in the infection cycle of AcMNPV. (A) Infection is initiated by the ingestion of a virus occlusion body (OB). This consists of multiple virus nucleocapsids surrounded by a single lipid envelope (ODV) embedded in a protein matrix formed by the virally encoded polyhedrin protein. (B) The occlusion body is dissolved by the alkaline environment of the insect mid-gut, releasing ODV which initiates a primary infection in the midgut epithelial cells. (C) Virus enters cells and replicates in the nucleus. Two different forms of infectious virus are produced in infected cells. Budded virus (BV) is released at the cell surface and mediates systemic infection of the insect via the tracheal system, and ODV remains embedded in occlusion bodies. (D) Late stages of virus infection trigger liquefaction of the host, releasing the environmentally stable proteinaceous occlusion bodies. Polyhedrin protein is nonessential for the infection of cells in continuous culture in the laboratory and its high level of synthesis makes its promoter ideal for the high-level production of recombinant protein. Reproduced from (Roy & Noad 2012).

Despite extensive studies on the BV life cycle, several steps remains poorly characterised. The means by which BV delivers its genomic material into the nucleus of the infected cells, is yet to be defined. Understanding such mechanisms will help to enhance the effectiveness of using BV in protein expression, gene therapy, and vaccine production as nuclear importation is a vital event in the BV life cycle.

3.3.3. AcMNPV cell entry

Tjia et al. 1983, first found that BVs can be internalised by mammalian cells and at least some of the viral DNA reached the nucleus (Tjia et al. 1983). The mechanism of entry of AcMNPV into cells to infect insect cells and transduce mammalian cells is yet
to be fully understood. Cell entry of AcMNPV has been studied mainly in insect cells by using extracellular BV (Figure 51). Infectious AcMNPV particles, in BV form, enter susceptible insect cells through receptor-mediated endocytosis. It has been found that the BV GP64 envelope fusion protein is involved in the initial AcMNPV attachment on the cell surface; GP64 is acquired when the nucleocapsids bud through the host cell plasma membrane. Following receptor-mediated internalisation of AcMNPV, GP64 undergoes conformational change into a fusion-competent state at low pH, and the nucleocapsid is released into the cytoplasm after cell fusion. However, recent studies suggest that AcMNPV enters cells through direct fusion with the plasma membrane at low pH (Wang et al. 2014). GP64 is also involved in secretion of viral particles from infected cells as GP64-null viruses exhibited around 98% reduction in viral budding. GP64 derived from AcMNPV was shown to induce transduction of mammalian cells by HaSNPV (*Helicoverpa armigera* single nucleopolyhedrovirus), a virus that does not transduce mammalian cells.

The range of mammalian cell types transduced by HaSNPV expressing the GP64 of AcMNPV was consistent with those transduced by AcMNPV (Lang et al., 2005). Tani et al. 2003, reported that AcMNPV entry was inhibited when mammalian cells were treated with purified lipids, and virus entry was significantly low when mutant hamster cell lines deficient in phospholipid synthesis were used (Tani et al. 2003). Data from Kaname et al. 2001, suggest that the interaction of GP64 with phospholipids on the cell surface thus plays an important role in BV entry into mammalian cells, as GP64 interacts with CD55/decay-accelerating factor (DAF) in a lipid raft and confers resistance to serum inactivation (Tani et al. 2003). The lipid raft is a cholesterol-rich microdomain on the cell surface that plays crucial roles in signal transduction, protein sorting, and membrane transport. Although further studies are needed to clarify the involvement of cholesterol in the lipid raft in the internalisation of BV into mammalian cells, data from Kataoka et al (2001) suggest that cholesterol participates in the internalisation of BV but not in the binding to mammalian cells and that BV is
internalised into cells through clathrin-dependent but caveola-independent endocytosis or macropinocytosis. Heparan sulfate was shown to be essential for gene transduction by BV into mammalian cells as a reduction in transduction was observed when heparan sulfate was removed from cell surface (Nasimuzzaman 2014). Moreover, GP64 is essential for BV binding, internalisation and release in mammalian cells (Kataoka et al. 2012).

![Diagram of baculovirus internalisation into mammalian cells](image)

**Figure 51: Internalisation of baculovirus into mammalian cells.** Baculovirus binds to a not-yet-identified cellular receptor(s) present in the lipid raft. This association induces cellular remodeling through signal transduction. In clathrin-mediated endocytosis, baculovirus is internalized into the clathrin-coated pit. In macropinocytosis, filopodia formed by actin dynamics wrapped the baculovirus into a macropinosome. The viral genome is released from the endosome or macropinosome through membrane fusion induced by low pH. Reproduced from (Kataoka et al. 2012)

### 3.3.4. Baculoviruses as Expression Vectors

The first report of introducing site-specific modifications into the AcMNPV genome was in the early 1980s. The ability of BV to mediate foreign gene expression raised great interest in the use of BVs for recombinant protein production (Smith et al. 1983). In biomedical research, BVs are better known as potent protein expression vectors. Baculovirus expression systems are one of the most powerful and versatile eukaryotic expression system available, allowing expression of heterologous genes from various
sources including fungi, plants, bacteria, viruses, insect and mammalian cells. Baculoviruses offer several advantages over other expression vector systems.

Baculoviruses have a restricted host range limited to specific invertebrate species and are essentially non-pathogenic to mammals and plants. They thus exhibit a good safety profile. Recombinant baculoviruses are easily generated and propagated. They can be scaled up with consistent reproducibility for the large-scale production of biologically active recombinant products. Baculoviruses can be propagated in insect hosts which post-translationally modify peptides, polypeptides and proteins in a manner similar to that of mammalian cells. Baculoviruses provide high levels of recombinant gene expression. In many cases the recombinant proteins are soluble and easily recovered from infected cells late in infection when host protein synthesis is diminished. Baculoviruses are propagated in cell lines ideal for suspension cultures, allowing the production of recombinant proteins in large-scale bioreactors (Kelly et al. 2007).

The baculovirus expression system relies on the fact that the BV polyhedrin gene is non-essential for virus replication in insect cells grown in culture. Polyhedrin is the major structural component of the occlusion bodies. Recombinant baculovirus is produced by replacing the coding sequence of the polyhedrin gene by a recombinant gene or other cDNA. The promoter from the polyhedrin, p10 or basic protein AcMNPV gene drives the expression of the foreign gene. Recombinant baculoviruses produced in this way are polyhedrin or polyhedral-negative viruses. The size of the BV nucleocapsids is flexible permitting easily insert of foreign genes, thus rBV particles can accommodate large amounts of foreign DNA (Rohrmann 2013).

Understanding viral cellular cytopathogenesis allows efficient production of higher BV stocks and high quality recombinant product that is non-degraded and free of cell debris. Host gene expression is terminated upon cell infection with rBV allowing higher recombinant mRNA and protein production. Depending on the type of infected cells, strain of BV, metabolic condition of the culture and growth medium used, timing of the infection cycle, changes in cell morphology vary. Spodoptera frugiperda (Sf) cells
infected with recombinant AcMNPV were used as a standardised example to study the morphological changes during rBV infection. Following endocytosis of one or more rBV into Sf cells, nucleocapsids pass through the cytoplasm to the nucleus where the capsid contents are released. Within 30 minutes of infection, viral RNA is detectable.

![Budded virus infection cycle in cultured cells](image)

**Figure 52: Budded virus infection cycle in cultured cells.** Reproduced from (Au et al. 2013).

During the first 6 hours of infection, cellular structure changes, normal cellular functions decline extensively, and early-phase proteins become evident. Several normal cell functions terminate and infected cells stop dividing. Within 6 to 24 hours post infection, virions are assembled and recombinant viruses begin to bud from the infected cell. Infected cells increase in diameter and demonstrate abnormal enlargement of nuclei.

During the very late phase of infection, 20 to 36 hours after infection, recombinant proteins are expressed in place of the naturally occurring polyhedrin protein. Cells in both monolayer and suspension cultures display decreased density. Infected cells continue to increase in diameter and have enlarged nuclei. Vacuoles begin to form in the cytoplasm and the cell nuclei demonstrate granularity. A cytopathic effect in the form of plaques can be identified under a microscope as regions of decreased cell
density, or by eye examination of regions of differential refractivity. Recombinant proteins are produced at levels ranging between 0.1% and 50% of the total insect cell protein. However, it is difficult to precisely predict the efficiency of gene expression. The initiation of virus production and budding occurs at approximately 8 to 10 hours post-infection, while protein expression under control of the polyhedrin promoter occurs approximately 20 to 24 hours post-infection (Rohrmann 2013).

3.3.5. Construction of recombinant baculoviruses

To produce a rBV the coding sequence of the polyhedrin gene is replaced by the coding region of the gene to be expressed, the polyhedrin gene promoter drives expression of the foreign gene. Recombinant baculoviruses produced in this way are polyhedrin negative viruses (Au et al. 2013).

3.3.5.1. Generating a Recombinant Virus by Homologous Recombination

To generate a rBV via homologous recombination, heterologous genes are cloned into a transfer plasmid, which contains sequences that flank the non-essential polyhedrin gene in the virus genome. The virus genome and transfer plasmid are simultaneously introduced into insect cells (co-transfection) allowing recombination between homologous sites, transferring the heterologous gene from the plasmid vector to the virus DNA resulting in a rBV. The virus genome then replicates and produces recombinant virus which can be harvested as budded virus in the culture medium; 0.1% to 1% of the resulting progeny are recombinant (Smith et al. 1983; Rohrmann 2013). The recombinants are identified by altered plaque morphology. Detection of the desired occlusion-minus plaque phenotype against the background of greater than 99% wild-type parental viruses is difficult. Plaques formed by occlusion body-negative (recombinant) viruses are different from those of occlusion body-positive (wild-type) viruses. Newer modified AcMNPV allow either colour selection to identify recombinants
or permit positive survival selection for recombinants rendering the occlusion body-based visual screening method obsolete (Rohrmann 2013).

New methods have been developed to generate rBVs for target protein expression in insect cells avoiding time-consuming plaque purification steps. Detection of rBV was almost 30% higher when the parental virus was linearised at one or more unique sites located near the target site for insertion of the foreign gene into the BV genome. An even higher percentage of recombinants - 80% or more - were obtained from linearised viral DNA missing a portion of the essential open reading frame (ORF) 1629 of the polyhedrin gene (Figure 53). Such methods prevent non-recombinant virus from replicating in insect cells. Non-essential chitinase (chiA) gene is deleted to improve production of secreted and membrane-targeted proteins. Additional deletion of the v-cath gene, a cathepsin-like cysteine protease, resulted in significantly improved quality and yield for most target proteins. Further deletions of nonessential genes (p10, p24, and p26) result in further improvement of protein expression. One or more of these deletions can be used in recombinant virus production (Rohrmann 2013).


Recombinant baculoviruses generated using this method benefit from using BV DNAs contain an artificial sequence in place of the polyhedrin (polh) coding sequencing, which is co-transfected with a compatible transfer plasmid containing the target coding sequence into insect cells. In the cells, homologous recombination restores the viral
ORF1629 and the target coding sequence replaces the artificial sequences. Only rBV can replicate, producing a homogeneous population of recombinants (Rohrmann 2013).

### 3.3.5.2. Generating a Recombinant Virus by Site-Specific Transposition

Luckow et al. 1993, developed a faster approach for generating rBVs based on site-specific transposition with Tn7 to insert foreign genes into a BV shuttle vector (bacmid) DNA propagated in *E. coli* (Luckow et al. 1993). This generates a rBV shuttle vector (bacmid) which can be replicated in *E. coli* as a large plasmid which is infectious when introduced into insect cells. Bacmids contains a low-copy number mini-F replicon which allows autonomous replication and stable segregation of plasmids at low copy number, a selectable kanamycin resistance marker, attTn7 - the target site for the bacterial transposon Tn7, and LacZα peptide, all inserted into the polyhedrin locus of AcMNPV (Bao et al. 1991).

Tn7 inserts at a high frequency into the single attTn7 site located on the *E.coli* chromosome and into DNA segments carrying attTn7 on a plasmid. Recombinant bacmids are generated by transposing a mini-Tn7 element from a donor plasmid to the mini-attTn7 attachment site on the bacmid. The Tn7 transposition proteins are provided by a helper plasmid which encodes the transposase and confers resistance to tetracycline. The helper plasmid provides the Tn7 transposition function in trans (Barry 1988). Colonies containing recombinant bacmids are identified by antibiotic selection and blue/white screening, since the transposition results in disruption of the lacZα gene. The resulting recombinant bacmid is purified and used to transfec insect cells to generate rBVs. The steps to generate a rBV by site-specific transposition using the BAC-TO-BAC Baculovirus Expression System are outlined in Figure 54. This method, can obtain pure stocks of recombinant virus within 7 to 10 days (Bao et al. 1991).
Using site-specific transposition has two major advantages over homologous recombination. It is essentially a one-step purification and amplification process, since recombinant virus DNA isolated from selected colonies is not mixed with parental virus. Multiple rounds of plaque purification are not required and identification of the recombinant virus is easier. It also can provide a rapid and simultaneous isolation of multiple recombinant viruses. This feature is particularly valuable for expressing protein variants in structure/function studies (Bao et al. 1991).

### 3.3.6. Baculovirus and mammalian cell transduction

Baculoviruses were shown to be capable of entering into various mammalian cells without replication. As the spectrum of cell types permissive to BV transduction expands, a wider range of potential applications of BV vectors are receiving increasing attention. Baculoviruses can transduce several types of cells including cell lines.
originating from cells of human, rodent, porcine, bovine, and even fish origin (Table 10). Together with dividing cells, BVs are capable of transducing non-dividing cells, such as PK1 cells arrested in the S phase (van Loo et al. 2001). Primary cells have also been transduced with BVs, such as human neural cells (Sarkis et al. 2000), pancreatic islet cells (Ma et al. 2000), and rat articular chondrocytes (Ho et al. 2004). Baculoviruses are also capable of transducing mesenchymal stem cells (MSC) derived from human umbilical cord blood and bone marrow (Ho et al. 2005), as well as MSC-derived adipogenic, osteogenic, and chondrogenic progenitor cells (Ho et al. 2006).

The cell type can affect BV transduction efficiencies considerably with ranges vary from 95% for BHK cells (Wang et al. 2005) to lower than 10% for NIH-3T3 cells (Cheng et al. 2004). The cellular differentiation state also plays an important factor as transduction efficiency range from around 30% for undifferentiated human neural progenitor cells up to 55% for differentiated neural cells when infected at the same multiplicity (Sarkis et al. 2000). The transduction efficiency is very high for adipogenic and osteogenic progenitors, but is relatively low for chondrogenic progenitors (Ho et al. 2006).

<table>
<thead>
<tr>
<th>Human cells</th>
<th>References</th>
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<tr>
<td>HeLa</td>
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<td>(Condreay et al. 1999)</td>
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<tr>
<td>MRC5</td>
<td>(Palombo et al. 1998; Yap et al. 1997)</td>
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<tr>
<td>MG63</td>
<td>(Condreay et al. 1999)</td>
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<td>KATO-III (gastric cancer)</td>
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</tbody>
</table>
Baculoviruses can serve as a vehicle to efficiently deliver and express genes to mammalian cells. However, these transgenes cannot be expressed unless they are driven by a mammalian promoter such as the CMV IE promoter. Various mammalian promoters have been cloned into BV vectors to drive gene transcription in mammalian cells (Table 11). Spenger et al., compared transgenic expression of several promoters including Simian virus 40 (SV40), CMV, RSV, and a cellular promoter (human ubiquitin C) in a range of cell types including CHO, COS-1, and HEK293 cells. Results indicated that CMV and RSV promoters were the most active in all cell lines tested, followed by the ubiquitin C promoter, while SV40 promoter was the weakest of them all (Spenger et al. 2004).

Table 11: Promoter used in Baculovirus expression system

<table>
<thead>
<tr>
<th>Promoter</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rous sarcoma virus long terminal repeat (RSV-LTR) promoter</td>
<td>(Boyce &amp; Bucher 1996)</td>
</tr>
<tr>
<td>Cytomegalovirus immediate early promoter CMV-IE</td>
<td>(Hofmann et al. 1995; Sollerbrant et al. 2001)</td>
</tr>
<tr>
<td>Simian virus 40 (SV40) promoter</td>
<td>(Spenger et al. 2004)</td>
</tr>
<tr>
<td>Hybrid chicken -actin promoter (CAG)</td>
<td>(Shoji et al. 1997; Stanbridge et al. 2003)</td>
</tr>
<tr>
<td>Hepatitis B virus (HBV) promoter/enhancer</td>
<td>(Delaney &amp; Isom 1998)</td>
</tr>
<tr>
<td>Human -fetoprotein promoter/enhancer</td>
<td>(Park et al. 2007)</td>
</tr>
<tr>
<td>Human ubiquitin C promoter</td>
<td>(Spenger et al. 2004)</td>
</tr>
<tr>
<td>Hybrid neuronal promoter</td>
<td>(Wang &amp; Wang 2005)</td>
</tr>
<tr>
<td>Drosophila heat shock protein (hsp70)</td>
<td>(Viswanathan et al. 2003)</td>
</tr>
</tbody>
</table>

Baculoviruses do not replicate in mammalian cells and thus have an excellent safety profile combined with being well-tolerated by cells. The Baculovirus vector BacMam has been successfully used as a transient expression vector in cell based assays, live cell imaging and stem cell biology research. Because of its ability to transduce mammalian cells without viral replication, BVs are considered as promising candidates for gene therapy and vaccine vectors in mammalian cells. The BacMam system combines the advantages of viral transient expression, ease of generation of protein and a broad cell tropism; enabling rapid, efficient and flexible protein expression in various mammalian cells (Dukkipati et al. 2008).
To introduce genes into mammalian cells, a standard transduction process is used. BacMam particles are taken up by endocytosis or macropinocytosis and released for transcription and expression following migration to the nucleus. Gene expression begins within 4–6 hours of transduction and is at near maximum level within 24 hours of transduction. Depending on transduction efficiency, cell type and cell division rate, the transgene remains detectable from 5 to 14 days (Airenne et al. 2013).

Using BacMam technology for gene expression in mammalian cells offers several advantages. Baculoviruses can transduce a broad range of cell types, including primary and stem cells with high transduction efficiency. Recombinant baculoviruses are capable of expressing large gene inserts in mammalian systems where insert with more than 38Kbp, and simultaneous delivery of multiple genes has been demonstrated. Studies indicate that BacMam transduction produces a highly reproducible and titratable expression. As BVs do not replicate in mammalian cells, minimal microscopy is needed for observable cytopathic effects. In most cases, BVs are handled under biosafety level 1, while biosafety level 2 may apply depending on the transgene expressed (Airenne et al. 2013).

### 3.3.7. Generation of VLPs using baculovirus expression

Virus-like particles (VLPs) present viral antigens in a more authentic conformation than monomeric structural proteins. VLPs mimic the structure of virus particles and display excellent adjuvant properties, being capable of inducing innate and cognate immune responses. Depending on the biological function of the VLPs different expression systems can be applied. Baculoviruses can express VLPs in bacteria, yeast, insect and mammalian cells (Table 11) (Liu et al. 2013). Insect cells have fast specific growth rates allowing large-scale protein production under controlled culture conditions. Expression systems like bacteria and yeast achieve higher production scales. However, those systems are simple and VLPs produced from such systems do not match the degree of complexity of those produced from insect cells nor mammalian cells which form much more diverse proteins. Insect cells are relatively easy to culture.
for those with experience and do not require tedious cell culture adaptation procedures as in mammalian cells. They can grow in the absence of serum, which is a highly desirable feature when rescuing VLPs from intensive purification processes. Production yields of insect cells are significantly higher and faster when compared with mammalian cell-based VLP production. Production processes can be shortened to approximately 12 weeks from identification of a new strain. VLPs expressed from insect cells have similar glycosylation patterns similar to those such as wild type influenza VLPs. The insect expression system offers several advantages over mammalian expression systems (Liu et al. 2013). However, not all VLP produced in insect cells produce the correct configuration of protein. This relates to the fact that insect cells have a different pattern of post-translational modifications than mammalian cells (Davis & Wood 1995). Insect cells perform simpler post-translational glycosylation based on enriched mannose (Wu & Passarelli 2010).

The differences in essential mechanisms between insect and mammalian cells, such as glycosylation and folding of heterologous proteins post translation, may hinder the formation of VLPs, especially structurally complicated VLPs. Improved insect cells lines – that perform mammalian-like post-translation modifications – are becoming available. VLPs induce potent humoral and cellular immune responses as they present high-density B-cell epitopes responsible of antibody production and intracellular T-cell epitopes. Recombinant VLPs can serve as an excellent candidate for vaccine and diagnostic purposes. The underlying mechanism of efficacious prophylactic vaccines is generation of neutralising antibodies against specific epitopes. In the case of VLPs, this depends on correct antigen conformation and effective antigen presentation on the VLP surface (Liu et al. 2013).
<table>
<thead>
<tr>
<th>Virus</th>
<th>Classification of VLP</th>
<th>Cells</th>
<th>Recombinant proteins</th>
<th>Expression strategies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feline leukaemia virus</td>
<td>Enveloped VLP</td>
<td>Sf9 cells</td>
<td>Gp85 and gag</td>
<td>Co-infection</td>
</tr>
<tr>
<td>Norwalk virus</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>Capsid protein</td>
<td>Single infection</td>
</tr>
<tr>
<td>Porcine parvovirus</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>VP2</td>
<td>Single infection</td>
</tr>
<tr>
<td>Bluetongue virus</td>
<td>Non-enveloped VLP</td>
<td>Sf21 cells</td>
<td>VP2, VP6, VP7 and NS1</td>
<td>Co-expression</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>Non-enveloped VLP</td>
<td>Sf21 cells</td>
<td>VP0, VP3 and VP1</td>
<td>Co-infection</td>
</tr>
<tr>
<td>Human papillomavirus</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>L1 and L2</td>
<td>Co-expression</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>Non-enveloped capsid</td>
<td>Sf21 cells</td>
<td>VP23, VP5, VP22a, VP21&amp;VP24, VP26 and VP19C</td>
<td>Co-infection</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>VP2, VP6, VP7 and VP4</td>
<td>Co-infection</td>
</tr>
<tr>
<td>Porcine parvovirus (LCMV)</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>VP2 containing LCMV epitope</td>
<td>Single infection</td>
</tr>
<tr>
<td>African horse sickness virus</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>VP3 and VP4</td>
<td>Co-infection</td>
</tr>
<tr>
<td>Human immunodeficiency virus</td>
<td>Enveloped VLP</td>
<td>Sf9 cells</td>
<td>Gag and gp120</td>
<td>Co-expression</td>
</tr>
<tr>
<td>Human severe acute respiratory syndrome coronavirus</td>
<td>Enveloped VLP</td>
<td>Sf21 cells</td>
<td>Spike, membrane and envelope proteins</td>
<td>Co-infection</td>
</tr>
<tr>
<td>Human astrovirus</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>Complete ORF2</td>
<td>Single infection</td>
</tr>
<tr>
<td>Enterovirus-71</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>P1 and 3CD</td>
<td>Co-expression or co-infection</td>
</tr>
<tr>
<td>Feline calicivirus</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>Capsid</td>
<td>Single infection</td>
</tr>
<tr>
<td>Simian virus 40</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>VP1, VP2 and VP3</td>
<td>Single infection or co-infection</td>
</tr>
<tr>
<td>Rift Valley fever virus</td>
<td>Enveloped VLP</td>
<td>Sf9 cells</td>
<td>Gn, Gc and N</td>
<td>Co-expression</td>
</tr>
<tr>
<td>Porcine circovirus</td>
<td>Non-enveloped VLP</td>
<td>Tn cells</td>
<td>VP2</td>
<td>Single infection</td>
</tr>
<tr>
<td>Avian influenza virus</td>
<td>Enveloped VLP</td>
<td>Sf9 cells</td>
<td>HA, NA and M1</td>
<td>Co-expression</td>
</tr>
<tr>
<td>Ebola virus</td>
<td>Enveloped VLP</td>
<td>Sf9 cells</td>
<td>VP40 and GP</td>
<td>Co-infection</td>
</tr>
<tr>
<td>Encephalomyocarditis virus</td>
<td>Non-enveloped VLP</td>
<td>Sf9 cells</td>
<td>P1–2A-3C</td>
<td>Single</td>
</tr>
</tbody>
</table>
Currently, numerous VLP-based vaccine candidates have entered preclinical testing or clinical trials and many are in the proof-of-concept stage. To date, only a few fully VLP-based vaccines against viral infections have been licensed for human use (Liu et al. 2013).

Recombivax HB® (Merck) and Engerix®-B (GSK) are licensed against hepatitis B virus (HBV). Both are Non-enveloped VLPs composed of HBV surface antigen (HBsAg) harvested and purified after being expressed in yeast (Saccharomyces cerevisiae) infected with BV. Both vaccines induce the production of the same antibodies to HBsAg and have similar immunogenic properties that may allow for interchangeability.

GlaxoSmithKline’s Cervarix™ (GSK) is a VLP-based bivalent human papillomavirus vaccine against cervical cancer. Composed of HPV16/18 formulated in L1 ASO4 adjuvant and produced using a rBV expression system in an insect cell line. Hecolin® is a vaccine against hepatitis E virus (HEV) developed by Xiamen Innovax Biotech Co., Ltd (China), based on recombinant HEV ORF2 truncated protein HEV 239 (aa368-aa606) expressed from BV in an E.coli expression system. FluBlok®, a seasonal influenza subunit vaccine for adults is a recombinant trivalent hemagglutinin (rHA) vaccine produced in insect cell culture using the BV expression system. It is tailored annually to provide protection against the latest strains of influenza by containing the corresponding hemagglutinin (HA) antigens.
3.3.8. **Immune response and potential as gene therapy vectors and vaccine vectors**

The interest in using BVs as vectors for both in vitro and in vivo gene delivery has increased. Several studies indicated that BVs can be used for in vivo gene transfers among different tissue types. Airenne et al., showed that BVs can be used for in vivo gene transfer in rabbit carotid artery with efficiency comparable to adenoviruses (Airenne et al. 2000). Huser et al., studied gene transfer of complement-resistant BV vectors into complement-sufficient neonatal rats by injection into their livers (Hüser et al. 2001). Studies have been conducted to understand the efficiency of BV gene delivery to rat brain (Sarkis et al. 2000; Lehtolainen et al. 2002; Wang & Wang 2005) and several in vivo mouse studies have targeted BV gene transfer in different tissue types including brain, skeletal muscle, cerebral cortex and testis and liver (Sarkis et al. 2000; Pieroni et al. 2001; Tani et al. 2003; Hoare et al. 2005).

Other advantages include the broad range of mammalian cells capable of transduction; their nontoxic and non-replicative nature; large packaging capacity; ease of production and so BVs appear to be a promising tool for gene therapy (Table 13). However, for BV-mediated in vivo gene therapy, contact between BVs and serum complement results in the rapid inactivation of the virus. Sandig et al. 1996, described the potential limitation caused by the complement system in a systemic or intraportal application as well as in direct injection of BVs into the rodent liver parenchyma (Sandig et al. 1996). This phenomenon was not observed when the BV was injected into rodent brain. Within the brain the complement level may be insufficient to affect gene transfer (Lehtolainen et al. 2002). Hofmann & Strauss showed that BVs most likely activate the classical pathway of the C system and assembly of very late C components is required for inactivation of the vector (Hofmann & Strauss 1998). Hoare et al., further showed that both classical and alternative pathways are involved in the inactivation and suggested that naturally occurring IgM antibodies with high affinity for BVs may be partially responsible for the inactivation (Hoare et al. 2005).
Table 13: Comparison of Baculovirus and Other Viral Vectors

<table>
<thead>
<tr>
<th>Features</th>
<th>Retroviral</th>
<th>Lentiviral</th>
<th>Adenoviral</th>
<th>AAV</th>
<th>Baculoviral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ease of preparation</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Packaging capacity</td>
<td>7–7.5 kb</td>
<td>7–7.5 kb</td>
<td>Up to 30 kb</td>
<td>3.5–4 kb</td>
<td>&gt;38 kb</td>
</tr>
<tr>
<td>Route of administration</td>
<td>Ex vivo</td>
<td>Ex/in vivo</td>
<td>Ex/in vivo</td>
<td>Ex/in vivo</td>
<td>Ex/in vivo</td>
</tr>
<tr>
<td>Vector genome forms</td>
<td>Integrated</td>
<td>Integrated</td>
<td>Episomal</td>
<td>Episomal</td>
<td>Episomal</td>
</tr>
<tr>
<td>Gene expression duration</td>
<td>Short</td>
<td>Long</td>
<td>Short</td>
<td>Long</td>
<td>Short</td>
</tr>
<tr>
<td>Tropism</td>
<td>Dividing cell</td>
<td>Broad</td>
<td>Broad</td>
<td>Broad</td>
<td>Broad</td>
</tr>
<tr>
<td>Immune response</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Preexisting immunity</td>
<td>Unlikely</td>
<td>Unlikely</td>
<td>Yes</td>
<td>Yes</td>
<td>Unlikely</td>
</tr>
<tr>
<td>Safety</td>
<td>Integration may induce oncogenesis</td>
<td>Integration may induce oncogenesis</td>
<td>Inflammatory response, toxicity</td>
<td>Inflammatory response, toxicity</td>
<td>High</td>
</tr>
</tbody>
</table>

Various studies have been conducted to alleviate complement inactivation. Treating with complement inhibitors such as a functional antibody-blocking complement component 5 (C5) or cobra venom factor (CVF) can enhance the survival of BVs in the presence of serum (Hofmann & Strauss 1998). Generation of complement-resistant BVs represents a more cutting-edge approach by the display of a regulator that blocks complement at the central step of both the classical and alternative pathways on the viral envelope such as decay-accelerating factor (DAF) (Hüser et al. 2001). Alternatively, a more frequently used approach is to pseudotype BVs with VSV-G. VSV-G-modified virus enhances gene transfer efficiency into mouse skeletal muscle in vivo with the transgene expression lasting 178 days in DBA/2J mice and 35 days in BALB/c and C57BL/6 mice (Pieroni et al. 2001).

Pseudotyped BVs exhibit greater resistance to inactivation by animal sera and can transduce the cerebral cortex as well as testes of mice by direct inoculation (Tani et al. 2003). Tang et al. 2011, suggested that immunised BALB/c mice with purified influenza
VLPs produced from BV-transduced mammalian cells, or directly immunised with pseudotyped BacMam BV survived without any signs of sickness or weight loss. Both induced influenza-specific immune responses and provided mice with sterilising protection against influenza challenge (Tang et al. 2011). Baculoviruses are considered safe, but more studies are required to confirm their safety for in vivo and ex vivo applications. Gronowski et al., was the first to study the immune response against BVs in mammalian cells. He reported that administration of BVs in vitro and in vivo induced the production of IFN α/β from both human and murine cell lines. This IFN-stimulation resulted from the live virus and not due to the presence of viral RNA or DNA (Gronowski et al. 1999). Abe et al., found that BV DNA, not the gp64 glycoprotein, induced the innate immune system activation and produced IFN-α via the Toll-like receptor 9 (TLR9)/MyD88-dependent signaling pathway. The production of inflammatory cytokines were severely reduced in peritoneal macrophages (PECs) and splenic CD11c⁺ dendritic cells (DCs) derived from mice deficient in MyD88 or TLR9 after cultivation with AcMNPV. In contrast, a significant amount of IFN-α was still detectable in the PECs and DCs of these mice after stimulation with AcMNPV, suggesting that a TLR9/MyD88-independent signalling pathway might also participate in the production of IFN-α by AcMNPV (Abe et al. 2005).

It has been suggested that IFN induction requires an interaction between the BV envelope protein, gp64, and the receptors on the corresponding cell membrane (Kataoka et al. 2012). This finding provides further emphasis that viral-induced IFN production is not always dependent on viral replication. Administration of BVs in vivo protects mice from death when challenged with a lethal dose of FMDV (Molinari et al. 2010). However, the mechanisms by which viruses interact with a host cell and initiate IFN production are not well understood. Beck et al., demonstrated that upon BV exposure, production of cytokines in hepatocytes is up-regulated. Baculoviruses had an antagonistic effect on cytochrome P450 (CYP) induction processes and expression profiles which may alter the detoxification capabilities of the liver (Beck et al. 2000).
Airenne et al., found that in vivo administration of BV to rabbit carotid artery resulted in signs of inflammation (Airenne et al. 2000). Abe et al., inoculated mice with rBV by intramuscular, intradermal, i.p., and intranasal routes using a rBV expressing haemagglutinin (HA) of influenza virus under the control of the CAG promoter (Abe et al. 2003). Innate immune responses and the level of protection were found to depend on the route of administration. Mice were inoculated with rBV by intramuscular, intradermal, i.p., and intranasal routes and then were challenged with a lethal dose of the influenza virus. Higher titers of anti-hemagglutinin Ab resulted from intramuscular or i.p. immunisation compared to intradermal or intranasal administration. However, intranasal immunisation conferred sufficient protection from the lethal challenge of the influenza virus.

Baculoviruses have been found to have immune-stimulatory capacities to promote the release of inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-12, in a murine macrophage cell line, RAW264.7. Facciabene et al., have determined the induction of humoral and cell-mediated immune responses to the E2 glycoprotein of hepatitis C virus (HCV) and to carcinoembryonic antigen (CEA) in mice vaccinated with rBV vectors expressing the putative structural component of the HCV virions or the epithelial tumor antigen. Their findings agreed with the finding that VSV-G-pseudotyped BVs reduced the minimal dose required to elicit a measurable T cell response tenfold. This indicates that VSV-G-pseudotyped viruses provide more efficient immunogen expression in transduced cells and avoid complement inactivation (Facciabene et al. 2004).

Although BVs seems to be a promising candidate as a vaccine vector against infectious diseases as well as vectors for in vivo human gene therapy, further investigation of the mechanisms of viral uptake and induction of immunity are needed, particularly in understanding which cytokines are induced by BVs, and how these cytokines modulate cellular and humoral immunities in vivo.
3.4. **Material and methods:**

Genetically Modified Organisms approval for this study was obtained for the laboratory and the project. The lab permissions and the GMO permission were granted by the University of Manchester safety committee, the GMO Establishment license# 541 (p5603-4).

3.4.1. **Virus like particle expression utilising the T7 RNA Polymerase/Promoter system**

BacMam system one allowed expression of VLPs by transducing mammalian cells with five different rBVs bearing the T7 RNA polymerase system. BacMam-Gag/Tat encodes for both the group-specific antigen (Gag) that forms the main structural proteins, and transactivator (Tat) a positive regulator of transcription; BacMam-Pol/Vif/Vpr expresses polymerase (Pol), encodes reverse transcriptase (Rt), protease (Pro) and integrase (Int) together with viral infectivity factor (Vif) and virus protein R (Vpr), (required for the virus replication). To extend the limited cell tropism inherent in SIV, the VLP was pseudotyped with VSV-G. BacMam-VSV-G/Rev encodes both VSV-G and regulator of expression of virion (Rev) which is essential for viral replication. BacMam-IgG1b12 was designed to encode for antibody gene sequence to be packed as a core sequence of the VLP. Genes of interest in all four rBVs were driven by the T7 promoter in the presence of the T7 polymerase. The fifth recombinant, BacMam-T7 is responsible for the expression of T7 RNA polymerase in high levels, protein expression was driven by CMV IE.

3.4.2. **Virus like particle expression using a CMV Promoter**

BacMam system allowed expression of VLPs by transducing mammalian cells with four different rBVs using CMV to drive the expression of all genes of interests. BacMam-Gag/Tat encodes for both the group-specific antigen (Gag) that forms the main structural proteins, together with transactivator (Tat) a positive regulator of transcription. BacMam-Pol/Vif/Vpr expresses polymerase (Pol), which codes for
reverse transcriptase (Rt), protease (Pro) and integrase (Int); together with viral infectivity factor (Vif) and virus protein R (Vpr), both required for the virus replication. To extend the limited cell tropism inherent in SIV, the VLP was pseudotyped with VSV-G. **BacMam-VSV-G/Rev** encodes both VSV-G and regulator of expression of virion (Rev) which is essential for viral replication. **BacMam-IgG1 b12** was designed to encode for the antibody gene sequence to be packed as a core sequence of VLP. Genes of interest in all four rBVs were driven by the CMV IE promoter.

### 3.4.3. Molecular cloning

All genes of interest were driven either by the T7 promoter in the presence of the T7 RNA polymerase or by under the CMV IE gene promoter. The final product of both the systems should be the production of a pseudotype virus like particle (VLP) encoding the IgG1b12 sequence in its core. The VLP was of SIVmac239 origin. Expression cassettes used contained two common genes (gag, pol) and five additional accessory genes (tat, rev, vpx, vpr and vif).

![Figure 55: pOET6 BacMAM map](https://oetltd.com/wp-content/uploads/2015/07/pOET6-vector-map.pdf)

**Figure 55: pOET6 BacMAM map.** Reproduced from [https://oetltd.com/wp-content/uploads/2015/07/pOET6-vector-map.pdf accessed on 09/05/2018].

pOET6 BacMAM (Oxford Expression Technologies # 200107) was the primary BV transfer vector used for expression of foreign genes in mammalian cells. All plasmids were designed using SnapGene software (GSL Biotech, Chicago, IL), in conjunction
with data held in the NCBI GenBank database. pOET6 BacMam transfer vector was purchased from Oxford Expression Technologies (Oxford, UK). pT7-Vpx_MVA, pPol-Vpr-Vif_MVA and pGag_Tat_MVA shuttle vectors were provided by Dr Tom Blanchard University of Manchester. pLF-IgG1b12-LacZ-D4 and pLF-VSV-G-Rev-D4 were transfer vectors developed in the MVA work (refer to sections 2.5.1.5 and 2.5.1.4)

For amplification of plasmid DNA the following *E. coli* strains were used:

Either: NovaBlue GigaSingles Competent Cells (71227-3, Novagen.UK). NovaBlue is a K-12 strain derivative that offers high transformation efficiency, genotype: endA1 hsdR17(fK12− mK12+) supE44 thi-1 recA1 gyrA96 relA1 lac F[proA’B’ lacZΔM15::Tn10 (TcR)].

Or: NEB 10-beta Competent a derivative of DH10B (NEB #C3019L). It is T1 phage resistant and endonuclease I (endA1) deficient. NEB 10-beta electrocompetent *E. coli* cells are Δ(ara-leu) 7697 araD139 thiA ΔlacX74 galK16 galE15 e14-φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC).

### 3.4.3.1. DNA extraction and purification from bacterial cells

Plasmid DNA was isolated using the QIAPrep Spin Miniprep Kit (QIAGEN #27104). Following the manufacturer instructions, an overnight *E. coli* culture was pelleted by centrifugation. Bacterial cells were then lysed by alkaline lysis followed by adsorption of DNA onto silica in the presence of high salt. Impurities were washed with the supplied washing buffer and pure plasmid DNA eluted in a small volume of elution buffer. The concentration of the plasmid DNA was determined using the ND1000 NanoDrop Spectrophotometer (Labtech, Uckfield,UK).
3.4.3.2. Preparation of vector and insert: Restriction digest

In a total volume of 50μl: 1μg of DNA, 5μl of 10xNEBuffer and 1μl (10units) enzyme were mixed. Both incubation times and temperatures were enzyme dependent.

3.4.3.3. DNA End Modification

3.4.3.3.1. Dephosphorylation

1 μL (1 unit/μL) of recombinant shrimp Alkaline Phosphatase (rSAP) (NEB #M0371) was added to each pmol of DNA (about 1 μg of a 3 kb plasmid). After incubation at 37°C for 30 minutes, the reaction was stopped by heat inactivation at 65°C for 5 minutes.

3.4.3.3.2. Blunting/End-repair

Quick Blunting Kit (NEB #E1201) was used to fill in (5’→3’) and ‘chew back’ (3’→5’). For up to 5μg DNA a total reaction of 25μL was prepared including 5 μL of 10X Blunting Buffer, 5 μL of 1 mM dNTP Mix and 2 μL of Blunt Enzyme Mix. The reaction mix was then incubated at room temperature for 30 minutes, followed by an inactivation step of heating at 70°C for 10 minutes.

3.4.3.4. PCR Using Q5® High-Fidelity DNA polymerase

Inserts from a PCR product were amplified using Q5 High-Fidelity DNA Polymerase (NEB # M0491). Conditions for optimal performance were used following the manufacturer's structures (Table 14, Table 15 and Table 16). NEB Tm Calculator (https://tmcalculator.neb.com/#/main) was used to determine optimal annealing temperatures.
Table 14: Reaction Setup

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>25 µL REACTION</th>
<th>50 µL REACTION</th>
<th>FINAL CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Q5 Reaction Buffer</td>
<td>5 µL</td>
<td>10 µL</td>
<td>1X</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.5 µL</td>
<td>1 µL</td>
<td>200 µM</td>
</tr>
<tr>
<td>10 µM Forward Primer</td>
<td>1.25 µL</td>
<td>2.5 µL</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>10 µM Reverse Primer</td>
<td>1.25 µL</td>
<td>2.5 µL</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>variable</td>
<td>variable</td>
<td>&lt; 1,000 ng</td>
</tr>
<tr>
<td>Q5 High-Fidelity DNA Polymerase</td>
<td>0.25 µL</td>
<td>0.5 µL</td>
<td>0.02 U/µL</td>
</tr>
<tr>
<td>5X Q5 High GC Enhancer (optional)</td>
<td>(5 µL)</td>
<td>(10 µL)</td>
<td>(1X)</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>to 25 µL</td>
<td>to 50 µL</td>
<td></td>
</tr>
</tbody>
</table>

Table 15: Thermocycling Conditions for a Routine PCR

<table>
<thead>
<tr>
<th>STEP</th>
<th>TEMP</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>98°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>25–35 Cycles</td>
<td>98°C *50–72°C 72°C</td>
<td>5–10 seconds 10–30 seconds 20–30 seconds/kb</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4–10°C</td>
<td></td>
</tr>
</tbody>
</table>

Table 16: Recommended amounts of DNA template for a 50 µL reaction

<table>
<thead>
<tr>
<th>DNA</th>
<th>AMOUNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Genomic</td>
<td>1 ng–1 µg</td>
</tr>
<tr>
<td>Plasmid or Viral</td>
<td>1 pg–1 ng</td>
</tr>
</tbody>
</table>
Table 17: PCR primers used for inserts amplification

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Target</th>
<th>Tm</th>
<th>Direction</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ TAAGCAGCTAGCCACCATGGGCGTGAGAAA 3’</td>
<td>Gag</td>
<td>69.5</td>
<td>Forward</td>
<td>NheI</td>
</tr>
<tr>
<td>5’ TGCTTGAATTCATCACTACTAGTGGTCTC 3’</td>
<td></td>
<td>62.2</td>
<td>Reverse</td>
<td>EcoRI</td>
</tr>
<tr>
<td>5’ TAAGCAGATACGGCCCTCTCCCTCC 3’</td>
<td>IRES</td>
<td>70.9</td>
<td>Forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>5’ TGTTATCTAGATCATGGTGGTGGCCATA 3’</td>
<td></td>
<td>66.9</td>
<td>Reverse</td>
<td>XbaI</td>
</tr>
<tr>
<td>5’ TAAGGCACCCGGGAATCATCTGGCCAGGGCG 3’</td>
<td>Tat</td>
<td>73.6</td>
<td>Forward</td>
<td>XmaI</td>
</tr>
<tr>
<td>5’ TGCTTATCTAGAAGGGGACCATGGAGACC 3’</td>
<td></td>
<td>69.5</td>
<td>Reverse</td>
<td>XbaI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Target</th>
<th>Tm</th>
<th>Direction</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ TGCTTAGCTAGCCACCATGGGAGAGACC 3’</td>
<td>Rl-Int</td>
<td>70.9</td>
<td>Reverse</td>
<td>NheI</td>
</tr>
<tr>
<td>5’ AAGCAGAATTTCTTAGGGCCACCTCTCTC 3’</td>
<td></td>
<td>63.2</td>
<td>Forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>5’ TAAGCAGATACGGCCCTCTCCCTCC 3’</td>
<td>IRES</td>
<td>70.9</td>
<td>Forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>5’ TGCTTATCTAGATCACCATGGTGGTGGCCATA 3’</td>
<td></td>
<td>66.9</td>
<td>Reverse</td>
<td>XbaI</td>
</tr>
<tr>
<td>5’ TAAGCATCTAGACCATGGAGAGGAGAAGGAG 3’</td>
<td>Vif</td>
<td>68.2</td>
<td>Forward</td>
<td>XbaI</td>
</tr>
<tr>
<td>5’ TGCTTACCAGGGAATGAGGACGATGC 3’</td>
<td></td>
<td>70.9</td>
<td>Reverse</td>
<td>XmaI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Target</th>
<th>Tm</th>
<th>Direction</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ TAAGCAGCAGCTAGCCACCATGGGCGTGAGGA 3’</td>
<td>VSV</td>
<td>66.8</td>
<td>Forward</td>
<td>XhoI</td>
</tr>
<tr>
<td>5’ TGCTTAGCTAGCCACCATGGGAGAGACC 3’</td>
<td></td>
<td>68.1</td>
<td>Reverse</td>
<td>NheI</td>
</tr>
<tr>
<td>5’ TAAGCAGAATTTCTTAGGGCCACCTCTCTC 3’</td>
<td>IRES</td>
<td>70.9</td>
<td>Forward</td>
<td>EcoRI</td>
</tr>
<tr>
<td>5’ TGCTTATCTAGATCACCATGGTGGTGGCCATA 3’</td>
<td></td>
<td>66.9</td>
<td>Reverse</td>
<td>XbaI</td>
</tr>
<tr>
<td>5’ TAAGCATCTAGACCATGGAGAGGAGAAGGAGA 3’</td>
<td>Rev</td>
<td>66.8</td>
<td>Forward</td>
<td>XbaI</td>
</tr>
<tr>
<td>5’ TGCTTACCAGGGAATCAGTCTGGGGGCTT 3’</td>
<td></td>
<td>72.2</td>
<td>Reverse</td>
<td>XmaI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Target</th>
<th>Tm</th>
<th>Direction</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ TAAGCATCTAGACGGCGGAGCAGTCGTCTGCGGGA 3’</td>
<td>IgG1 b12</td>
<td>73.3</td>
<td>Forward</td>
<td>XbaI</td>
</tr>
<tr>
<td>5’ TGCTTACCAGGGAATCAGTCTGGGGGCTT 3’</td>
<td></td>
<td>68.2</td>
<td>Reverse</td>
<td>Smal</td>
</tr>
</tbody>
</table>
3.4.3.5. Agarose gel electrophoresis and gel extraction

3.4.3.5.1. Agarose gel electrophoresis

PCR products and DNA digests were analysed by gel electrophoresis on the basis of their molecular weight. Fragment sizes were determined in relation to the bands of a mixture of standard DNA fragments (marker ladder) on the gel. DNA fragments in agarose gels were visualised with UV light using Invitrogen™ SYBR™ Safe DNA Gel Stain (ThermoFisher Scientific # S33102) or Gel Red (Biotium Inc., Hayward, USA) following manufacturer’s guidelines. 6x Gel Loading Dye was mixed with each DNA sample (NEB # B7024S). Either 1kb DNA ladder or 100 bp DNA ladder molecular weight standards (Figure 25; NEB #3232L, NEB # 3231L) were used for DNA band size approximation. The percentage of agarose used in preparing the gel was determined according to the expected band sizes. The percentage of agarose in preparing the gel was determined according to the expected band sizes (Table 5). UltraPure agarose (ThermoFisher Scientific # 16500500) was used in preparing the agarose gels. PCR products and DNA digests were analysed by gel electrophoresis on the basis of their molecular weight. Fragment sizes were determined in relation to the bands of a mixture of standard molecules (marker ladder) on the gel.

3.4.3.5.2. Purification of vector and insert by DNA extraction from agarose gels

Following gel electrophoresis of DNA from both vector and insert, bands of interests were excised and purified using the Monarch® DNA Gel Extraction Kit (NEB #T1020S). Following the manufacturer’s protocol, DNA fragments were excised using a scalpel (Disposable scalpel number 21, Ref # 0507. Swann-Morton, Sheffield, UK) after visualization under UV light transillumination (Syngene, Cambridge, UK). Dissolving buffer was added to the slice; DNA was then adsorbed on a silica membrane, and purified DNA eluted in the provided Elution Buffer.
3.4.3.6. Ligation of vector and insert

Ligation of DNA fragments was performed using Instant Sticky-end Ligase Master Mix (NEB #M0370S) containing a T4 DNA ligase. The ligation mix was prepared by combining 20–100 ng of vector with a 3 to 5 fold molar excess of insert, the volume was adjusted to 5µl with nuclease free water. 5µl of Instant Sticky-end Ligase Master Mix was added to the ligation mix.

In the case of blunt ends, ligation was made using the Quick Ligation Kit (NEB, M2200). The ligation mix was prepared by combining 50 ng of vector with 3 to 5 fold molar excess of insert DNA, the volume was then adjusted to 10 µL with nuclease-free water. 10 µL of 2X Quick Ligation Buffer was added together with 1 µL of Quick T4 DNA Ligase followed by thorough mixing. The reaction mix was then centrifuged briefly and incubated at room temperature (25°C) for 5 minutes.

The amount of vector and insert in the ligation mix was calculated using NEBBioCalculator (http://nebiocalculator.neb.com/#/main).

3.4.3.7. Transformation

Competent Cells were transformed with plasmid DNA according to the manufacturer’s instructions. Briefly 1-2.5 µl of plasmid DNA or ligation mix (50-100ng DNA) were suspended in 50µl competent cells and incubated on ice for 5 minutes. After a heat shock at 42°C for 30 seconds the cells were incubated on ice for two minutes. The competent cells were mixed with 250µl SOC medium (Super optimal broth medium, Hanahan, 1983) and incubated at 37°C for one hour at 300rpm. Following the one hour recovery step, the cells were plated (100µl and 150µl) on selective LB-agar plates with the respective antibiotic (ampicillin [100mg/ml] or kanamycin [25mg/ml]).
3.4.3.8. Sequencing

Sanger nucleotide sequencing was used for confirmation of each cloning experiment. Sequencing was performed using plasmid and gene specific primers (Table 18), spanning the region of plasmid and gene insertion. Big Dye Terminator v1.1 cycle sequencing reagents (Applied Biosystems) were used for the sequencing reactions, cleaned up using ethanol precipitation and placed on the ABI 3130 genetic analyser for sequence reading. Post sequencing analysis was performed using ReCALL (beta v3.03) software (http://pssm.cfenet.ubc.ca/account/login). Consensus sequences were aligned in SnapGene software (http://www.snapgene.com) to ensure gene insertion in the correct reading frame. The sequencing primers were designed manually following common primer designing guidelines and subsequently ordered from Eurofins/MWG Operon (Ebersberg, Germany).

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Target</th>
<th>Tm</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' GTTCAATTACAGCTCTTAAGGC 3'</td>
<td>General Backbone</td>
<td>56.5</td>
<td>Forward</td>
</tr>
<tr>
<td>5' CTCACATTGCCAAAAAGACGGCAA 3'</td>
<td>General IRES</td>
<td>60.6</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' CCGAACCGGGGACGTGGT 3'</td>
<td>General IRES</td>
<td>65.5</td>
<td>Forward</td>
</tr>
<tr>
<td>5' GTATCTTATCATGTCTGGC 3'</td>
<td>General Backbone</td>
<td>52.4</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' CCACAAGCCCTCCAACACC 3'</td>
<td>heavy chain</td>
<td>61.0</td>
<td>Forward</td>
</tr>
<tr>
<td>5' TTGCAGATGTAGGTCTGG 3'</td>
<td>heavy chain</td>
<td>53.7</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' TCGGCCGCTCCTCGTTGATGATGT 3'</td>
<td>GAG</td>
<td>67.9</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' GAGGCCGCGACTGGGACCTGCA 3'</td>
<td>GAG</td>
<td>71.3</td>
<td>Forward</td>
</tr>
<tr>
<td>5' GCCAGTAGTGTCCACCACCA 3'</td>
<td>RtInt</td>
<td>61.0</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' TGGGAGCAGTGGTGGACCGA 3'</td>
<td>RtInt</td>
<td>63.5</td>
<td>Forward</td>
</tr>
<tr>
<td>5' TGATGTCCATGGAAATAGGG 3'</td>
<td>VSV</td>
<td>55.3</td>
<td>Reverse</td>
</tr>
<tr>
<td>5' GGCTATGTGGATCTAACCTCA 3'</td>
<td>VSV</td>
<td>55.9</td>
<td>Forward</td>
</tr>
</tbody>
</table>
3.4.3.9. Recombinant BacMam plasmids construction

3.4.3.9.1. Study I: Expression under the T7 RNA Polymerase/Promoter system

Five different recombinant plasmids were constructed, DNA fragment (derived from plasmids) containing the gene(s) of interest together with excess DNA were cloned into pOET6. Successful clones were subjected to a further restriction digest step were excess DNA was removed. Overhang ends were blunted using a Quick Blunting Kit (NEB #E1201) and then ligated to form the desired BacMam pOET6 vector (Figure 56).

Figure 56: Cloning method for the first study utilising T7 promoter
3.4.3.9.2. Study II: BacMam expression utilising a CMV promoter

Four different recombinant plasmids were constructed; primers with appropriate restriction sites were used to direct a uni-directional clone into BacMam pOET6 vector. PCR products were purified by running the DNA on an agarose gel either by excising the band or by using a spin column (NEB #T1020S) to purify the PCR mix directly, they were then digested with the appropriate restriction enzyme.

![Diagram](image_url)

**Figure 57:** Cloning method for the second study utilising CMV promoter

3.4.3.10. Generation of recombinant baculoviruses

Either BacMagic-3 DNA kit (Merck Millipore #72350) or flashBAC ULTRA (Oxford Expression Technologies #100304) was used in generating all rBVs. Insect SF9 cells (provided by Dr E. McKenzie, University of Manchester) were used for BV multiplication and rBV generation. Cells were grown in Grace’s medium supplemented with 10% heat inactivated fetal calf serum (FCS) (Life Technologies). Sf9 cells were cultured in Grace’s medium until 80% confluent. The cells were then harvested and seeded into 6-well plates (1x10^6 cells/well) and incubated at 28° C. Recombinant transfer vectors were co-transfected with linearised baculovirus DNA into Sf9 cells using ESCORT IV transfection reagent (Sigma-Aldrich #L3287) according to the manufacturer’s
instructions. The supernatant was harvested after one week and then amplified into a 50mL high titre stock.

3.4.3.11. Quantitative PCR (qPCR) titration

Initial titration of recombinant viruses was performed by Oxford Expression Technologies Ltd. (Oxford, UK), using their baculoQUANT quantitative PCR (QPCR) titration service. All further recombinant viruses were quantified using a real-time PCR assay, run in-house by a laboratory colleague Mr Ben Brown. The real-time PCR assay used previously published primers and probes (Hitchman et al. 2007), in combination with Taqman™ Fast Universal PCR master mix (Thermofisher Scientific, UK) and run on the StepOne™ real-time PCR system (Thermofisher Scientific, UK). A baculovirus standard curve calibrated in pfu/mL was included in each run, and all recombinant viruses were run in duplicate, with the mean average calculated to give the quantity for each virus.

3.4.3.12. Transduction of mammalian cells

293 human embryo kidney (HEK) cells (Public Health England, Porton Down, Salisbury, UK # 85120602), Human foetal lung fibroblast (MRC-5) cells (Public Health England, Porton Down, Salisbury, UK # 05090501), HeLa (ECACC, Public Health England, Porton Down, Salisbury, UK # 93021013) and African green monkey kidney cell line (Vero) cells (Public Health England, Porton Down, Salisbury, UK # 84113001) were cultured either in minimum essential media (MEM) or Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% FCS and 2mM glutamine. For transduction, mammalian cells were seeded onto 6-well plates, T75, or T175 flasks at a density of 5 x 10⁴ cells/cm². After 24 hours, cells at approximately 70% confluence were washed with (Phosphate buffer saline) PBS and transduced with transduction solution composed of non-concentrated virus and PBS as a surrounding solution in a ratio of 1:4 (Figure 58). In the first study, dual transduction was carried out each time with BacMam T7 being a fixed component. In the second study, cells were transduced with
either single recombinant BacMam or with all recombinant viruses combined. Cells were incubated at 27°C for 4-6 hours with mild shaking. Transduction solution was then replaced with culture medium containing 3 mM sodium butyrate (Sigma-Aldrich # B5887) as a histone deacetylase (HDAC) inhibitor. After 24 hours of incubation at 37°C, the medium was replaced with fresh medium. Cultures were examined for GFP expression using fluorescence microscopy. 72 hours post transduction, cells were collected for analysis.

**Figure 58: Transduction of mammalian cells with baculovirus.**

### 3.4.3.13. Immunohistochemistry

Vero and MRC-5 cells were seeded in 6-well plate at a density of 5 x 10^4 cells/cm². 24 hours post seeding, cells at approximately 70% confluence were transduced with dual BacMam viruses bearing BacMam T7 as a fixed component. Immunohistochemistry (IHC) was conducted for the first study only. At 24 hours after transduction, cells were washed and fixed in absolute ethanol for an hour at room temperature. Cells then washed with water for 5 minutes and immersed in 0.5% hydrogen peroxide in methanol for 30 minutes, followed by three times washing step each for 2 minutes in Tris-buffered saline (TBS). Cells were incubated with either anti-gp64 antibody (AcV5) or a specific primary antibody targeting the protein of interest for 30 minutes at room temperature (Table 19). Cells were washed thoroughly with TBS and incubated with
secondary antibody corresponding with its primary antibody for 30 minutes at room temperature, a TBS wash was then used to stop the reaction. VECTOR NovaRED Peroxidase (HRP) Substrate Kit (Vector Laboratories # SK4800) was used. NovaRED reagents were added to each well until faint red-brown colour developed. Cells were washed with distilled water before examined under the microscope.

3.4.3.14. Western Blot Analysis

3.4.3.14.1. Protein lysis

72h post transduction, uninfected (negative control) and infected cell monolayers were washed twice with cold 1X PBS and were then harvested using a 25 cm cell scraper, and pelleted by centrifugation at 700g for 5 minutes at 4°C. The supernatant was discarded and the cell pellet of each sample was re-suspended in 500 μl of lysis buffer [150 mM NaCl (Fisher Scientific #S/3160/6); 25 mM Tris-HCl (Formedium #TRIS01); 1% Triton-X-100 (Sigma-Aldrich #X100); Proteinase Inhibitor Cocktail 1:5000 (Thermo-Scientific #78425); Benzonase 1:10,000 (Novagen #70746); 5 mM Imidazole (Sigma Life Science #I0125-1KG); pH 8.0]. The cell lysate was incubated on ice for 30 minutes with pipetting up and down ten times every 10 minutes. Subsequently the lysate was clarified by centrifugation at 15,000g for 30 minutes at 4°C. The resultant lysate (500μl) containing the soluble protein was carefully transferred into a fresh 1.5 mL Eppendorf tube and used either immediately or stored at -80 Cº for further use.

3.4.3.14.2. Protein-electrophoresis

For the separation of proteins according to their size, 4-20% Mini-Protean TGX stain free gels (Bio-Rad, cat #456-8093) were used, according to the manufacturers’ guidelines. Cell lysate and culture supernatant were run for protein detection. Before loading the gel, samples were mixed in 1:1 ratio with loading buffer [62.5 mM Tris buffer (pH 6.8) (Formedium #TRIS01); 2% SDS (Fisher Scientific #1261-1680); 25% Glycerol (Sigma-Aldrich #G5516); 0.01% Bromophenol blue (Sigma-Aldrich #318744)]. Samples were denatured for 5 minutes at 95°C. After a brief pulse centrifugation, 15 μL
of each sample were loaded into each well. 10 μL of ColorPlus Prestained Protein Ladder, Broad Range (NEB #P7712S) (Figure 59) was used as marker.

![ColorPlus™ Prestained Protein Ladder, Broad Range (10-230 kDa)](image)

Figure 59: ColorPlus™ Prestained Protein Ladder, Broad Range (10-230 kDa)

Tris-glycine-SDS-buffer [25 mM Tris Base Ultra-Pure (Formedium #TRIS01); 192 mM Glycine (Fisher Scientific #G/0800/60); 0.1% Sodium Dodecyl Sulphate (Fisher Scientific #1261-1680)] was used as running buffer. A standard protein-gel was run at 200 V and 150 W for 40 minutes. Gels were then used for Coomassie blue staining (Instant Blue protein stain, Expedeon #ISB1L) or Western blot analysis.

### 3.4.3.14.3. **Protein Blotting**

For Western blot analysis the Trans-Blot-Turbo transfer system (Bio-Rad, Hertfordshire, UK) with Trans-Blot Turbo transfer packs (Midi format, 0.2 μm PVDF, Bio-Rad #170-4157) was used according to the manufacturers’ instructions. Blotting was conducted in a Trans-Blot-Turbo transfer starter system blotter. The membrane was subsequently blocked with 5% semi-skimmed milk (Dried skimmed milk powder, Marvel, Premier Foods Group Ltd., London, UK, in 0.2% Phosphate buffer saline with Tween 20 (PBS-T)) for one hour. Primary antibody of choice was added (1:1,000 dilution) and incubated overnight at 4°C with gentle agitation (Table 19). The following day, the membrane was washed 3x5 minutes with 0.2% PBS-T. Combatable secondary antibody was added in a 1:3000 dilution and incubated at room temperature
for one hour under gentle agitation. Following 4x5 minutes washing steps (3x 0.2% PBS-T, 1xPBS buffer), the membrane was covered in enhanced chemiluminescent-reagent (Amersham ECL Western Blotting Detection Reagent, GE Healthcare #RPN2106) for 5 minutes and then exposed in a chemiluminescence imaging platform (Syngene, GeneGnome). To predict the location of a protein in relation to a molecular weight standard on a gel or a blot, the size of each protein was estimated from the protein sequence using SnapGene (Table 20).

Table 19: List of primary and secondary antibodies

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Provider</th>
<th>Ref no</th>
<th>Host</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-gp64 antibody [AcV5]</td>
<td>Abcam</td>
<td>Ab91214</td>
<td>Mouse</td>
<td>1/1000</td>
</tr>
<tr>
<td>Monoclonal antibody to SIV gag p27</td>
<td>NIBSC</td>
<td>ARP396/397</td>
<td>Mouse</td>
<td>1/1000</td>
</tr>
<tr>
<td>Rabbit anti-SIVmac tat</td>
<td>NIBSC</td>
<td>ARP4006</td>
<td>Rabbit</td>
<td>1/1600</td>
</tr>
<tr>
<td>Anti-Pol (SIVmac239)</td>
<td>Immune Technology</td>
<td>IT-001-025</td>
<td>Rabbit</td>
<td>1/1000</td>
</tr>
<tr>
<td>Anti-VSV-G tag antibody (Biotin)</td>
<td>Abcam</td>
<td>Ab34774</td>
<td>Rabbit</td>
<td>1/500 - 1/2000</td>
</tr>
<tr>
<td>Rabbit Anti-Human IgG H&amp;L (HRP)</td>
<td>Abcam</td>
<td>Ab6759</td>
<td>Rabbit</td>
<td>1/5000 - 1/25000</td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit Anti-Mouse IgG H&amp;L (HRP)</td>
<td>Abcam</td>
<td>Ab6728</td>
<td>Rabbit</td>
<td>1/3000</td>
</tr>
<tr>
<td>Goat Anti-Rabbit IgG, Horseradish peroxidase (HRP) Conjugate</td>
<td>Novex (life technologies™)</td>
<td>A16096</td>
<td>Goat</td>
<td>1/3000</td>
</tr>
</tbody>
</table>

Table 20: Protein sizes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag</td>
<td>57 kDa</td>
</tr>
<tr>
<td>Tat</td>
<td>14.6 kDa</td>
</tr>
<tr>
<td>VSV-G</td>
<td>57.5 kDa</td>
</tr>
<tr>
<td>Rev</td>
<td>12.6 kDa</td>
</tr>
<tr>
<td>Vpr</td>
<td>11.4 kDa</td>
</tr>
<tr>
<td>Pol (RtInt)</td>
<td>98.2 kDa</td>
</tr>
<tr>
<td>Vif</td>
<td>25.3 kDa</td>
</tr>
<tr>
<td>IgG</td>
<td>Light chain</td>
</tr>
<tr>
<td></td>
<td>26 kDa</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>52.7 kDa</td>
</tr>
<tr>
<td>T7</td>
<td>98.9 kDa</td>
</tr>
</tbody>
</table>
3.4.3.15. Ultracentrifugation and Transmission Electron Microscopy (TEM)

In the second study, the supernatant from 72 hours post transduction HEK239 cell culture was collected and centrifuged at 700g for 5 minutes at 4°C. The supernatant was collected and subjected to ultracentrifugation. Beckman Ultra-Clear centrifuge tubes (Cat # 344057) were sterilised for 15 minutes by exposure to UV light in a biological safety cabinet. A total of 30 mL viral supernatant was centrifuged at 90,000 g for 90 minutes at 4°C in a Beckman SW50.1 swinging bucket rotor lined with a Beckman Ultra-Clear centrifuge tube. To obtain accurate balanced volumes in each tube, supernatant was loaded while measuring on a weighing balance to ensure that all tubes would be in similar final volumes. Following centrifugation, the medium was carefully disposed of into a bleach-filled container. Pellets were gently resuspended in 200µL of PBS by pipetting 20 times using a P200 pipette, care being taken to minimise the creation of foam. The sample was then sent to the Electron Microscopy Facility, University of Manchester. The sample was incubated for 1 minute on carbon coated 400 Mesh Grids Copper (Agar scientific # AGS160-4). This was followed by washing 5x in distilled water to remove phosphate ions, then negatively stained using 1% uranyl acetate dissolved in distilled water (pH 4.2 to 4.5) for 30 second followed by an air drying step. The negatively stained specimen was examined using a 20-120 kV FEI Tecnai G2 Spirit BioTWIN Transmission Electron Microscope. Micrographs were prepared using an Orius SC1000 CCD camera system.
3.5. Results

3.5.1. Design and construction of BacMam transfer plasmids

3.5.1.1. Study I: T7 RNA Polymerase/Promoter system

3.5.1.1.1. pLF_T7_RNA-Polymerase

A summary of the cloning procedure is shown in Figure 60. DNA plasmid extracted from randomly selected colonies of transformed E.coli was subjected to restriction digestion to screen for positive clones (Figure 61, Figure 62 and Figure 63). Insert positive clones were confirmed by sequencing.

![Diagram](image-url)

**Figure 60: Schematic representation of pLF_T7_RNA-Polymerase cloning steps.** A fragment size 4562 bp containing T7 polymerase was excised from pT7-Vpx_MVA and inserted into pOET6 between the Acc65I and NotI sites. The resultant plasmid was then cleaned from excess DNA flanking the T7 polymerase cassette by cutting one end with Acc65I and BstBI, blunting and ligation. The second end was cut with Ascl and NotI, which was then blunted and ligated to form pT7 RNA polymerase. The T7 polymerase cassette was placed downstream of the CMV promoter.
Figure 61: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_RNA-Polymerase first step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were FseI and SwaI. Fragment sizes were 1869bp and 9130bp. All colonies are positive.

Figure 62: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_RNA-Polymerase second step. M is a 1kb DNA ladder. C is colony. Restriction enzyme used is Scal. Fragments sizes 3193bp and 6560bp. All colonies are positive.

Figure 63: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_RNA-Polymerase final step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used are FseI and Pmel. Fragment sizes were 2551bp and 6598bp. All colonies are positive.
3.5.1.1.2. \textit{pLF\_T7\_IgGb12-GFP}

A summary of the cloning procedure is shown in Figure 64. Plasmid DNA was extracted from randomly selected colonies and was subjected to restriction digestion (Figure 65 and Figure 66). Positive clones were confirmed by sequencing.

\textbf{Figure 64: Schematic representation of pLF\_T7\_IgGb12-GFP cloning steps.} The IgGb12 expression cassette with fragment size 6306bp encoding EGFP was removed from pIgGb12-GFP\_MVA and inserted into pOET6 between the \textit{XbaI} and \textit{HpaI} sites. The restriction fragment between \textit{PacI} and \textit{BglII} was deleted and overhanging sequences were filled-in/removed by blunt end modification to form the recombinant transfer vector pLF\_T7\_IgGb12-GFP.
Figure 65: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_IgGb12-GFP first step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were XbaI and HpaI. Fragment sizes were 7687bp, 2794bp, 2329bp and 399bp. Colony 4 (C4) is positive.

Figure 66: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_IgGb12-GFP second step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were SpeI and ScaI. Fragment sizes were 8199bp and 2656bp. Colony 1 (C1) is positive.
3.5.1.1.3. *pLF_T7_Rev-VSV-G*

A summary of the cloning is illustrated in Figure 67. Plasmid DNA was extracted from randomly selected colonies and was subjected to restriction digestion (Figure 68 and Figure 69). Positive clones were confirmed by sequencing.

![Diagram](image)

**Figure 67: Schematic representation of pLF_T7_Rev-VSV-G cloning steps.** A fragment size of 2785 bp containing VSV-G Rev was removed from pRev-VSV-G_MVA and inserted into pOET6 between BglII and NotI sites. Excess DNA was removed by cutting with SphI and Ascl to blunt and then ligate to form the recombinant transfer vector pLF_T7_Rev-VSV-G.
Figure 68: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_Rev-VSV-G first step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were Pmel and Ascl. Fragment sizes were 5805bp and 2304bp. All colonies are positive.

Figure 69: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_Rev-VSV-G second step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were Spel and NcoI. Fragment sizes were 6104bp and 1162bp. All colonies are positive.
### 3.5.1.4. pLF_T7_Gag_Tat

A summary of the cloning is shown in Figure 70. Plasmid DNA was extracted from randomly selected colonies and subjected to restriction digestion (Figure 71, Figure 72, Figure 73). Positive clones were confirmed by sequencing.

**Figure 70: Schematic representation of pLF_T7_Gag_Tat cloning steps.** A fragment of 3137 bp containing Gag-Tat from pGag-Tat_MVA with AsiSI and NotI ends was inserted into pOET6 between the AsiSI and NotI sites. The plasmid was then cleaned from excess DNA flanking the Gag-Tat cassette by cutting one end with SphI and Ascl, blunting and then ligation. The second end was cut with AsiSI and BglIII, which was blunted and ligated to generate transfer vector pLF_T7_Gag_Tat.
Figure 71: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_Gag_Tat first step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were Pmel and Ascl. Fragment sizes were 6816bp and 2314bp. All colonies are positive.

Figure 72: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_Gag_Tat second step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were BamHI and NsiI. Fragment sizes were 6520bp and 1753bp. Colony 3 (C3) is positive.

Figure 73: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_Gag_Tat final step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were Scal and KpnI. Fragment sizes were 4897bp and 2711bp. Colony 3 (C3) is positive.
3.5.1.1.5. \textit{pLF\textsubscript{T7}_Pol-Vpr-Vif}

A summary of the cloning is illustrated in Figure 74. Plasmid DNA was extracted from randomly selected colonies and subjected to restriction digestion (Figure 75, Figure 76, Figure 77). Positive clones were confirmed by sequencing.

\textbf{Figure 74: Schematic representation of pLF\textsubscript{T7}_Pol-Vpr-Vif cloning steps.} A fragment of size 4487 bp containing Pol-Vpr-Vif genes from pPol-Vpr-Vif\textsubscript{MVA} with \textit{AsiSI} and \textit{NotI} ends were inserted individually into pOET6 between the \textit{AsiSI} and \textit{NotI} sites. The plasmid was then cleaned from excess DNA flanking Pol-Vpr-Vif cassette by cutting one end with \textit{HpaI} and \textit{AscI}, blunting and ligation. The second end was cut with \textit{SpeI} and \textit{AsiSI}, which was blunted and ligated to generate transfer vector pLF\textsubscript{T7}_Pol-Vpr-Vif.
Figure 75: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_Pol-Vpr-Vif first step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were NsiI and BmaHI. Fragment sizes were 7573bp and 2907bp. Colonies 3, 4 and 5 (C3, C4 and C5) are positive.

Figure 76: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_Pol-Vpr-Vif second step. M is a 1kb DNA ladder. C is colony. Restriction enzyme used was SphI. Fragment sizes were 8422bp and 1300bp. All colonies are positive.

Figure 77: 1% agarose gel of DNA restriction digests product cloning of pLF_T7_Pol-Vpr-Vif final step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were NsiI and BamHI. Fragment sizes were 6815bp and 2399bp. Colonies 2 and 4 (C2 and C4) are positive.
3.5.1.2. Study II, CMV promoter

3.5.1.2.1. pLF_CMV-Gag-IRES-Tat, pLF_CMV-RtInt-IRES-Vif and pLF_CMV-VSV-IRES-Rev

To form the pLF_CMV-Gag-IRES-Tat, pLF_CMV-RtInt-IRES-Vif and pLF_CMV-VSV-IRES-Rev constructs, 3 different genes of interest were inserted into each plasmid. NheI and EcoRI was used to insert Gag, Rt-Int separately into 2 different pOET6; while NheI and XhoI used to insert VSV-G into a 3rd different pOET6. EcoRI and XbaI was used to insert IRES in each of the resulting plasmids. Finally, XmaI and XbaI were used to insert Tat, Vif and Rev into each plasmid. A summary of the cloning results is shown in Figure 78. Plasmid DNA was extracted from randomly selected colonies and subjected to restriction digestion (Figure 79). Positive clones were confirmed by sequencing.

Figure 78: Schematic representation of pLF_CMV-Gag-IRES-Tat, pLF_CMV-RtInt-IRES-Vif and pLF_CMV-VSV-IRES-Rev cloning steps.
Figure 79: 1% agarose gels of DNA restriction digests product cloning of pLF_CMV-Gag-IRES-Tat, pLF_CMV-Rtint-IRES-Vif and pLF_CMV-VSV-IRES-Rev. M is a 1kb DNA ladder. C is colony. pLF_CMV-Gag-IRES-Tat digested with HindIII fragment sizes 6870bp and 2115bp. pLF_CMV-VSV-IRES-Rev digested with Acc65I fragment sizes were 7927bp and 985bp. pLF_CMV-Rtint-IRES-Vif was digested with AsiSI and BamHI and fragment sizes were 8597bp and 1995bp.

3.5.1.2.2. pLF_CMV-IgG1b12

Results from the first BacMam study indicated that the BacMamT7-IgG1b12 failed to express IgG, further investigation revealed that the IgG1b12 expression cassette contained critical mistakes.

Figure 80: Highlights of the inaccuracy in the pLF_T7_IgG1b12

1) ECMV IRES Missing 22bp + Contain an extra ATG
2) HTLV-1 IRES Is not an actual IRES
Sequencing analysis showed that the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) inserted between the light and heavy chain was missing 22bp and contained an extra ATG (Figure 80). This was likely to effect IRES activity particularly in bicistronic construct.

Transfer plasmid contains the correct EMCV IRES sequence were designed and manufactured by Eurofin Genomics, Germany. Unfortunately Eurofin couldn’t generate the plasmid in one piece. The plasmid was prepared as Fragment A and B and then re-assembled. Several stages were needed to join Fragment A and B together. First, Fragment A was cloned into pOET6 between the EcoRI and Nhel restriction sites Nhel is located in two locations one of which is directly upstream of EcoRI. Fragment A was excised by cutting with EcoRI first then with Nhel to eliminate the second Nhel (Figure 81).

To confirm cloning, plasmid DNA was extracted from randomly selected colonies and subjected to restriction digestion (Figure 82). Second, primers were designed to introduce appropriate restriction sites to clone fragment B into pOET6+Fragment A between EcoRI and XmaI (Figure 83). To confirm cloning, plasmid DNA was extracted from randomly selected colonies and was subjected to restriction digestion (Figure 84). Finally, the resulting plasmid pOET6+Fragment A+B contained a gap between fragment A and B, which would interfere with protein expression. To correct this, the fragment between XhoI was replaced with the correct one from pIgG+P2A (Figure 87). plgG+P2A was constructed by replacing a fragment located between AflII and BstAPI. This fragment was excised from pCorrect_seq1 and cloned into the pOET6-IgG12 vector (Figure 85). To confirm cloning, plasmid DNA was extracted from randomly selected colonies and was subjected to restriction digestion (Figure 86 and Figure 88).
Figure 81: Schematic representation of pOET6+A. (A) The sequence and minimum energy structure of the EMCV IRES, highlight motifs of interest for bicistronic protein expression. Adapted from (Bochkov and Palmenberg, 2006). (B) EcoRI and Nhel position within fragment A plasmid. (C) pFragmentA. (D) pOET6+FragmentA.
Figure 82: 1% agarose gel of DNA restriction digest product cloning of pLF_CMV-IgG1b12 first step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were Acc65I and BglII. Fragment sizes were 5347bp, 2359bp and 1237bp. All colonies are positive.

Figure 83: Schematic representation of (A) fragment B (B) pOET6+A+B.

Figure 84: 1% agarose gel of DNA restriction digest product cloning of pLF_CMV-IgG1b12 second step. M is a 1kb DNA ladder. C is colony. Restriction enzymes used were HindIII and NotI. Fragment sizes were 6085bp, 3901bp, 1087bp and 735bp. Colonies 2, 4, 5 and 6 (C2, C4, C5 and C6) are positive.
Figure 85: Schematic representation of (A) fragment located between AflIII and BstAPI from plgG+P2A (B) pLF_T7_IgG1b12 (C) plgG+P2A.

Figure 86: 1% agarose gel of DNA restriction digest product of plgG+P2A. M is a 1kb DNA ladder. C is colony. Restriction enzymes used was Xhol. Fragment sizes were 7364bp and 2978bp.
Figure 87: Schematic representation of the final step of pLF_CMV-IgG1b12 cloning.

<table>
<thead>
<tr>
<th>Step 4</th>
<th>bp</th>
<th>M</th>
<th>C1</th>
<th>C2</th>
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<th>C7</th>
<th>C8</th>
<th>C9</th>
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Figure 88: 1% agarose gel of DNA restriction digest product cloning of pLF_CMV-IgG1b12 final step. M is a 1kb DNA ladder. C is colony. Restriction enzyme used was SacI. Fragment sizes were 10352bp and 1423bp. Colonies 7, 8, 9, 10 and 12 (C7, C8, C9, C10 and C12) are positive.
3.5.2. *Generation of recombinant BacMam viruses*

Recombinant baculoviruses were generated by co-transfection of insect cells with the triple-cut linear BacPAK6 virus DNA (BacMagic™-3 DNA Kit or flashBAC™ULTRA) and transfer plasmids containing expression cassettes. Homologous recombination within the insect cells restores ORF 1629 allowing the recombinant virus to replicate, removes the BAC sequences and inserts the foreign gene under control of the promoter chosen in the transfer plasmid. Re-circularised DNA replicates to produce recombinant budded virus that is harvested from the co-transfection medium and becomes the seed stock (P1) of recombinant virus.

![Image](image.png)

*Figure 89: Sf9 cells visualised under the microscope 6 days after infection with BacMam pOET6_T7. Extensive cytopathic effect (CPE), of granular cells with enlarged nuclei is evident and provides evidence of successful infection (X40).*
3.5.3. Quantitative (qPCR) titration

Table below lists the titration results for both the first and second study.

Table 21: Titration results

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titre</th>
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<tbody>
<tr>
<td><strong>First Study</strong></td>
<td></td>
</tr>
<tr>
<td>BacMam T7_IgGb12-GFP</td>
<td>1.16x10^8 qpfu/ml</td>
</tr>
<tr>
<td>BacMam T7_RNA-Polymerase</td>
<td>1.06x10^8 qpfu/ml</td>
</tr>
<tr>
<td>BacMam T7_Gag_Tat</td>
<td>1.1x10^8 qpfu/ml</td>
</tr>
<tr>
<td>BacMam T7_Rev-VSV-G</td>
<td>9.9x10^7 qpfu/ml</td>
</tr>
<tr>
<td>BacMam T7_Pol-Vpr-Vif</td>
<td>1.2x10^8 qpfu/ml</td>
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<tr>
<td><strong>Second Study</strong></td>
<td></td>
</tr>
<tr>
<td>BacMam CMV-gGb12-GFP</td>
<td>5.7x10^7 qpfu/ml</td>
</tr>
<tr>
<td>BacMam CMV-Gag_Tat</td>
<td>1.17x10^8 qpfu/ml</td>
</tr>
<tr>
<td>BacMam CMV-Rev-VSV-G</td>
<td>8.7x10^7 qpfu/ml</td>
</tr>
<tr>
<td>BacMam CMV-Pol-Vpr-Vif</td>
<td>1.15x10^8 qpfu/ml</td>
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3.5.4. Immunohistochemistry

3.5.4.1. Study I

MRC5 and HEK cells were transduced with either wild type BV or by dual transduction with recombinant BacMam bearing BacMam T7_RNA-Polymerase as a fixed component. Brown colouration (positive) inside the cells was observed in all cells when anti-gp64 antibody was used (Figure 90 and Figure 91). However, when specific antibodies detecting targeted proteins, i.e. Pol, Gag and VSV, were used, colour changes were not detected. This suggests that BV had entered the cells but protein expression did not occur. Staining was made 48 hours post-transduction and the infection rate was 1:4.
Figure 90: Immunohistochemistry results for the first study, MRC5 cells. Anti-gp64 antibody was used as the primary antibody. Brown colouration (positive) inside the cells was observed in all transduced cells confirming BV entry. Negative uninfected cells shows no colour changes.
Figure 91: Immunohistochemistry results for the first study, HEK cells. Anti-gp64 antibody was used as the primary antibody. Brown colouration (positive) inside the cells was observed in all transduced cells confirming BV entry.

### 3.5.4.2. Study II

MRC5 cells were transduced with either wild type BV or with BacMam CMV-Gag_Tat. Brown colouration (positive) inside the cells was observed in all cells when anti-gp64 antibody was used (Figure 92). However, when specific antibodies detecting Gag were used colour changes were only observed in cells transduced with recombinant virus. This suggests that BV entered cells and expressed protein of interest. Staining was made after 48 hours and infection rate was 1:4.
Figure 92: Immunohistochemistry results for the second study, MRC5 cells. Brown colouration (positive) inside the cells was observed in all transduced cells when anti-gp64 antibody was used as primary antibody. However, when specific antibodies detecting Gag were used colour changes were only observed in cells transduced with recombinant virus. This suggests that BV entered cells and expressed protein of interest (X40). Negative uninfected cells shows no colour changes.
3.5.5. Green-fluorescent protein (GFP) expression

Green-fluorescent protein (GFP) was incorporated within the expression cassette of IgG1b12. GFP expression was used to study expression efficiency. The IgG1b12 expression cassette combines the expression of heavy and light chains in a single cassette together with GFP in a tri-cistronic setup. EMCV IRES elements were used to combine the reporter gene GFP with antibody sequence. GFP expression proves the activity of the EMCV IRES integrated into all recombinant BacMam constructs.

Monolayers of VERO and HeLa cells were co-transfected with BacMam CMV-gGb12-GFP. 1-3 days after transfection, GFP expression was visualised under an inverted fluorescent microscope (Optika-XDS-3FL). Green fluorescent signal was not clearly confirmed as observation of all transfected cells shows very week fluorescent signals (Figure 93). Before transfection, all cells showed negative green fluorescent signals.

Figure 93: Transfection of mammalian cells with BacMam pOET6_IgGb12-GFP recombinant plasmid. (A) Vero cells, Infection rate 1:4, 24 hour post transfection. (B) Hela cells, Infection rate 1:4, 24 hour post transfection.
3.5.6. Western Blot

3.5.6.1. Study I

To confirm the expression of the various recombinant proteins, western blots were performed on cell extracts from HEK cells transduced with dual infection and recombinant BacMam bearing BacMam T7_RNA-Polymerase as primary virus. No protein was detected in all cell extracts. SDS-PAGE analysis of cell extracts from HEK cells transduced with wild type virus and with dual transduction with recombinant BacMam, showed similar results (Figure 94). Results from both western blot and SDS-PAGE indicate the system failed to express the proteins of interest.

<table>
<thead>
<tr>
<th>BacMam T7_Rev- VSVG + BacMam T7_RNA-</th>
<th>BacMam T7_Gag_Tat + BacMam T7_RNA-</th>
<th>BacMam T7_Pol- Vpr-Vif + BacMam T7_RNA-</th>
<th>Wt Baculovirus</th>
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Figure 94: SDS-PAGE analysis of cell extracts from transduced HEK cells. M is marker. L is cell lysis (soluble). P is cell pellet (insoluble).

3.5.6.2. Study II

Western blots were performed on cell extracts from HEK cells transduced with recombinant BacMam virus. Cells were either transduced with a single recombinant BacMam or co-transduced with all 4 rBVs (BacMam CMV-gGb12-GFP, BacMam CMV-
Gag-Tat, BacMam CMV-Rev-VSV-G and BacMam CMV-Pol-Vpr-Vif). It was difficult to find antibodies targeting all of the genes of interest.

SIVmac239 Gag protein was analysed in HEK cells using specific monoclonal antibodies to SIV gag p27. The predicted size for the Gag protein, calculated from the deduced amino acid sequence, was 57kDa. Target Gag protein expressed from both individual and co-transduced cells (Figure 95 and Figure 96) demonstrate that BacMam CMV-Gag-Tat expressed Gag protein to a higher level when individually transduced. As expected, Gag was found in higher abundance in the supernatant.

As shown in Figure 96, BacMam CMV-Pol-Vpr-Vif expressed SIVmac239 Pol proteins in HEK cells. Targeted Pol proteins were detected using specific anti-Pol (SIV/mac239) polyclonal antibody. The predicted size for the Pol proteins, calculated from the deduced amino acid sequence, was 98.2kDa.

VSV glycoproteins were expressed successfully from BacMam CMV-Rev-VSV-G as Figure 96 shows a band of predicted size of 57.5kDa, the size calculated from the
deduced amino acid sequence. VSV-G was detected using specific Anti-VSV-G tag antibody.

<table>
<thead>
<tr>
<th>BacMam CMV-Rev- VSVG</th>
<th>BacMam CMV.Gag- Tat</th>
<th>BacMam CMV.Pol- Vpr-Vif</th>
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**Figure 96**: Demonstration of protein expressed from HEK cells. (A) Western blot results from HEK cells transduced with BacMam CMV-Rev-VSV-G, arrow point to VSV-G at 57.5kDa. (B) Western blot results from HEK cells transduced with BacMam CMV-Gag-Tat, arrow point to Gag at 57kDa. (C) Western blot results from HEK cells transduced with BacMam CMV-Pol-Vpr-Vif, arrow point to Gag at 98.2kDa.

**Figure 97**: Demonstration of IgG1b12 expressed from HEK cells. Western blot results from HEK cells transduced with BacMam CMV-gGb12-GFP or co-transduced with all 4 rBv, arrows point to heavy chains at 52.7kDa, light chains at 28kDa and total antibody at ~150kDa.

IgG1b12 expression was analysed in HEK cells using anti-Human IgG targeting both heavy and light chain. The antibody was expressed successfully from BacMam CMV-gGb12-GFP in both individually or co- transduced HEK cells. Western blot analysis was performed on reduced and denatured total IgG1b12 in 1X SDS loading buffer with 2-mercaptoethanol. Antibodies dissociated into heavy and light chains as results of the
reducing conditions. 2-mercaptoethanol causes reduction of disulphide bonds. Three bands appear in Figure 97 corresponding to the heavy chains (52.7kDa), light chains (26kDa) and total antibody which should be ~150kDa but migrate at lower mw and appears with size ~135kDa due to the effect of reduced condition.

3.5.7. Electron microscopy

VLP expression was analysed in HEK cells co-transduced with all 4 rBVs (BacMam CMV-IgGb12-GFP, BacMam CMV-Gag_Tat, BacMam CMV-Rev-VSV-G and BacMam CMV-Pol-Vpr-Vif). VLPs were successfully expressed and visualised under TEM. Figure 98 shows un-enveloped VLPs with variable sizes. It is believed that the VSV-G was lost during the ultracentrifugation step. The variability in the size of VLPs produced is suspected to be caused by the fragility of the VLPs. The background contains large amounts of protein again suggesting that VLP were degraded during the high-speed centrifugation.

Figure 98: TEM microphotographs of the VLP sample generated by BacMam system. (A) X30000, (B) X23000, (C) X18500 and (D) X23000. Figures show un-enveloped icosahedral VLPs. The background contains large amounts of protein resulted from degraded VLPs during high-speed centrifugation. However, these proteins might be unassembled Gag polyprotein or other polyproteins such as Tat, Pol, VSV-G, Vif, Rev and IgG1b12.
3.6. Discussion

The BacMam system combines the advantages of transient viral expression, ease of protein generation, and a broad cell tropism; enabling rapid, efficient and flexible protein expression in various mammalian cells. In this project a Baculovirus Retroviral Hybrid Vector was developed, in which BV was used to deliver SIV like particles into mammalian cells. The first BacMam system allowed expression of VLPs by transducing mammalian cells with five different rBVs bearing the T7 RNA polymerase system (BacMam-T7_Gag/Tat, BacMam-T7_Pol/Vif/Vpr, BacMam-T7_VSV-G/Rev, BacMam-T7_IgG1 b12 and BacMam-T7 RNA polymerase). The research hypothesis is illustrated in Figure 99.

![Figure 99: Schematic representation of the hybrid dual-vector system (baculovirus/retrovirus system) generating HIV-neutralising monoclonal antibody, utilising a T7 system. A retroviral vector transcription was integrated into the double-stranded DNA genome of rBVs. (A) In a first step, co-transduction of a packaging cell leads to transcription of the retroviral expression cassette. The transcripts serve as genomic RNA and are packaged by the nascent protein components constitutively produced by the packaging cells. (B) In a second step, the resulting retroviral vector particles infect target cells (neighbouring cells). Transduction results in the expression of the gene of interest (mAbs) from the integrated provirus under the control of the nuclear transcription signals.](image-url)
It was speculated that co-transduction of mammalian cells with the five different recombinants BacMam would result in the formation and the production of simian virus like particles. The VLPs were designed to encode HIV-neutralising monoclonal antibody sequences to function as a simian retroviral gene therapy agent. VLPs are attenuated and thus not fully replication-competent retroviruses. These virus particles were designed to be pseudo-typed with vesicular stomatitis virus glycoprotein (VSV-G). The VLPs infect neighbouring cells and insert IgG1b12 sequences into the cells genome. Neutralising antibody will then be produced from transduced cells.

To generate recombinant BacMam plasmid vectors, a BacMam pCMV-DEST vector was used. However, all cloning attempt failed to produce a recombinant vector. Investigation showed that the vector backbone contains ccdB. CcdB codes for the toxic protein (CcdB) that acts as a DNA gyrase poison, locking the DNA gyrase with broken double stranded DNA and ultimately causing cell death. Although cloning using restriction enzyme was possible, the choice of cloning for this plasmid is Gateway® technology in which insertion completely replaces CcdB with the investigator’s insert of interest. Traditional cloning failed to remove CcdB, thus cells could not propagate. A different plasmid was therefore considered. Baculovirus transfer vector pOET6 was used for construction of pT7_BacMam and to obtain rBVs carrying genes of interests under the control of the T7 promoter. Construction pT7_BacMam transfer plasmids using pOET6 was a challenge as all of genes of interests were from MVA work which used a different set of restriction enzymes. Expression cassettes were extracted from MVA transfer plasmids with extra sequences flanking the gene of interest. DNA fragment containing the gene of interest and excess DNA was cloned into pOET6. Successful clones were subjected to further restriction digests steps, out of frame DNA was removed forming the desired BacMam pOET6 vector (Figure 56). Generating BacMam transfer plasmids and construction of recombinant BacMam viruses were successful and confirmed by restriction digestion, sequencing and qPCR. However, protein expression was not detected with any of the 5 rBVs. HEK cells were transduced.
with wild type virus or with dual transduction with recombinant BacMam and BacMam-T7polymerase. SDS-PAGE analysis shows similar protein expression of recombinant BacMam and wild type baculovirus (wtBV). To confirm that virus entry was not the cause of lack of protein expression, transduced cells were subjected to IHC. To validate the result 2 different cell lines were used MRC5 and HEK cells. Both indicated that WT and rBVs entered the cells but protein expression failed to occur. Positive results were detected when anti-gp64 was used while negative results were reported when specific antibodies targeting the protein of interest were used. The transduction rate of both wtBV and BacMam viruses were similar and there were no noticeable differences between the densities of the peroxidase stain after treating the cells with anti-gp64. An immunofluorescence analysis from BacMam_IgG1b12 encoding eGFP was negative.

The prokaryotic T7 RNA polymerase transcription mechanism has been shown to be utilised for transient expression in eukaryotic cells. This was first demonstrated by using vaccinia virus (Fuerst et al. 1986) and later extended to systems comprising the expression of T7 RNA polymerase in avian and mammalian cells such as the recombinant fowlpox virus (Britton et al. 1996). A recombinant baculovirus expressing T7 RNA polymerase in insect cells has been constructed and offers an alternative for transient expression system (Poelwijk et al. 1995; Polkinghorne & Roy 1995). Depending on the cell type, different expression levels of T7 RNA polymerase were obtained even though the same MOI was employed (Yap et al. 1997). In the present work the results indicated that the T7 RNA polymerase system failed to express detectable levels of proteins. It is known that transcripts made by T7 RNA polymerase are mostly uncapped (Elroy-Stein et al. 1989), resulting in low efficiency of in vivo translation of the transcripts. Yap et al (1997) suggested that the susceptibility to BV infection and the efficacy of translation in different cells could be reasons for the discrepancy in expression levels. However, no significant change in the expression rate was detected when translation efficiency of the uncapped transcripts was improved.
using an IRES sequence of picornavirus. This is in contrast to the study reported by Poelwijk et al. (1995) on the CAT activity involving the translation of uncapped mRNA in insect cells. Low expression of a reporter gene was observed in insect cells using the hybrid BV–T7 system (Polkinghorne & Roy 1995). In our study we used HEK cells and MRC5 cells, both have a very high BV transduction efficiency and high protein expression rate. In agreement with previous studies, the hybrid BV–T7 system seemed to be an unreliable system of expression in mammalian cells. Baculovirus transcription occurs in the nucleus after transfection of an expression cassette. It was suggested that efficient transcription could be achieved when T7 RNA polymerase is functional in the nucleus. In addition, host cells were thought to provide a nuclear capping enzyme by which T7 transcripts can be modified leading to to higher translation efficiency (Lieber et al. 1989). However, this study together with the finding from Yap et al., 1997 confirm that no significant increase in protein expression was observed in cells infected with enzymatically active T7 RNA polymerase. The present results confirm that translation of the produced mRNA by T7 promoter is inefficient, most likely because of the absence of RNA 5’ cap structure. To overcome this hybrid BV–T7 system limitation, a different system was developed.

The second system developed in our study allows the production of VLPs by transducing mammalian cells with four different rBVs (BacMam-Gag/Tat, BacMam-Pol/Vif/Vpr, BacMam-VSV-G/Rev and BacMam-IgG1 b12), under the control of cytomegalovirus immediate early gene promoter. The research procedure is illustrated in Figure 100. pOET6 was used for construction of pCMV_BacMam to obtain rBVs carrying reporter genes under the control of CMV promoter. All genes of interest were re-cloned to be driven by the same promoter. Recombinant plasmids pLF_CMV-Gag- IRES-Tat, pLF_CMV-RtInt-IRES-Vif and pLF_CMV-VSV-IRES-Rev, were constructed using several cloning steps including PCR amplification and traditional restriction digest cloning.
Further analysis of the IgG1b12 expression cassette from the first study indicated that pLF_T7-IgGb12-GFP contained a faulty encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES), and the BacMamT7-IgG1b12 failed to express IgG (Figure 80). Following sequencing analysis it was found that the IRES inserted between the light and heavy chain lacked a 22bp sequence just before the first ATG and contained an extra 12th ATG at the end of the IRES sequence (Figure 80). This poor design affected the IRES activity particularly in bicistronic constructions. The data reiterates the importance of the native EMCV IRES configuration for protein optimum expression in bicistronic contexts.
The studies pointed to the involvement of secondary structures in the recognition of functional ribosome entry sites, and the importance of the distance between the polypyrimidine track and the initiating AUG sequence. In the case of the Encephalomyocarditis virus-derived IRES, the second AUG located 22 nucleotides downstream of the UUUCC sequence, present in the poly pyrimidine-rich track at the 3’ of the IRES, has been identified as the authentic viral initiation codon (Kaminski et al. 1994; Ohlmann & Jackson 1999; Jang & Wimmer 1990). The preferred IRES (viral bases 273-845) for optimum activity is illustrated in Figure 81-A (Bochkov & Palmenberg 2006). Virus mapping experiments have suggested that ribosome recognition may actually be enhanced by including the first few codons of the polyprotein ORF (López de Quinto & Martínez-Salas 1998). The extra length is not required for ribosome recognition, but it can provide a useful spacer between the A and B cistrons, allowing the IRES to fold without undue structural constraints. Placing the IRES in a position too close to the A cistron or immediately adjacent to a 5’cap is likely to fail or interfere with translation from the upstream ORF (Martin et al. 2006). Studies have demonstrated that the EMCV IRES predominant AUG (the 11th AUG) cannot be modified without strongly affecting the overall efficiency of the IRES-dependent expression (Martin et al. 2006). Placing the IRES immediately after the light chain sequence and including a 12th ATG in the original (faulty) design resulted into the formation of an attenuated IRES which was not ideal for maximal protein synthesis.

Including HTLV-1 IRES between the heavy chain and the eGFP in the IgG1b12 cassette was not optimum. An early study of the mechanism of translation initiation of HTLV-1 mRNA suggested the presence of an IRES within its 5’ UTR. This IRES was successfully used in the construction of tricistronic viral vectors (Olivares et al. 2014). However, when these data were re-examined, the existence of an IRES was dismissed, suggesting that translation initiation of the HTLV-1 mRNA was exclusively cap dependent. Stringent RNA and protein analyses determined the 5’ UTR of human T-cell leukemia virus type 1 (HTLV-1) exhibit a 5’ proximal post-transcriptional control
element (PCE) activity, but not IRES activity. Bolinger et al. 2007 show that the 5’ UTR of HTLV-1 does not support IRES activity in bicistronic RNA. Stringent bicistronic reporter assays using plasmid transfection, RNA transfection of synthetic RNAs, and RNA analysis identified a lack of IRES activity in the 5’ UTR of HTLV-1. Although, Attal J et al, 1996 and 2000 identified IRES-like activity from HTLV-1 R expressed adjacent to the SV40 early gene leader. Bolinger et al. 2007 identified that bicistronic reporter activity was detectable in the HTLV-1 UTR, but that this was attributable to heterogeneous F-luc transcripts, rather than authentic IRES activity. On the other hand, Olivares et al. 2014 contradicted these findings and suggested that the HTLV-1 mRNA harbours an IRES within its 5’UTR. However, the HTLV-1 IRES is dependent on ribosomal protein S25 for full activity. These studies failed to definitely indicate capacity of the HTLV-1 UTR to promote translation of the following coding sequences in capped monocistronic RNAs. To avoid issues in translation, a replacement IRES was used in the design.

Figure 101: Encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) sequence.
Transfer plasmid containing the corrected sequence was designed (Figure 102) and sent to Eurofin genomics, Germany, to be manufactured. Unfortunately, despite multiple attempts over a period of more than 10 months Eurofins were unable to provide the plasmid in one piece. The plasmid was therefore provided as Fragment A and B that needed to be assembled into one continuous sequence. Joining the two fragment was not straightforward and several stages were needed to join Fragment A and B (detailed in section 3.5.1.2.2).

Several tests were done to validate protein expression in rBVs carrying reporter genes under the control of a CMV promoter. To confirm that rBV entered mammalian cells and expressed the protein of interest, IHC were performed on MRC5 cells that were either transduced with wtBV or with BacMam CMV-Gag-Tat. Results confirm both virus entry and protein expression. The transduction rate was equal between the different recombinant viruses in each cell type, based on the intensity of the brown colour resulting after treatment with peroxidase. For protein production, and with it confirmation of the activity of EMCV IRES, HEK Cells were either transduced with a single recombinant BacMam or co-transduced with all 4 different rBVs together (BacMam CMV-gGb12-GFP, BacMam CMV-Gag-Tat, BacMam CMV-Rev-VSV-G and BacMam CMV-Pol-Vpr-Vif). For protein analysis, western blots were performed on cell extracts for Gag, Pol, VSV-G and IgG1b12 polyprotein expression using specific primary antibodies. Unfortunately, due to the lack of available primary antibodies, Tat, Vif and Rev polyproteins could not be confirmed by western blot. Analysis by SDS-
PAGE showed a clear band at the expected size for Tat, suggesting the successful expression of that protein. For the Vif and Rev polyproteins, SDS-PAGE analysis did not produce clear bands at the expected size. This suggests either a complete lack of protein expression, or the expression was at a low level, preventing the detection by SDS-PAGE. For those proteins that currently lack a commercially available primary detection antibody, future work could include the production of antibodies for these proteins. Of the proteins confirmed by WB, the exact location of protein production varied. Although detected in cell lysate, IgG1b12, Gag and VSV-G were most abundant extracellularly i.e. in the supernatant. However, Pol was mainly intracellular.

To investigate IgG1b12 expression and integration into mammalian cells, HEK, VERO and HeLa cells were transduced with BacMam CMV-gGb12-GFP or co-transduced with all 4 recombinant BacMam viruses. The IgG1b12 expression cassette is composed of LC, HC and eGFP driven by a CMV promoter and flanked by LTRs. Green fluorescent signal was observed in all transduced VERO and HeLa cells, proving that the expression of IgG1b12 was successful. Cell extracts from transduced HEK cells was subjected to western blot, results indicated that IgG1b12 was detected in both single and co-transduced cells. Detection of both heavy and light chain antibodies separately on the SDS-PAGE demonstrated production of the antibody proteins. Results from IHC together with western blot indicated that IgG1b12 was expressed when cells were transduced with BacMam CMV-gGb12-GFP alone as well as when co-transduced with all 4 rBVs. This suggests that antibody secretion occurred without integration of the antibody expression cassette into the cellular genome, where expression was driven by CMV promotor. Or perhaps, that the LTRs alone are sufficient for integration regardless of the presence of Rt, Integrase and protease. However, the evidence so far for a role of the LTR in integration is circumstantial (Hindmarsh & Leis 1999). Determination of whether or not the antibody sequence is integrated within the host cell genome is clearly critical. If stable integration of the antibody gene is not achieved then sustained intracellular production of antibody will not be accomplished.
TEM was used to analyse the morphological aspect of the VLPs and measure the individual particle size per sample. Virus like particle expression was analysed in HEK cells co-transduced with all 4 rBV s. Upon expression, the Gag polyprotein of SIV spontaneously assembles giving rise to enveloped VLPs. The results indicate that the great majority of Gag present in cell culture supernatants have shown to be correctly assembled and have been successfully expressed and secreted as VLPs. Figure 98 shows VLPs visualised under TEM. It should be noted that in presence of viral protease, SIV maturation (process by which the Gag polyprotein is cleaved into its distinct subunits and the VLP is reorganised) will take place. Viral particles generated were smaller than the expected mature virion size (110-130 nm) (Gentile et al. 1994). Configuration of the visualised VLPs presents an angular outline forming icosahedral structures. The background contains large amounts of protein perhaps suggesting that the VLPs were degraded during high-speed centrifugation. However, these proteins might be unassembled Gag polyprotein or other polyproteins such as Tat, Pol, VSV-G, Vif, Rev and IgG1b12.

Our study confirms that mammalian cells can be transduced with multiple different rBV s simultaneously. Co-transduction ensures that monocistronic BVs can be easily and rapidly produced if new genotypes or serotypes appear. In agreement with previous studies we found that the MOIs do not affect the transduction rate or the protein expression. The choice of the surrounding solution during transduction has a considerable effect on transduction efficiency. PBS was used as a surrounding solution and the volumetric ratio of diluted virus and PBS was 1:4, following recommendation from Sung et al. 2014.

These proof of principle studies not only establishes the feasibility of antibody gene transfer, but also investigates the efficiency of SIV like particles production in mammalian cells using BacMam technology, to support their candidacy as potential vaccines and/or immunotherapy agents.
3.7. Future work

Successful transduction of various mammalian cells has been demonstrated with different rBVs. Protein expression and VLP expression was detected. To further validate these results it will be necessary to confirm that the VLPs contain all of the desired proteins and the IgG1b12 expression cassette encoded into its core. Following infection of cells with the VLP confirmation of the integration of the antibody sequence into the mammalian genome and determination of the site of integration is necessary. This can be done most efficient by sequencing the entire genome of the VLP infected host cells. Antibody was detected when cells were transduced with BacMam CMV-gGb12-GFP only, suggesting that direct transduction rather transduction mediated by VLPs may allow a more direct route to cellular delivery of the antibody sequence into the mammalian genome.

The need of the Rt integrase and associated proteins for successful integration would then be questionable. The possibility that antibody secretion can be carried out without integration needs to be investigated. A key aspect is to confirm the intake of the antibody sequence by VLPs and inserting it into the mammalian genome. This can be investigated by infecting mammalian cells with purified VLPs and monitor the expression of IgG1b12. Technically if the VLPs encode the antibody expression cassette within its core, IgG1b12 will be secreted. Whole genome sequencing can be used to confirm the insertion. We also, need to monitor the expression of the antibody to examine whether it is a stable integration and for how long IgG1b12 will be expressed. Mok et al. 2007, found that the stability of HIV-1-based vector gene expression is extremely durable, with over 80% of selected cell clones persistently expressing transgene at 18 months post infection (Mok et al. 2007).

Analysis of SIV integration shows a striking tendency to integrate within transcription units (73% of the mapped integration events), but no propensity toward integration in any specific region of the transcription units (Hematti et al. 2004). The effect of antibody secretion on cells quality and growing should not be neglected. Also, the
neutralising effectivity of the antibody secreted must be determined to validate the protection effect of our system. Our current results indicated that IgG1b12 was detected when cells were transduced with BacMam CMV-gGb12-GFP alone as well as when co-transduced with all 4 rBV. The possibility of delivering the Ab sequence into mammalian cells can be accomplished using single BV encoded LRTs must be investigated.
4. Chapter 4

General discussion and future work

The HIV pandemic continues despite international effort to control it. Progress has been made in reducing AIDS-related deaths (by nearly 50% since the peak of the epidemic), but each day more than 5,000 people are newly infected (World Health Organization Guideline 2016). Development of a vaccine to aid control of the pandemic has been in progress since 1984 but to date, despite enormous international effort, the only vaccine trial to show promise (RV144) protected only 31.2% of study participants. A multiplicity of antiviral drugs for the treatment of HIV infection have been developed but their effective use relies on complex specialist laboratory support (HIV viral load testing, antiviral drug resistance testing, therapeutic drug monitoring) and advanced medical care of patients to manage associated drug toxicities. In addition, high costs of the drugs place a heavy economic burden on health systems and restrict their application in the countries where highest rates of HIV infection are seen. Vaccines have been our most effective means to protect us from most of the world’s deadliest viral infectious diseases. However, there are many confounding issues that prevent the production of a sufficiently effective HIV vaccine (Morrow et al. 2012; Esparza 2001). Challenges include HIV’s extreme antigenic variability; lack of understanding of immune correlates for protection; limitations of available animal models; and the constraints associated with the need for multiple large-scale clinical trials in different parts of the world.

The hyper mutation capability of HIV has proved an, as yet, insurmountable problem in developing a protective HIV vaccine. However, the discovery of individuals in high endemicity areas who are resistant to HIV infection, and the recognition of so called ‘elite responders’ to HIV infection (individuals who very effectively suppress HIV replication after initial infection), led to description of the so-called ‘broadly reactive’ neutralising antibodies. It is believed that elicitation of broadly neutralising antibodies
(bNAbs) is a key requirement of a successful HIV vaccine. These antibodies have been shown capable of neutralising the infectivity of a wide range of HIV strains. Passive administration of bNAbs has been shown to provide protection from infection after intravenous, vaginal, rectal, and oral virus challenge in non-human primate models (Mascola et al. 1999; Hessell et al. 2010; Hessell et al. 2009). Moreover, several studies have demonstrated that in a non-human primate (macaques) model vaccine-induced bNAb responses can confer complete protection against homologous SHIV challenge (Barnett et al. 2008; Bogers et al. 2008; Pal et al. 2006). This suggests that a vaccine capable of eliciting sufficient levels of bNAb against HIV-1 could prevent the establishment of infection. In order to be effective, such vaccination would be required to elicit neutralising antibodies that bind to the highly variable Env glycoprotein and neutralise a broad range of primary isolates in humans. Animal model studies show that high serum concentrations of bNAbs are required to provide adequate protection (Walker & Burton 2010). While passive immunisation may work, its administration echoes many of the high cost, technological demand, and complex medical support that are seen in deployment of HIV antiviral drugs. These problems could be surmounted if the gene for bNAbs could be delivered intracellularly.

The present project used different unique hybrid vectors to insert HIV-neutralising monoclonal antibody sequences into the human genome using a simian retroviral gene therapy agent pseudo-typed with vesicular stomatitis virus glycoprotein (VSV-G). Required genes to form SIV like particles encoding the neutralising antibody sequence were encoded in earlier developed poxvirus split-vectors or into a baculovirus expression system. Protein expression from either system was expected to result in the production of pseudotyped replication incompetent retroviruses like particles. The retroviral particles were in turn expected to deliver a gene for the expression of HIV neutralising antibody intracellularly and then mediate its integration into the human genome. The inserted genetic materials were composed of an expression cassette carrying a CMV promoter and the neutralising anti-HIV antibody gene with the aim of
establishing constant expression of the neutralising monoclonal antibody over a prolonged period within the cell. The VLPs were intentionally designed to be of a simian origin to increase the safety profile of the VLPs. Wild type SIV replicates in and infect several species of non-human primates but does not infect or replicate in humans (Sakuma & Takeuchi 2012). To add a further level of safety, the VLP genes were attenuated and lacked the ability to replicate in any type of mammalian cell.

One essential element in any HIV vaccine is to be able to elicit broadly neutralising antibodies (bNAbs) that can inhibit the infection of diverse HIV strains (Montero et al. 2008; Schiffner et al. 2013). Thus far, VLPs in vaccine production have limited success in developing a successful vaccine against HIV. Env has been considered as the principal antigen for HIV VLP vaccine development. However, Env has several features that limit the accessibility to the neutralising epitopes which strongly affect the development of bNAbs. The heavy glycosylation resulting in the formation of a glycan shield on the surface of Env trimer severely reduce the access to protein epitopes (Wei et al. 2003; Sagar et al. 2006; van Gils et al. 2011; Pancera et al. 2014). Moreover, Env trimer hides its conserved neutralising sites, such as epitopes in the CD4-binding site and the membrane-proximal external region (MPER), and only fully exposes them when bind to CD4 and undergo conformational changes (Pancera et al. 2014; Frey et al. 2008; Montero et al. 2008). In addition, the low density of native Env spikes displayed on the viral surface, as well as the contamination with non-functional Env, also contribute to the inaccessibility of neutralising epitopes (Burton & Mascola 2015; Klein & Bjorkman 2010; Liu et al. 2008).

Another way of producing HIV VLP is by using Gag polyprotein which is capable of self-assembly. HIV-1 Gag can serve as a platform for displaying Env antigen as well as being a promising HIV-1 vaccine component for prophylactic and therapeutic purposes (Buonaguro et al. 2001; Deml et al. 2005; Rovinski et al. 1995; Sakuragi et al. 2002). It has been reported that HIV-1 Gag-VLPs were capable of priming significant cellular responses as well as antibody response with cross-clade in chronically HIV-infected
patients and in non-human primates (Tsunetsugu-Yokota et al. 2003; Paliard et al. 2000; Pastori et al. 2012). Studies demonstrated that Gag-VLP efficiently induced the activation and maturation of DCs as well as the production of pro-inflammatory cytokines (Chang, Suzuki, Suzuki, et al. 2012; Chang, Suzuki, Yamamoto, et al. 2012). Although very promising, a huge hurdle in using VLPs for vaccination purposes is that the elected immune responses are not sufficient for full protection against divergent HIV strains. The system developed in this thesis overcomes all of the above limitation by incorporating the bNAb sequence inside the core of the VLPs.

The VLPs developed in this study contain both HIV Gag and Pol genes and these together with other attenuated proteins can be predicted to stimulate cell mediated immune responses. Such responses are thought to be important in controlling HIV infection, as Gag and Pol are relatively conserved proteins. Stimulation of CD8+ T cells may produce an important second line of defence, by lowering viral load in infected vaccinees, potentially interrupting the transmission of viral escape mutants selected by a vaccine-induced antibody response (Migueles & Connors 2015) (Figure 103).

**Figure 103: Requirements for vaccine-induced protection and control.** Vaccine-induced, HIV-specific, broadly neutralising antibodies at mucosal surfaces are thought to represent an important first line of defence against HIV infection. Vaccine-induced, HIV-specific CD8+ T cell responses may have an important role complementary to that of HIV-specific antibodies that involves eliminating cells infected by neutralization-resistant viral escape mutants (red virions) or viruses that were able to cross nonintact epithelia. Reproduced from (Migueles & Connors 2015).
The use of immunotherapy delivered through genetic manipulation allows in vivo delivery of broad and potent NAbs without the logistical complexities of traditional immunotherapy procedures. The concept has been explored in other studies for example Lewis et al. used an adeno-associated virus (AAV) vector to deliver the IgG1 b12 gene into mouse muscle and found that the antibody molecules imparted neutralisation activity in sera for over six months (Lewis et al. 2002). This approach was also tested in a nonhuman primate model by delivering neutralising immunoadhesins (antigen-binding variable fragment domains of Fabs fused to the Fc fragment of a rhesus IgG2 molecule) into macaques (Johnson et al. 2009). The immunoadhesins were expressed in the macaque muscle myofibers, and serum neutralisation activity was sustained for over one year. More importantly, sterilising protection against SIV challenge was achieved in six out of nine immunised monkeys, and all nine monkeys were protected from AIDS. Studies indicated that the human monoclonal antibody IgG1 b12 can prevent SHIV SF162 infection in rhesus macaques. However, the dose of antibodies that is required to achieve complete neutralisation was suggested to be extremely high, which is unlikely to be achieved through current immunisation techniques (Hessell et al. 2007; Hessell et al. 2009; Moldt et al. 2012; Parren et al. 2001). Encouraged by these results, we have selected IgG1b12 to be our bNAb of choice. Evidence from antibody binding to infected cells suggests that b12 may recognise a native conformation of gp120 more effectively than other antibodies directed against the CD4 binding site (Burton et al. 1994). IgG1b12 can neutralise almost 50% of clade B viral strains and approximately 30% of non-clade B strains (Yang & Wang 2014). Moreover, the IgG1b12 antibody was found to neutralise approximately 35% of circulating isolates (Kwong et al. 2011).

However, with the increase number of studies on bNAb several new broadly neutralising Abs have shown greater neutralisation potency and breadth than the first generation of bNAbs (Klein et al. 2013; Yang & Wang 2014; Kwong et al. 2011). One such antibody the human monoclonal antibody VRC-HIVMAB060-00-AB (VRC01)
which has shown considerable promise when used for passive immunisation, providing immediate protection against HIV infection. Thus for future work VRC01 will be considered as replacement antibody. Similar to IgG1b12, VRC01 is a bNAb that targets the CD4-binding site of the HIV envelope glycoprotein. However, VRC01 has a higher neutralisation effect, it can neutralise up to 90% of a broad panel of 190 group M HIV envelope pseudotyped viruses with a mean 50% inhibitory concentration (IC50) of 0.33 μg per millilitre (Wu et al. 2010). Passive administration of VRC01, has been shown to prevent HIV transmission in animal models and is now being tested in clinical trials of vertical and horizontal transmission in humans (Bar et al. 2016).

Two different approaches were used to express the protein of interests. In the first scheme, transient gene expression in mammalian cells was based on either recombinant MVA or recombinant baculovirus synthesising T7 RNA polymerase under the control of a CMV promoter. The T7 RNA polymerase has strict specificities for promoter and terminator respectively but can produce high levels of transcriptase activity. The localisation of T7 RNA polymerase in cytoplasm gives higher levels of protein expression than nuclear localisation. Yap et al. 1997, found that nuclei from HepG2 cells infected with baculovirus encoding T7 RNA polymerase exhibited very low activity which was indistinguishable from that found in uninfected cells (Yap et al. 1997).

Several features make MVA a favourable candidate as an efficient vector for gene delivery or vaccination. The viral genome has a high capacity (up to 25 kb) for recombinant DNA insertion. It also contains a strong poxviral promoter that ensures efficient recombinant DNA expression. Another advantage is the lack of persistence or genomic integration in the host due to its cytoplasmic replication. Although MVA-572 and MVA-I721 strains have been shown to replicate in some human cell lines and in immunodeficient mice (Verheust et al. 2012). As a vaccine, MVA has proven safety and immunogenicity in subjects infected with HIV and MVA based vectors and vaccines are easily produced. For large-scale manufacture, the MVA vector is
generally produced in chick embryo fibroblast (CEF) cells. Recently, AGE1.CR and EB66 cell lines have been developed for improved MVA production. A serious problem in this project was a lack of availability of specific pathogen free (SPF) fertile eggs to allow propagation of MVA. Further problems were encountered with the stability of genetic insertion in MVA. A deletion site was used for insertion which resulted in unstable constructs where the gene or genes of interest were repeatedly lost. To circumvent this problem the use of baculovirus as a gene vector was explored.

Since Hofmann et al. and Boyce and Buchner reported, that recombinant baculoviruses can enter mammalian cells and express proteins of interest when driven by mammalian promoters, several studies have deepened our understanding of BacMam technology and its utility (Hofmann & Strauss 1998; Boyce & Bucher 1996). Different commercially available systems are available for generation of recombinant baculoviruses. These gene delivery vectors can be easily manipulated and produced. Baculoviruses have broad cell type specificity, are suitable for both transient and stable gene transfer, and, if mass application is envisaged, are cost-effective in comparison to chemical gene transfer procedures. Recombinant baculoviruses are non-replicative in mammalian cells and can simultaneously deliver multiple genes. Many commonly used cell lines as well as primary cultures are efficiently transduced with little to no microscopically observable cytopathic effect or cytotoxicity. Baculoviruses have the capacity for encoding significant amounts of additional genetic material within their genome. A recombinant baculovirus containing up to a 38 kb insert can be generated and propagated in a genetically stable manner in insect cells. The synthetic methods for construction of defined genes and their introduction to shuttle plasmids to permit construction of modified baculoviruses are well established. Surprisingly, publications reporting application of utilising BacMam technology in immunisation challenge/experiments against pathogens are still limited.

In the present project transduction of mammalian cells using baculoviruses encoding defective retrovirus genes, caused the formation of virus like particles. Production and
expression of VLPs occur in the targeted cell. Thus glycosylation, protein folding and particle assembly will not be affected. Also, in comparison with other schemes, such as production of VLP in bacteria, yeast and insect cells, contamination with foreign cellular proteins is eliminated. Studies have shown that it is almost impossible to produce VLPs without contaminant cellular proteins, such virus/host complexes may produce complex immunogenicity (Cho 2000; Levy 2007).

The VLPs released infected neighbouring cells. In these infected cells reverse transcription of the genome of the retrovirus construct followed by integration of the resultant cDNA into the host cell genome brings about production of HIV antibody - driven by a viral CMV IE promoter - both intra and extra cellularly. This genetic manipulation could provide a safe, sustainable vaccine to protect those who have not yet encountered HIV from infection (protective vaccination). Additionally, it holds the promise of immunotherapy for those already infected with the virus by neutralising the infectivity of virus released during HIV infection (immunotherapy). This could replace antiviral drug treatment for the majority of those infected with HIV.

Production of such a novel vaccine/therapeutic would provide an easily delivered and affordable global treatment. The advantages of this approach are the development of a vaccine that is stable and easily transported on cold chain transport as baculoviruses may be freeze dried (a major advantage for developing countries with limited infrastructure eliminating the need for ‘cold-chain’ transport and storage of vaccine in zones with high atmospheric temperatures); baculoviruses do not replicate within human cells thus the problems of pre-existing immunity seen with some gene transfer vectors (e.g. adenovirus) are avoided; a single dose of the vaccine would result in the development of long term, stable, production of antibody in vivo; significant therapeutic application (immunotherapy) in those already infected, as an adjunct, or better replacement for antiviral chemotherapy (avoiding the necessity for continuous monitoring of the infection, drug toxicity and the large economic costs of drug therapy); providing a realistic, affordable tool in the global effort to control the HIV pandemic.
One limitation of gene therapy using retroviruses is that the integrase enzyme can insert the genetic material of the virus in any arbitrary position in the genome of the host. If genetic material happens to be inserted in the middle of one of the original genes of the host cell, this gene will be disrupted (insertional mutagenesis). If the gene happens to be one regulating cell division, uncontrolled cell division (i.e., cancer) can occur. This problem has recently begun to be addressed by utilising zinc finger nucleases or by including certain sequences such as the beta-globin locus control region to direct the site of integration to specific chromosomal sites. Full understanding of the safety of such process is important. It is known that natural retroviral infection with HIV produces such direct oncogenesis with exceptional rarity and only in the context of continuous HIV replication.

As discussed before, results from this project indicate the expression and secretion of IgG1b12 from transduced cells (refer to section 3.5.6). Since the antibody was detected when cells were transduced with BacMam CMV-gGb12-GFP only, the possibility that antibody secretion was carried out without integration should be investigated first. The resultant antibody titre should be measured together with antibody neutralisation effectivity. Assessment of IgG1b12 potency and evaluation of its inhibitory activity should be carried in vitro using TZM-bl cells where the IC90s of IgG1b12 against HIV-1 infection is derived. The effect of antibody secretion on tissue viability should not be neglected. Transduced cells should be passaged and monitored for any morphological and functional changes.

To validate our results we have to confirm the integration of the antibody sequence into the mammalian genome and determine the site of integration. This can be done by whole human genome sequencing and using other methodologies such as Linear - amplification mediated PCR (LAM-PCR) to catalogue retroviral insertions. Evidence from studies concerned with retroviral integration patterns indicated that SIV integration shows a significant tendency to integrate within transcription units (73% of the mapped integration events), but no propensity toward integration in any specific region of the
transcription units (Hematti et al. 2004). Retroviral gene integration into the host cell chromosome raises an ongoing concern of the risk of insertional mutagenesis and oncogene activation. These fears were particularly raised after early gene therapy clinical trial-treated patients with gene therapy for X-linked severe combined immunodeficiency syndrome (SCID). Two out of 11 patients involved in the clinical trial developed T cell leukaemias 3 years following transplantation of autologous bone marrow CD34+ cells transduced with a standard retroviral vector expressing the common γ chain (γc) transgene. However, insertional mutagenesis has not been observed in replication defective retroviral vector-mediated gene transfer, either in animal models or in clinical trials (Schmidt et al. 2002; Kiem et al. 2004). Reassuring results from long term clinical and molecular follow up of over 200 patients enrolled in clinical trials utilising retroviral vectors to transduce hematopoietic progenitor cells showed no development of leukaemia (Kiem et al. 2004).

The extended clinical follow-up and molecular analysis of vector insertional clonality report in nonhuman primates and dogs suggests a nonrandom pattern of insertion is unlikely to become dominant or be associated with haematologic abnormalities. Without a transgene to provide an abnormal proliferative or survival signal, the risk of leukaemogenesis appears to be very low (Moolten & Cupples 1992; Kiem et al. 2004). Thus it is very unlikely that the development of leukaemia in the children enrolled in the γC X-SCID trial resulted solely from a single or a few retroviral insertions per cell. Additional factors are more likely to be related in the development of leukemia in these patients. The underlying immunodeficiency perhaps resulted in an expanded lymphoid progenitor target cell population; the therapeutic gene itself, γc, might be a risk factor, when expressed from a recombinant retroviral vector under the control of the vector's LTR in patients with X-linked SCID; also, the patients might have expressed a constitutively activated cytokine receptor transgene that resulted in oncogene activation (Kohn et al. 2003).
In regard to the present BacMam expression system, a simpler route to the desired vaccine/therapeutic may be possible. Instead of using 4 different baculovirus constructs to transduce mammalian cells all necessary genes could be inserted within one baculovirus genome. Further, instead of assembling a defective retrovirus within the mammalian cell and using this to produce a retrovirus-antibody gene delivery system, the baculovirus could be used to directly deliver the antibody gene to the host cell without the intermediary step of production of a defective retrovirus to achieve gene delivery. The baculovirus would be modified with surface molecules from VSV to achieve broad cell tropism. Modification of the coat of the baculovirus (pseudotyping) is readily achieved through addition of VSV glycoprotein genes to the baculovirus genome using standard molecular biological approaches. Pseudotyping of the recombinant baculovirus with VSV-G will increase cell tropism as well as gene delivery. Also, prevent possible virus inactivation by complement reported in vivo studies with unmodified baculoviruses (Tani et al. 2003; Pieroni et al. 2001; Barsoum et al. 1997), as envelope modification of the baculovirus can change its immunogenic properties (Kaikkonen et al. 2011).

To avoid antibody overproduction, integration of a silencing gene alongside the antibody gene would be used to control any potential for antibody over production. However, direct in vivo gene transfer has been found to permit high and sustainable production of circulating ectopic MAb without inducing an anti-idiotypic response sufficiently robust to exert a neutralising effect (Noël et al. 2002). Thus, such system is very unlikely to trigger anaphylaxis. Many regulatable systems are currently under development and some have been used successfully for in vivo preclinical applications (Goverdhana et al. 2005). The tetracycline regulatory system (Gossen & Bujard 1992) is the most widely used and versatile system. The Tet-regulatable system can be used to reduce unwanted gene expression. A chimeric protein incorporating a modified TetR fused to transcriptional silencing domains of the regulated protein. Co-expression of both rtTA proteins and regulated protein will produce insufficient heterodimerise due to
modifications on the dimerising surfaces of the TetR component (Freundlieb et al. 1999).

The aim of our study is to elicit high titres of HIV neutralising antibodies in vaccinated individuals, over a prolonged period with a constant protection level. Upon encouraging results, the study will progress to preclinical pharmacological and toxicological testing. A pharmacodynamic study of the potential vaccine will be conducted to evaluate immunogenicity using a humanised mouse model of HIV infection, to provide “proof of concept” information to support a clinical development plan.
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