

Exploiting Pre-Existing Viral Immunity for B Cell Vaccination Against Endogenous Antigens

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A thesis submitted for the degree of Doctor of Philosophy
to the Faculty of Clinical Pharmacology, University of Oxford.

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Abstract

Cancer vaccines are a novel method of treating cancer by harnessing the patient's own immune system to recognise and attack malignant cells. Viral vectors can be used to encode tumour antigens to be presented to the immune system. However this field is hindered by immunity to the viral vectors themselves, which are often more immunogenic than tumour antigens.

This thesis describes the production of a novel cancer vaccine based on murine leukaemia retrovirus (MLV), where the enveloped vaccine particles express only tumour antigens on the surface with internal viral proteins physically shielded from B cells. This is designed to stimulate B cells specific for the weak tumour antigens by exploiting T cell recognition of strong viral MHC class II epitopes to confirm their activation.

Having cloned tumour antigens HER2 and 5T4 into the genome of MLV, the recombinant virus particles were purified and characterised thoroughly to ensure correct expression and presentation of tumour antigens. A vaccination schedule was also optimised, involving a homologous prime-boost strategy. Several prime vaccines were trialled with different results, highlighting the importance of priming CD4⁺ T cells and avoiding off-target effects. *In vivo* testing of the vaccine particles in BALB/c mice showed that mice with pre-existing immunity to MLV (those that were "primed" beforehand) had higher antibody responses against the tumour antigen than non-primed mice. This supports the hypothesis that the presence of viral-specific T cells will amplify the immune response to the tumour antigen after the subsequent "boost" vaccination, based on the requirement for T cell help for B cell activation and antibody production.

This thesis demonstrates that tolerance to endogenous tumour antigens can be overcome by using a self-adjuvanting viral vaccine particle. This method of homologous prime-boosting has important implications for cancer vaccination and immunotherapy.

Declaration of Authentication

All the work presented in this thesis was performed by me, and in no way forms part of another thesis in this or any other university. Contributions made by others have been acknowledged in this thesis. This work was performed under the supervision of Professor Leonard Seymour, Department of Oncology, Division of Medical Sciences, University of Oxford.

Suet Ling Wong

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List of Abbreviations

/	per
5' and 3'	5 prime and 3 prime ends of RNA or DNA molecule
21G	21 gauge needle size
23G	23 gauge needle size
25G	25 gauge needle size
293A	293 cells subclone (human endothelial kidney transformed with Ad5 DNA)
5T4	5T4 trophoblast glycoprotein
Ab	antibody
ACK	ammonium chloride potassium
Ad	adenovirus
ADCC	antibody dependent cellular cytotoxicity
AdZ5	non-replicating adenovirus serotype 5
Ad5T4	non-replicating adenovirus serotype 5 expressing 5T4 transgene
AdHER2	non-replicating adenovirus serotype 5 expressing HER2 transgene
Akt	also known as protein kinase B
ALP	alkaline phosphatase
ANOVA	analysis of variance
APC	antigen presenting cell
BCA	bicinchoninic acid
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BCR	B cell receptor
BHK-21	baby hamster kidney fibroblasts
bp	base pair
BSA	bovine serum albumin
°C	Celsius
CD20	cluster of differentiation 20 glycoprotein
CD4	cluster of differentiation 4 glycoprotein
CD4+	CD4 positive
CD40	cluster of differentiation 40 glycoprotein
CD40L	CD40 ligand
CD52	cluster of differentiation 52 glycoprotein
CD8	cluster of differentiation 8 glycoprotein
CD8+	CD8 positive
CD80/86	cluster of differentiation 80/86 glycoprotein
CEA	carcinoembryonic antigen
CFA	Freund's complete adjuvant
CIAP	calf intestinal alkaline phosphatase
CLIP	class II-associated invariant chain peptide
CMV	cytomegalovirus
ConA	concanavalin A
CP	cytoplasmic domain
CPE	cytopathic effect
CsCl	caesium chloride
CT26	mouse colon carcinoma cells
Cu	copper ion
Da	Dalton

DC	dendritic cell
dH ₂ O	deionised water
DM1	microtubule polymerisation inhibitor
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ds	double-stranded
EC	extracellular
ECL	enhanced chemiluminescence
EDTA	ethylenedinitrilotetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunosorbent spot
EMCV IRES	encephalomyocarditis virus internal ribosome entry site
Env	envelope
FACS	fluorescent activated cell sorting
Fc	fragment, crystallisable region
FCS	foetal calf serum
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
Fwd	forward
g	gram
<i>g</i>	gravitational-force
GC	germinal centre
GFP	green fluorescent protein
HBV	Hepatitis B virus
HER2	human epidermal growth factor receptor 2 (also neu, ErbB2)
Hib	<i>Haemophilus influenzae</i> type b
His	histidine
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HPV	human papillomavirus
IC	intracellular
IFA	incomplete Freund's adjuvant
IFN- γ	interferon gamma
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IVC	individually ventilated cage
kDa	kilo Dalton
kb	kilobase
l	litre
LB	Luria-Bertani
M	molar
mAb	monoclonal antibody
MACS	magnetic activated cell sorting

MAPK	mitogen activated protein kinase
MCF-7	human mammary gland adenocarcinoma
MEM	minimum essential medium
MES	2-(N-morpholino)ethanesulfonic acid
MFD	maximum feasible dose
mg	milligram
ml	millilitre
MHC	major histocompatibility complex
MLV	murine leukaemia virus
MLV 5T4	MLV expressing 5T4
MLV EGFP	MLV expressing EGFP
MLV HER2	MLV expressing HER2
mRNA	messenger RNA
MVA	modified Ankara vaccinia
MW	molecular weight
µg	microgram
µl	microlitre
µM	micromolar
ng	nanogram
nl	nanolitre
nm	nanometre
NIH 3T3	mouse embryonic fibroblasts
OD	optical density
pAC3-EMD	DNA plasmid encoding WT MLV with GFP transgene
pAC3-YCD	DNA plasmid encoding WT MLV with yeast cytosine deaminase transgene
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
Pol	polymerase
PRR	pattern recognition receptor
PVDF	polyvinylidene fluoride
QPCR	quantitative polymerase chain reaction
Rev	reverse
rGFP	recombinant GFP protein
rHER2	recombinant HER2 protein
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription PCR
RT-QPCR	reverse transcription quantitative PCR
rpm	revolutions per minute
s.c	subcutaneous
SDS	sodium dodecyl sulphate
SKBr3	human mammary gland adenocarcinoma
SOC	standard of care
SP	signal peptide
ss	single-stranded
TAE	Tris base, acetic acid and EDTA

TCR	T cell receptor
TE	Tris base and EDTA
TEMED	tetramethylethylenediamine
TGF- α	tumour growth factor α
TLR	Toll-like receptor
Tm	melting temperature for DNA primers
TM	transmembrane
UV	ultra-violet
VEGF	vascular endothelial growth factor
VLP	virus-like particle
vp	virus particle
vp/ml	virus particles per ml
VSV-G	vesicular stomatitis virus G glycoprotein
WT	wild type
WT MLV	wild type MLV
YCD	yeast cytosine deaminase

1 Introduction

1.1 *Passive Cancer Immunotherapy*

1.1.1 Monoclonal antibody therapy

Cancer is one of the main causes of morbidity and death worldwide; in 2010, 28% of deaths in the UK were attributed to cancer¹. This figure is set to rise in the future, despite advances in existing cancer treatments. Conventional cancer therapies are faced with a variety of problems; surgery is unlikely to result in complete eradication of cancer if metastases are present, while traditional chemotherapy and radiotherapy have severe side effects as they both target rapidly dividing cells, with little differentiation between normal and cancer cells.

Immune therapy can treat cancer, using immune mediated mechanisms to target tumours. One such form of immune therapy is passive immunotherapy, which utilises immune components that are produced independently of a patient's own immune system. An example is the use of bone marrow transplants to treat leukaemias and lymphomas, where immune cells from a healthy donor attack the cancerous cells, known as the graft versus leukaemia effect². However the risk of relapse after transplantation is fairly high, and there is the possibility of donor cells attacking the recipient's healthy tissue (graft versus host effect). Recombinant cytokines have also been trialled, such as interleukin-2 (IL-2), which promotes the growth and differentiation of T cells. IL-2 therapy however has been associated with numerous side effects and low response rates, and on its own may not be an effective therapy³.

Another form of passive immunotherapy that has been widely utilised is antibody-based treatment; for example, intravenous immunoglobulin therapy was used for many years to treat infectious diseases before the discovery of antibiotics⁴. The use of monoclonal antibodies (mAb) to treat cancer patients was first demonstrated in 1980, where a mAb against a lymphoma-associated antigen was used to treat a patient with non-Hodgkin's lymphoma⁵. This provided an important proof of principle that antibodies could be used to decrease the number of circulating tumour cells resulting in minimal toxicity to the patient. Early drawbacks

in the development of mAbs included the use of immunogenic mouse mAbs, which soon resulted in the development of humanised antibodies to minimise any detrimental anti-mouse response. In 1997 the first mAb was approved specifically for the treatment of cancer – rituximab, a humanised anti-CD20 (cluster of differentiation 20) antibody targeted against follicular B cell non-Hodgkin's lymphoma⁶. Since then, a variety of mAbs have been generated with different specificities to various tumour antigens (Table 1.1)⁷.

MAbs can function through a variety of different mechanisms such as steric inhibition and neutralisation. The binding of mAbs to their targets can trigger different immune mechanisms through the complement system or Fc receptors⁸, and is a major contribution to the therapeutic effect of exogenous antibodies. The classical complement pathway is initiated when complement proteins bind to the antibody-antigen complex, leading to the activation of a complement cascade and tumour cell lysis, as well as the recruitment of immune effector cells such as macrophages⁹. The binding of the Fc region of mAbs to their cognate Fc receptors on macrophages and natural killer (NK) cells can promote phagocytosis or antibody-dependent cell-mediated cytotoxicity (ADCC) respectively¹⁰. ADCC is initiated when the Fc portion of antibodies coating a cell interacts with Fc receptors on NK cells, triggering the release of cytotoxic granules containing perforin and granzyme B¹¹. Perforin forms pores in membrane, allowing granzyme to enter and initiate a caspase cascade leading to apoptosis of the antibody-coated cells.⁹ ADCC is a major mechanism of action of mAbs, as mice lacking Fcγ receptors with mAb-sensitive tumours are resistant to rituximab and trastuzumab; therefore the recognition of mAbs by Fcγ receptors on innate immune cells contributes to the anti-tumour activity of mAbs.

Some mAbs have more specific mechanisms of action based on the function of their tumour antigen target. The use of trastuzumab prevents dimerisation of HER2 receptors and hence inhibition of unwanted cell proliferation signals¹². Bevacizumab is effective in reducing the blood supply to tumour nodules by sequestering vascular endothelial growth factor (VEGF) and halting tumour growth, as the inhibition of angiogenesis slows delivery of nutrients and oxygen to tumours. This mAb has a relatively limited half-life of 17 days¹³, although other mAbs such as cetuximab have even shorter half-lives of only 1 – 5 days¹⁴.

Although mAbs are effective treatments against cancer, they are rarely used as first-line treatments, as they are often used in conjunction with chemo- and radiotherapy. These conventional methods of cancer treatment may limit the immune-based mechanisms of mAbs.

Clinical toxicity may also be seen due to off-target effects with systemic use; for example, ipilimumab is associated with immune-related adverse events such as hepatitis and rashes¹⁵. Skin and colon biopsies from affected patients revealed immune-cell infiltration of tissues, similar to autoimmune reactions¹⁶. The synthesis of mAbs through hybridomas is time-consuming and labour intensive, and due to the limited half-lives of the antibodies circulating in patients, frequent re-administration and long-term treatment is often required. Treatment can also be expensive; for example, the basic NHS price for a typical patient on a 38 week course of trastuzumab can cost £15 000¹⁷.

Table 1.1 | Monoclonal antibodies in cancer therapy.

Antibodies can target a variety of antigens that are up-regulated or over-expressed in different cancers, either for treatment or diagnostic purposes. Table modified from Oldham & Dillman, 2008 and Leveque et al 2005.

Monoclonal antibody	Antibody target	Target location/function	Tumour type	Biological half-life
Rituximab	CD20	Found on mature B cells	B cell lymphoma	33 hours – 12 days
Trastuzumab	HER2/neu	Tyrosine kinase receptor	Breast	3 days– 28 days
Bevacizumab	VEGF	Angiogenesis	Colorectal	13 days – 19 days ¹⁴ or 17 – 21 days ¹³
Alemtuzumab	CD52	Found on mature lymphocytes, monocytes and dendritic cells	Chronic lymphocytic leukaemia, T cell leukaemia	6 days – 21 days
Cetuximab	EGFR	Growth factor receptor	Colorectal	1 day – 5 days
Ipilimumab	CTLA-4	Inhibitory signalling receptor on T cells	Melanoma	359 hours ¹⁵

1.1.2 Rationale for making a B cell vaccine

Despite the effectiveness of monoclonal antibodies as a treatment for cancer, the limitations associated with this form of passive therapy means that other treatments must be sought. One alternative is active immunotherapy, where the patient's own immune system can be utilised to target cancer cells. Active therapy can enhance endogenous anti-tumour immunity and target tumour antigens by cell-based or antibody responses. T cell vaccines against cancer have been thought to be more effective as cancer is a cellular disease and may require the action of cytotoxic CD8+ T cells. This has been supported by a correlation between prolonged patient survival and the presence of tumour-associated CD8+ cytotoxic T cells¹⁸. Despite the promise of an effective therapeutic cancer vaccine, initial attempts at generating T cell immunity have proved problematic, as dendritic cells (DC) must first be targeted for any antigen presentation to occur to T cells¹⁹. Although many T cell based vaccines are in clinical trials, only one, Provenge (Sipuleucel-T) has been approved in the US²⁰. Other T cell based vaccines have also failed to live up to their potential, leading to a focus on antibody-based mechanisms²¹. MAbs have already proven to be effective treatments, through receptor antagonism and triggering anti-tumour immune responses, but also have their drawbacks, as stated previously. A more appealing form of active immune therapy would be to vaccinate patients with a B cell targeted vaccine, in order to produce endogenous polyclonal antibodies against tumours.

1.2 B Cell Targeted Vaccination

1.2.1 The humoral immune response

Naïve B cells are activated in secondary lymphoid organs, such as the lymph nodes and spleen²². Small soluble antigens are able to diffuse directly into the lymphoid tissue and bind B cells; however larger antigens (more than 70 kDa) must be presented by other means. These large antigens are often bound to antibodies and complement, forming immune complexes^{23,24}, which are transported in the lymph to lymph nodes. Follicular dendritic cells²⁵ and macrophages lining the subcapsular sinus²⁶ bind these immune complexes on their surface by Fc and complement receptors²⁷, and transfer intact antigen to naïve B cells. Such events take place after the formation of an immunological synapse between the B cell receptor (BCR) and membrane-bound antigen on the surface of the antigen presenting cell (APC)²⁸. It is thought that reorganisation of the cell membrane leading to the establishment of the immunological synapse results in B cell spreading, allowing BCRs to bind more antigen from the surrounding environment²⁹. After this spreading phase, the B cell contracts, resulting in clusters of antigen-BCR complexes and subsequent BCR cross-linking. This cross-linking rapidly leads to internalisation of antigen by receptor-mediated endocytosis in aggregates³⁰, which allows for the concentration of small amounts of antigen compared to other methods such as phagocytosis and pinocytosis³¹.

Antigen presentation by B cells takes place after internalisation and is necessary for T cell-dependent B cell activation. Most professional APCs achieve this by the MHC class II pathway, although DCs are able to cross present via the MHC class I pathway too^{32,33}. Both pathways share similarities and differ only by the nature of the antigen presented; MHC class I present endogenous protein while MHC class II relies on exogenous protein. The antigen-BCR complex is cleaved in acidified endosomes and fuses with cellular compartments containing MHC class II molecules³⁴. The exchange of CLIP (class II-associated invariant chain peptide), a small fragment bound to the MHC class II binding groove, for the peptide antigen results in peptide loading of the MHC class II complex and its subsequent trafficking to the cell surface. B cells will then translocate to the border between B and T cell zones within secondary lymphoid organs for antigen presentation to take place between the two cell types³⁵.

Naïve T cells are also activated in the same way as B cells in secondary lymphoid organs. T cell receptors (TCRs) on activated CD4⁺ T cells can engage with their cognate peptide-MHC class II complex on the surface of activated B cells. The binding of CD40 on B cells to CD40 ligand (CD40L) on T cells leads to the upregulation of CD80/86 on B cells, which in turn bind to the constitutively expressed CD28 on T cells^{36,37}. This latter interaction is an important co-stimulatory signal for sustained T cell activation, leading to the expression of cytokines to enhance activation for both B and T cells. The interactions between CD80/86 and CD28, and CD40L and CD40 are important in providing the necessary co-stimulation for B cell proliferation and differentiation³⁸. This is shown in Figure 1.1.

Antibodies expressed by B cells have different subtypes depending on which cytokines (e.g. IFN γ , IL-4) are present in the local environment and/or secreted by follicular helper T cells. Naïve B cells first express BCRs on the cell surface; these are membrane bound IgM antibodies, and once activated, B cells are able to migrate out of the follicle and undergo class switching^{39,40}. This is carried out by DNA recombination through V(D)J recombination and non-homologous end joining for double-stranded DNA breaks. Different antibody classes lead to a variety of downstream effector mechanisms – for example, IgA neutralise bacteria in mucosal surfaces, while IgG can opsonise pathogens for Fc γ receptor-mediated phagocytosis or activate the complement pathway⁴¹. This is summarised in Table 1.2. B cells remaining in the follicle continue to proliferate, leading to the formation of a germinal centre (GC), a specialised lymphoid structure for developing long lived plasma cells and memory cells⁴². Activation by CD4⁺ T cells is crucial for this GC reaction. B cells within GCs are continuously exposed to antigen presentation by follicular DCs, allowing for affinity maturation by somatic hypermutation (point mutation by activation-induced cytosine deaminase and uracil DNA glycosylase) and clonal selection in order to generate B cells with high affinity BCRs. This process is summarised in Figure 1.2.

Although antibody production can be stimulated without T cell help, T cell-independent antibody secretion is limited to low-affinity IgM against non-protein antigens, for which there will be few memory B cells. Confirmation of B cells by CD4⁺ T cells, is a requirement for isotype switching and high-affinity antibody production to produce a long-lasting and effective B cell response³⁶. B cells can only be activated by CD4⁺ T cells that recognise epitopes from the same molecular complex that is internalised and presented by the B cell to T cells. This is known as “linked recognition”⁴³ where it is not required that both cells recognise the same epitope, or that the epitopes need to be covalently linked, only that the two epitopes enter the same B

cell³⁷. This has been studied in enveloped viruses such as hepatitis B virus (HBV) and influenza, where the external and internal antigens are not covalently linked but spatially separated by a phospholipid bilayer. When mice are first primed with HBV core antigen, and subsequently challenged with whole HBV particles, antibodies against both the surface and core HBV antigens are produced⁴⁴. However if the mice were challenged instead with separate surface and core HBV antigens, no antibodies against the surface HBV antigens were produced. Therefore both the core and surface HBV antigens must be present in the same virus particle, which must be internalised whole to enable both antigens to enter the same B cell, to present a range of epitopes to different T cells. In a related study using influenza, it was found that T cells in mice primed with influenza particles without haemagglutinin spikes on the surface led to the activation of haemagglutinin-specific B cells and the production of anti-haemagglutinin antibodies following virus challenge; this result could not be reproduced if challenged with purified haemagglutinin⁴⁵. Hence, B cell recognition and antibody production is restricted to exposed epitopes that are available for BCR binding, whereas T cells can be activated by internal or external epitopes. This is supported by the observation that for complex antigens such as viruses, T cells can recognise the internal viral proteins and activate B cells specific to surface proteins. This is known as the intermolecular help model⁴⁶.

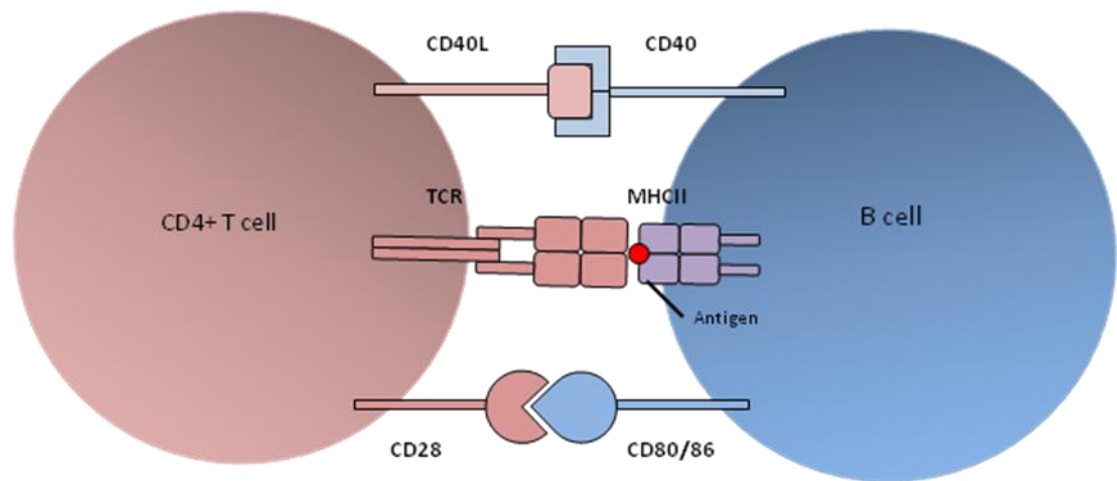


Figure 1.1 | T cell dependent activation of B cells after MHC class II presentation.

After TCR engagement of its cognate peptide-MHC class II complex, several co-stimulatory signals are required for full activation, namely the interactions between CD80/86 and CD28, and CD40 and CD40L.

Table 1.2 | Class and isotype switching in human and mice antibodies.

Different cytokines enable switching of the constant regions of immunoglobulins to enable specific effector functions. IgM immunoglobulins activate the complement system, while IgG and IgA antibodies function through neutralisation. Mouse IgG2a and IgG2b, and human counterparts IgG1 and IgG3 in particular engage in Fc receptor dependent responses such as ADCC, while murine IgG3 and human IgG2 bind to carbohydrate epitopes. IgE and murine IgG1 and human IgG4 immunoglobulins increase sensitisation to mast cells and are important for protection against extracellular pathogens.

Mouse

Effector function	Cytokine	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA	IgE
TH1	IFN γ	inhibits	inhibits	activates	activates	activates		inhibits
TH2	IL-4	inhibits	activates	inhibits	inhibits	inhibits		activates
	IL-5						activates	
T reg	TGF β	inhibits			activates		activates	

Human

Effector function	Cytokine	IgG1	IgG2	IgG3	IgG4	IgA	IgE
TH1	IFN γ	activates	activates	activates	inhibits		inhibits
TH2	IL-4	inhibits	inhibits	inhibits	activates		activates
	IL-5					activates	
T reg	TGF β					activates	

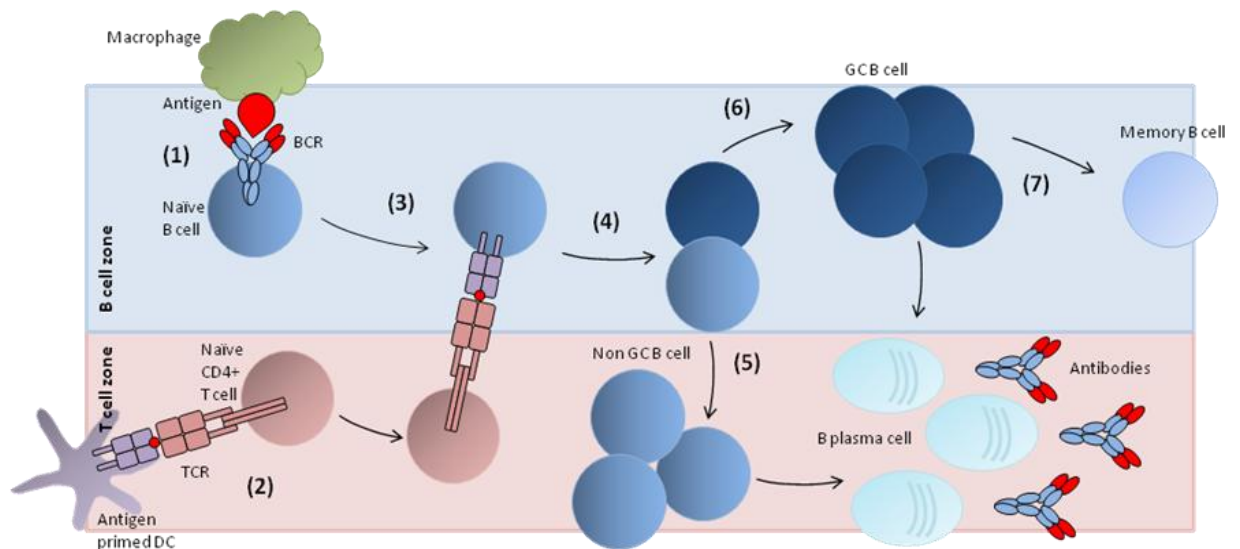


Figure 1.2 | B cell activation and antibody production.

(1) Naïve B cells receive antigen from macrophages in the follicles. (2) Naïve CD4+ T cells are primed by dendritic cells (DCs) and are activated. (3) B cells present antigen to CD4+ T cells by MHC-TCR binding. (4) B cells either remain in the germinal centre (GC) or migrate out of the follicle. (5) Non GC B cells undergo proliferation, antibody class switching and differentiation to become antibody-secreting plasma cells. Antibody sections coloured red indicate antigen-binding regions. (6) GC cells proliferate and undergo affinity maturation. (7) B cells can differentiate into memory or plasma cells. Figure modified from McHeyzer-Williams et al 2012.

1.2.2 Tumour antigens and tolerance

Malignant cells can express mutated gene products or have altered gene expression profiles. These changes result in the presentation of tumour antigens (Table 1.3), which was first demonstrated by immunising mice with tumours, leading to protection against subsequent tumour re-challenge⁴⁷. As most tumour antigens are derived from “self” or endogenous components, they are often poorly immunogenic due to the lack of pathogen-associated molecular patterns (PAMPs), which are required for the induction of innate and subsequently adaptive immune responses (Figure 1.3).

The poor immunogenicity of many tumour antigens is due to the processes that T and B cells undergo during development that exist to prevent autoimmunity (Figure 1.4). Both BCRs and TCRs have the capacity to bind any epitope due to the large number of possible rearrangements of their genes, therefore a large proportion of the BCRs and TCRs are self-reactive⁴⁸. These self-reactive cells are often deleted in the bone marrow or thymus respectively, in order to prevent autoimmune reactions⁴⁹. However a balance has to be found between deleting too many immune cells, as this would decrease the diversity of the BCR/TCR repertoire; hence it is possible for self-reactive cells to avoid deletion, in a process known as tolerance⁵⁰. T cells undergo positive and negative selection while in the thymus, which is a more stringent process than B cells, therefore few self-reactive T cells are present in the periphery. Conversely, some auto-reactive B cells are able to mature and migrate into the circulation; despite this, they remain unactivated (anergic) as they are unable to receive the required T cell help for full activation⁵¹.

In addition to tolerance mechanisms against tumour antigens, immune suppression of the local environment by tumours can dampen the responses of immune cells such as DCs. Although the immune system is thought to continuously monitor the body for abnormal cell development, this process can lead to selection of aggressive tumours capable of evading the immune system, known as cancer immunoediting⁵².

Table 1.3 | Types of tumour antigens.

Selected examples of the diverse range of tumour antigens and their origins. Table adapted from Emens 2008.

Type of antigen	Specific antigens	Tumour type
Universal	Telomerase, hTERT, mdm-2, survivin	All
Cancer testis	MAGE	Breast, bladder, lung
Tissue-specific differentiation products	PSA Tyrosinase, Mart-1/Melan-A,	Prostate Melanoma
Mutated gene products	β -catenin p53 bcr-abl	Colon Colon, breast, lung Leukaemia
Overexpressed self	HER2/neu Proteinase-3 Mucin-1	Breast Leukaemia Multiple myeloma, pancreas, gastric, breast
Idiotypes	Ig, TCR	Lymphoma

hTERT: human telomerase reverse transcriptase; mdm-2: mouse double minute 2 homologue; MAGE: melanoma associated antigen; PSA: prostate specific antigen; Mart-1/Melan-A: melanoma antigen recognised by T cells 1; HER2/neu: human epidermal growth factor receptor 2.

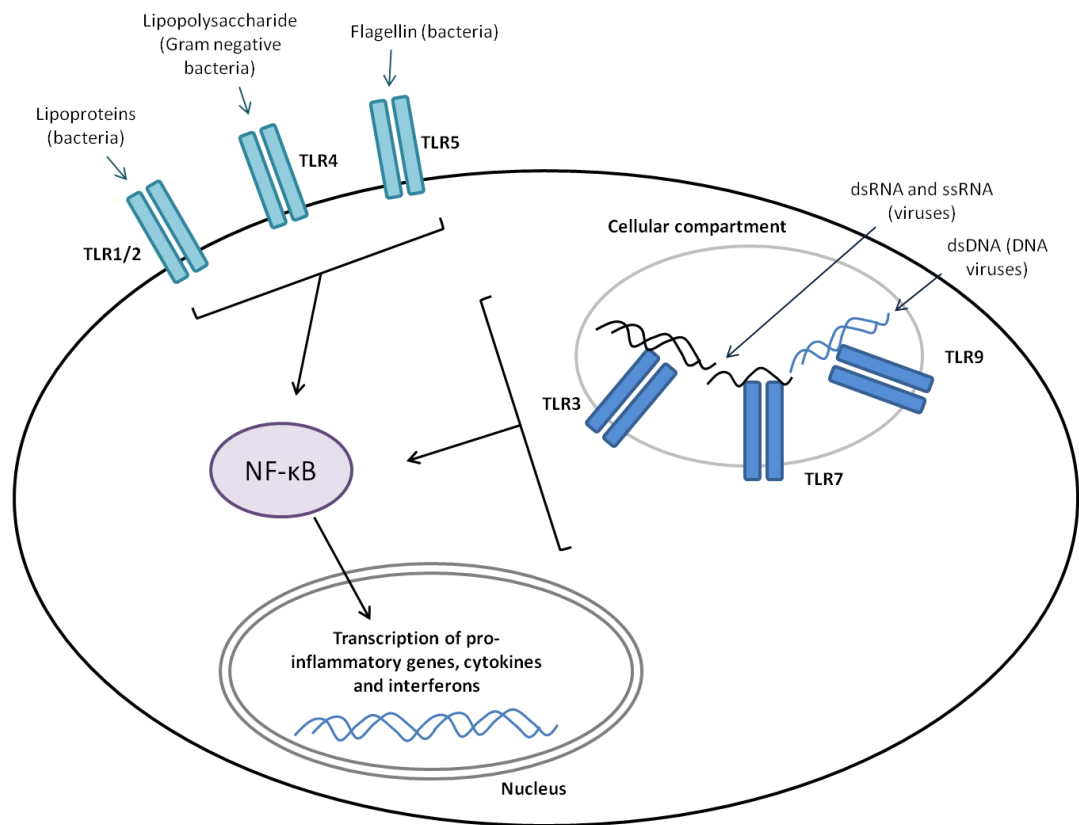


Figure 1.3 | Pathogen-associated molecular patterns (PAMPs) are recognised by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) leading to an inflammatory response.

Molecules containing PAMPs are recognised by PRRs such as TLRs (shown in diagram). TLRs 1, 2, 4 and 5 act on extracellular pathogens while TLRs 3, 7 and 9 recognise markers for intracellular pathogens. Dimerisation of TLRs leads to signalling via MyD88 or TRIF to activate NF-κB, leading to transcription of pro-inflammatory genes, cytokines and interferons. Other PRRs include Rig1-like receptors (RLRs) which recognise short double-stranded DNA. Ds: double-stranded, ss: single-stranded.

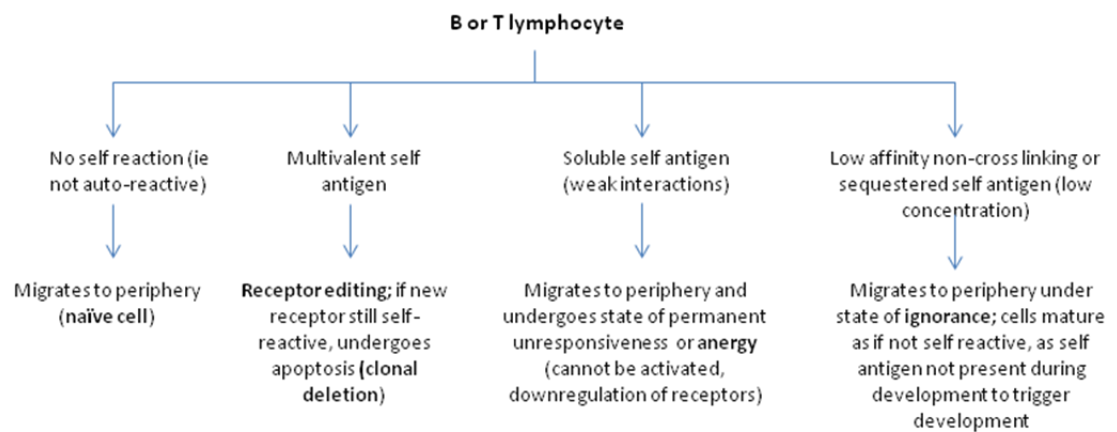


Figure 1.4 | Central tolerance in the development of lymphocytes.

When exposed to antigen during development in the lymphoid tissue, immature lymphocytes can follow different pathways depending on the nature of the antigen. If self-reactive lymphocytes manage to migrate to the periphery, they can undergo peripheral tolerance including somatic hypermutation (B cells) or downregulation by T regulatory cells.

1.2.3 Cancer vaccination

One method of cancer treatment is to vaccinate patients against their tumours using tumour specific antigens, similar to a vaccine for an infectious viral disease⁵³, where the patient's own immune system can be harnessed. Prophylactic vaccination has played an important role in the prevention of infectious diseases and reducing human mortality rates. The most successful licensed vaccines to date function by harnessing the antibody response through B cell stimulation⁵⁴. A clear example is the eradication of smallpox in 1980, declared by the World Health Organisation⁵⁵.

Cancer vaccinations can be in the form of tumour peptides, immune cells such as DCs pulsed with peptide, plasmid DNA or even tumour cells⁵⁶. Viruses can also be used as vectors for cancer vaccines, through genetic modification to allow expression of specific tumour antigens⁵⁷. Upon cell infection and transgene expression, tumour antigen peptides can be presented to the immune system to elicit CD4+ or CD8+ T cell stimulation⁵⁸. Adenovirus is a common vector for vaccination and gene therapy as its genome is well characterised, enabling the generation of safe, replication-defective vectors expressing various transgenes (discussed further in Section 1.4).

An alternative to using replication-defective viruses is to vaccinate with virus-like particles (VLPs). These are safer to use as they are non-infectious and do not contain any viral genetic information, yet are more immunogenic than protein-based vaccines alone⁵⁹. This is due to the display of antigens on the surface of the virus particle in a highly organised and dense formation, allowing for crosslinking of BCRs and B cell activation⁶⁰. VLPs can be taken up by professional APCs as exogenous or endogenous large antigens, and presented on MHC class I or II molecules to CD8+ or CD4+ T cells respectively⁶¹. The first VLP vaccine to be successfully licensed was the human papillomavirus (HPV) vaccine against cervical cancers caused by HPV^{62,63}. When administered prophylactically, the HPV vaccine leads to the generation of virus-neutralising antibodies⁶⁴ that prevent initial infection of HPV, thus reducing the chance of developing cervical cancer⁶⁵. Unlike most tumour antigens which are “self”, the HPV antigens will be recognised as “foreign”, due to the presence of PAMPs, hence the ease of vaccinating against HPV-related cancers. In designing a vaccine against HPV, it was found that expression of the major capsid protein L1 led to the self-assembly of empty capsids, which were identical to wild-type capsids of HPV⁶⁶. The HPV vaccine is highly effective, due to its optimal size (50 nm – 60 nm) allowing for transfer into the lymphatic system for immune cell activation, and its

ability to generate high titres of neutralising polyclonal antibodies from the highly repetitive and organised pentamer capsid structure⁶⁷.

Despite the success of the HPV vaccine for cervical cancer, other cancer vaccines based on VLPs have been slower to follow. While the anti-viral response against HPV is desirable against cervical cancer, the use of VLPs to display immunologically weaker endogenous antigens has resulted in the skewing of the immune response towards the VLP rather than the endogenous antigen. One such example has been to use murine polyomavirus containing a fusion protein between the internal viral capsid protein VP2 and the extracellular (EC) and transmembrane (TM) domains of human tumour antigen HER2. Although a single vaccination of the tumour antigen-VLP could confer protection against subsequent tumour challenge, this was likely to be due to cell-mediated immunity as there were HER2-specific T cells found by ELISPOT but no anti-HER2 antibodies were present in mouse sera. By contrast, mice were found to have high titres of antibodies against the VLP virus components⁶⁸. Another example is the display of tumour-associated cell lineage marker claudin-18 on the surface of a VLP composed of hepatitis B virus (HBV) core antigen. Despite the tumour antigen being prominently displayed on the surface of the VLP in a highly dense and organised form, both mice and rabbits vaccinated with the VLP showed higher titres of anti-HBV antibodies instead of anti-claudin antibodies⁶⁹. In a phase I clinical study, ten patients with metastatic breast cancer were vaccinated with a HER2 vaccine displayed on an influenza-based virosome. Although HER2 specific antibodies were confirmed in five out of ten patients, the majority of patients showed high titres of neutralising anti-viral antibodies, which were increased significantly from pre-existing titres⁷⁰. While the presence of viral antigens may be beneficial in generating a pro-inflammatory response (due to the presence of PAMPs) and recruiting viral-specific immune cells, it can also skew the immune response away from the immunologically weaker tumour antigen, and can have a detrimental effect on repeated vaccinations.

VLPs clearly present potent platforms for vaccination, and have been regarded as safe due to their non-infectious and non-replicative nature, in contrast to infectious replication defective viral vectors. However the display of weak non-viral antigens on the surface of VLPs is not guaranteed to generate high levels of antibodies targeted towards the weak antigen, due to the presence of the strong immunogenic viral antigens and the generation of neutralising anti-viral antibodies. These antibodies, if not already pre-existing, would be generated upon the first vaccination leading to all subsequent vaccinations to be ineffective. However, both the highly clustered display of weak antigens on the surface of VLPs and the immunogenicity of

viral proteins are required to act as an adjuvant for vaccination. It is therefore desirable to create a VLP which displays only weak antigens on the surface but still retains its core viral peptides, which are available for MHC presentation to T cells but not to BCRs on B cells. One such viral vector that can be used for this type of VLP is murine leukaemia virus.

1.3 Murine Leukaemia Virus

1.3.1 Life cycle of MLV

Murine leukaemia virus (MLV) is a gamma retrovirus and is among the simplest in the *Retroviridae* family, with a genome size of only 10 kilobases (kb). MLV was first isolated from murine tumours and was discovered as a causative agent for murine leukaemia⁷¹. It shares a similar structure to other members of the *Gammaretrovirus* genus, being 100 – 120 nm in diameter and spherical in shape⁷². The external surface of the virion is covered with the viral glycoprotein envelope (Env), a trimeric type I single transmembrane protein embedded in a lipid bilayer derived from the plasma membrane of a producing cell. The cytoplasmic tail of Env interacts with the N-terminus of the Gag protein (matrix), which is an important interaction for particle assembly. The C-terminus of Gag forms the nucleocapsid, while Pol polyproteins make up the remaining internal virus proteins – integrase, protease and reverse transcriptase. These interact with the RNA genome, which consists of a dimer of linear positive single strands⁷³ (Figure 1.5).

MLV infects cells by Env binding to its cognate receptor, thought to be Ram-1, a sodium phosphate symporter for amphotrophic MLV^{74,75}, which shows broad tissue expression⁷⁶. Upon receptor binding, Env undergoes large scale conformational changes resulting in the fusion of the virus membrane with the plasma membrane of the target cell. Once released into the cytoplasm of the cell, the viral RNA genome is copied by reverse transcriptase into dsDNA using cellular tRNA as a primer. This is able to enter the nucleus after breakdown of the nuclear membrane during mitosis. In this way, MLV is only able to productively infect dividing cells, unlike its fellow retrovirus HIV (human immunodeficiency virus), as MLV lacks any active nuclear import elements⁷⁷. Viral integrase incorporates the virus DNA into the host genome in three stages; first by processing the viral 3' strand to expose the 3' OH group for reaction, then by mediating strand transfer between the 3' viral DNA and cleaved cellular DNA. Stable integration is finally completed by cellular DNA repair proteins which fuse the free ends of the virus and host cell DNA⁷⁸. The proviral DNA is subsequently transcribed by cellular transcription and translation machinery. Viral RNA can be processed and exported from the nucleus as several different molecules – either as a full length transcript for packaging into virus progeny (making up more than 50% of the total viral export) or to be translated into Gag or Gag-Pol polyprotein (5% - 10% stop codon misread and polyprotein produced), which share the same

open reading frame, or as spliced mRNA to produce Env. Constitutive export elements bind to the RNA in order to force its export out of the nucleus and prevent any reverse transcription and re-integration into the cellular genome; while these elements have been found for other retroviruses (for example, Rev protein in HIV), they have not yet been identified for MLV. Gag/Pol and Gag proteins are trafficked to the cell membrane, assisted by host endosomal sorting complexes required for transport (ESCRT), aggregating in areas of lipid rafts for particle assembly and budding. Once the immature virion has left the cell membrane, the viral protease cleaves the polyproteins in order for the virus to mature and become infectious, and thus continue the retrovirus life cycle. This process is summarised in Figure 1.6.

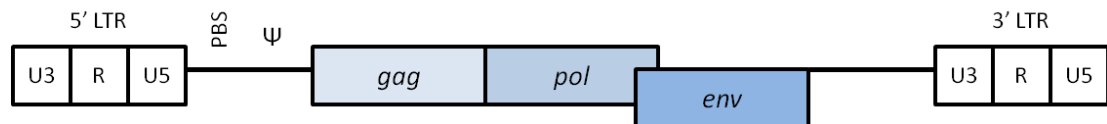


Figure 1.5 | MLV genome structure.

The proviral genome of MLV is similar to other retroviruses with 5' and 3' long terminal repeats (LTR) and an opening reading frame (blue boxes) containing the *gag*, *pol*, and *env* genes. PBS: primer binding site (for reverse transcription), Ψ : packaging signal. Figure adapted from Maetzig et al 2011.

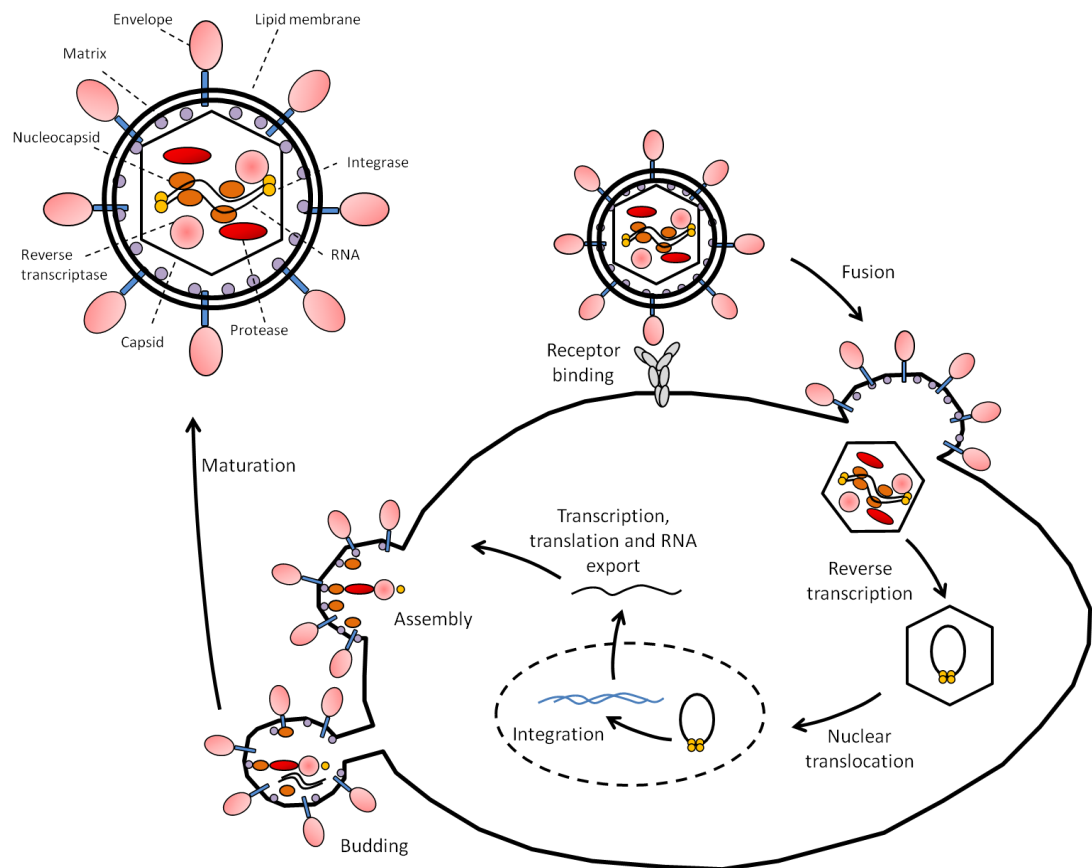


Figure 1.6 | MLV life cycle.

After receptor binding on the cell surface, the virus envelope fuses with the cell membrane leading to the release of the virus capsid. The ssRNA genome is reverse transcribed into dsDNA, which is integrated into the cellular genome. Transcription and translation of the proviral DNA takes place, resulting in viral proteins which assemble at the cell membrane and bud off as an immature virus particle. Maturation of the virion takes place outside of the cell, to complete the retroviral life cycle. Figure adapted from Maetzig et al 2011.

1.3.2 MLV as a vaccine vector

The use of MLV in cancer therapy largely harnesses its retroviral capabilities. Its relatively small and well characterised genome makes MLV amenable to genetic modification. Suicide genes such as cytosine deaminase can be encoded within the genome and integrated into infected cells. Cytosine deaminase converts the prodrug 5'-fluorocytosine to 5'-fluorouracil, leading to death of infected cells. The enzyme encoded in MLV is remarkably stable, as its gene is still present in the MLV genome after multiple serial passages in permissible cells⁷⁹, indicating that MLV has high genomic stability which should lend itself for use as a vaccine vector.

A major concern with the use of replication-competent MLV is its potential to infect and replicate in non-tumour cells. Although MLV is only able to replicate in rapidly dividing cells, making it permissible to tumour cell infection, there is still the possibility of off-target infections. A number of methods have been trialled to generate safer MLV vectors for use, such as the use of conditionally replicating MLVs where tissue-specific promoters are inserted upstream of the MLV genome to allow for replication in restricted cell types⁸⁰. Retrovirus-like particles can also be formed as MLV Gag expressed in cells can still bud from the membrane to form empty particles. Co-expression of MLV Env and Gag will allow the formation of particles capable of infection but are replication defective⁸¹. Other methods utilise DNA vaccination, where DNA encoding MLV Gag fused to a specific antigen is injected, and retrovirus-like particles displaying the desired antigen are produced in the host cell⁸². This has the advantages of being safer than working with infectious virus, and that DNA vaccines are easier to synthesise and scale up production.

The MLV particle is permissive to pseudotyping with various viral envelope proteins, which can insert into the membrane and form stable interactions with the MLV internal proteins. The glycoprotein of vesicular stomatitis virus (VSV-G) has often been pseudotyped into MLV particles, allowing for infection of a broader range of cell types⁸³. The envelope protein of MLV also has the potential for altered tropism through genetic modification. It has been shown that insertion of enhanced green fluorescent protein (EGFP) into the proline rich region of MLV Env allows for infection of cells and the expression of EGFP^{84,85}. This method of modifying MLV shows great promise for its use as a vaccine vector, as the envelope protein could be modified with an exogenous antigen yet still allow for correct MLV particle formation, as only Gag is required for virus budding.

1.3.3 Mechanism for proposed B cell recombinant vaccine

The main limitation of many B cell vaccines is that weak antigens fail to initiate T cell-dependent B cell activation, which is required for efficient antibody responses and memory⁴³. This is due to more rigid T cell selection processes which eliminate low avidity T cells in the thymus; however B cell selection is less stringent, hence the presence of B cells specific for weak antigens circulating in the periphery. It is unlikely that such B cells will be activated by T cells specific to the same weak antigen due to tolerance mechanisms; however these B cells may be activated by T cells of a different specificity, as implied by the intermolecular help model. An example is the *Haemophilus influenzae* type b (Hib) vaccine for children, where the bacterial polysaccharide is a weak antigen (T-cell independent) and is conjugated to an immunogenic protein carrier, in this case tetanus or diphtheria toxoid. B cells with polysaccharide-specific BCRs bind the vaccine by the carbohydrate moiety, and after internalisation and processing, peptide from the protein component can be presented by MHC class II to toxin-specific CD4+ T cells (present due to prior tetanus/diphtheria immunisation). These activate polysaccharide-specific B cells, resulting in long lasting protective immunity against Hib.

Similar approaches have been attempted for cancer vaccines by conjugating weak tumour antigens to stronger antigens, but the humoral immune response is dominated by the more immunogenic antigen, as both components of the vaccine are exposed for BCR binding (epitope dominance). For example, a carbohydrate antigen over-expressed on cancer cells was fused to a murine mAb and used to vaccinate Rhesus monkeys. High titres of anti-murine antibodies appeared 20 days after a single vaccination, whereas levels of anti-carbohydrate antigen failed to reach 1/1000th of the anti-murine antibody titre even after four doses of the vaccine⁸⁶. Likewise a phase I clinical trial of vaccination of HER2/CEA (carcinoembryonic antigen) fused to a bacterial heat labile toxin in the form of a DNA prime and adenoviral boost failed to elicit any cell-mediated responses via ELISPOTs, although high titres of anti-toxin antibodies and large numbers of anti-toxin cells were detected⁸⁷. This supports the idea that simply combining a highly immunogenic antigen with a weak antigen may not necessarily boost the immune response to the weaker antigen. Clearly, more sophisticated methods of linkage are required.

In order to direct the B cell immune response to poorly immunogenic antigens, it is desirable to link these weak antigens to strong viral antigens that are shielded from BCRs. This can be

done by presenting the weaker antigen on the surface of a particle with immunogenic T cell epitopes enclosed within. Viruses provide a unique opportunity to create such particles, where there will be no external viral components available for BCR binding and hence no neutralising anti-viral antibodies will be generated. As well as binding to TCRs via MHC class II complexes, the viral proteins and RNA genome can bind to Toll-like receptors (TLRs) and enhance the immune response to the weak antigens, acting as an adjuvant. The EC domain of the virus envelope protein must be removed and replaced with the weaker antigen in order to prevent any off-target immune responses to the virus envelope. Due to the chimeric nature of such a virion, the resulting vaccine particle will be non-infectious yet display epitopes in a highly organised fashion to enable BCR crosslinking and B cell activation to the poorly immunogenic antigen. This is summarised in Figure 1.7.

MLV is an ideal candidate for creating such a vaccine particle as it is a simple virus with only one external virus protein, Env glycoprotein. The EC domain of this can be substituted with a tumour antigen, allowing for presentation of the exogenous antigen on the surface of MLV in place of the native viral glycoprotein. However in order for the modified surface protein to be incorporated into the virus particle, the tumour antigen must be fused to the viral Env TM and cytoplasmic domain, which interact with Gag during particle assembly. These portions can be retained without affecting the immune response towards the surface antigen as they are inaccessible to BCRs, but can still be presented via the MHC pathway to T cells. Membranous tumour antigens would be preferable rather than cytoplasmic antigens, as the EC domain of these tumour antigens could be used to replace MLV Env, and would be predicted to fold properly and retain its native form upon exposure to the EC environment. This is important in order to elicit antibodies from the vaccine particle that can then bind the same tumour antigen on cancer cells. Also, as described previously, anti-tumour antibodies function by binding to the surface of tumour cells to initiate immune-mediated mechanisms of cell killing or antagonising signalling pathways; if polyclonal antibodies were generated by a vaccine to an internal antigen, it would be less likely to bind to its target as the intracellular (IC) environment would not be accessible to the antibodies.

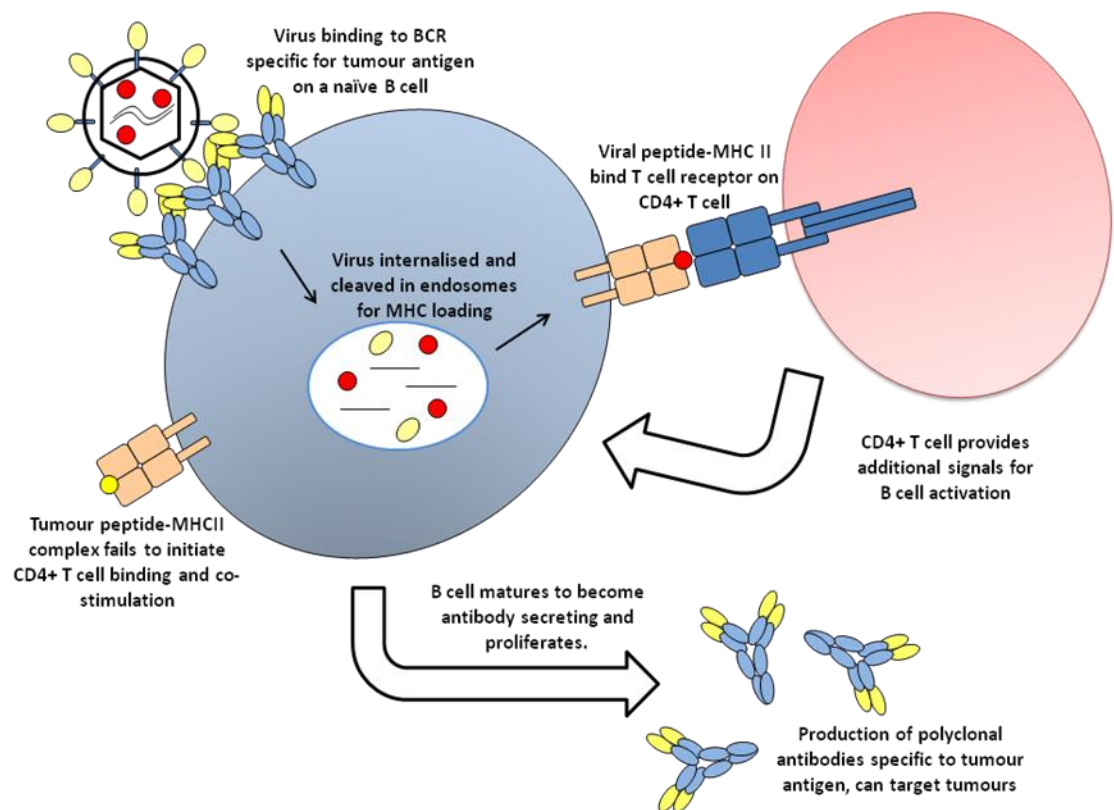


Figure 1.7 | Mechanism for proposed B cell cancer vaccine.

Membrane-bound antibodies on naïve B cells bind to tumour antigens (shown in yellow) on the surface of the virion, triggering internalisation of the particle. Both viral and tumour antigens are cleaved in endosomes. Viral peptides (red) are loaded onto MHC class II for presentation to CD4+ T cells. T cells with viral-specific TCRs bind to viral peptide-MHC class II complexes and activated tumour antigen-specific B cells, leading to a polyclonal antibody response against the tumour antigen (indicated in as yellow antigen binding domains on BCR and soluble antibodies). Tumour peptides (yellow) presented on MHC class II molecules fail to recruit T cell help.

1.4 Adenovirus

1.4.1 Life cycle of adenovirus

Adenoviruses are a diverse group of non-enveloped viruses with icosahedral nucleocapsids of 90 nm, containing a double-stranded DNA genome of around 34 – 43 kb (Figure 1.8), leading to its use as a common vector in research. They are able to infect a wide range of vertebrate hosts; human adenoviruses consist of 57 serotypes alone. Human adenoviruses are common pathogens found in the respiratory and gastrointestinal tracts. Although most adenoviral infections are mild, severe morbidity can occur in immune suppressed patients, military recruits and young children⁸⁸.

All serotypes of human adenovirus enter the cell by binding to CAR (coxsackie virus and adenovirus receptor) on the cell surface⁸⁹, with the exception of group B human adenovirus which binds CD46⁹⁰. Integrins act as co-receptors for adenovirus binding, and this interaction leads to endocytosis via clathrin-coated pits of the adenoviral particle into an endosome⁹¹. Acidification of the endosome results in disassembly of the viral particle, and the release of the virus into the surrounding cytoplasm. The virus harnesses cellular microtubules for transport to nuclear pore complexes, where the viral capsid is furthered disassembled and viral DNA is released. This enters the nucleus through pores in the nuclear membrane. Virus DNA transcription, replication and packaging takes place, characterised by the early phase (non-structural proteins) and late phase (structural proteins). The linear genome is made up of five early units (E1A, E1B, E2 - 5) and five late units (L1 – L5), as well as several accessory genes⁹². After expression of E1A, the E1A protein then activates transcription of the other early viral genes through interaction with cellular transcription factors⁹³.

While the early regions encode products important for viral replication, the late units encode the structural proteins required for virus assembly, such as the hexon (encoded by L3). The products of the early phase region are required to alter the expression of host proteins for adenovirus replication, activating transcription of other viral genes, and preventing premature apoptosis of the host cell before viral replication and assembly is complete. New virion particles are subsequently assembled, consisting of 240 trimers of hexon protein and 12 pentamers of penton base; other minor capsid proteins are required to bind the viral shell together and also to form a compact nucleoprotein complex with the viral DNA genome⁹². The

newly completed adenovirus particles are released into the extracellular environment by cell lysis. The life cycle of adenovirus is summarised in Figure 1.9.

1.4.2 Current limitations of adenovirus as a vaccine vector

The small genome size of adenovirus makes it amenable to genetic modification. Deletion of the E1A region renders adenoviruses replication-defective, as E1A is important for activating the transcription of the other viral genes. The E3 region may also be deleted, as it is non-essential for virus replication but allows viral escape through interference of the MHC class I presentation pathway.

Although the use of adenovirus for gene therapy and vaccination is associated with high transgene expression and anti-tumour antigen responses, it is also linked to high anti-vector responses, partially due to pre-existing anti-adenovirus immunity which most humans acquire through exposure to wild type adenovirus in the environment⁹³. This pre-existing immunity can be composed of both neutralising antibodies and cell-mediated immunity against adenovirus^{94,95}, resulting in the clearance of adenoviruses from the circulation before it can exert a therapeutic effect. Even if any viruses are successful in cell infection and transgene expression, the highly immunogenic viral proteins will skew the immune response away from the weaker tumour antigens. Alternative measures to circumvent this have included the development of rare or non-human adenoviruses as vectors^{96,97} and the use of different adjuvants in order to enhance the immune response to the tumour antigen⁹⁸. Other strategies include heterologous prime-boosting, where the same antigen is encoded by different vectors such as DNA/adenovirus combinations⁸⁷ or adenovirus in conjunction with a different virus, such as Semliki Forest Virus⁹⁹.

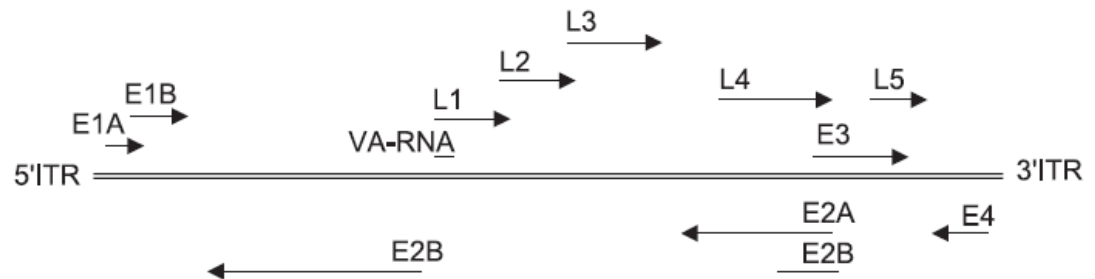


Figure 1.8 | Adenovirus genome.

The linear genome is flanked by inverted terminal repeats (ITR). E indicates early genes, L indicates late genes. VA: virus-associated. Arrows indicate direction of transcription which make full use of the small genome size. Figure adapted from Tatsis et al 2004.

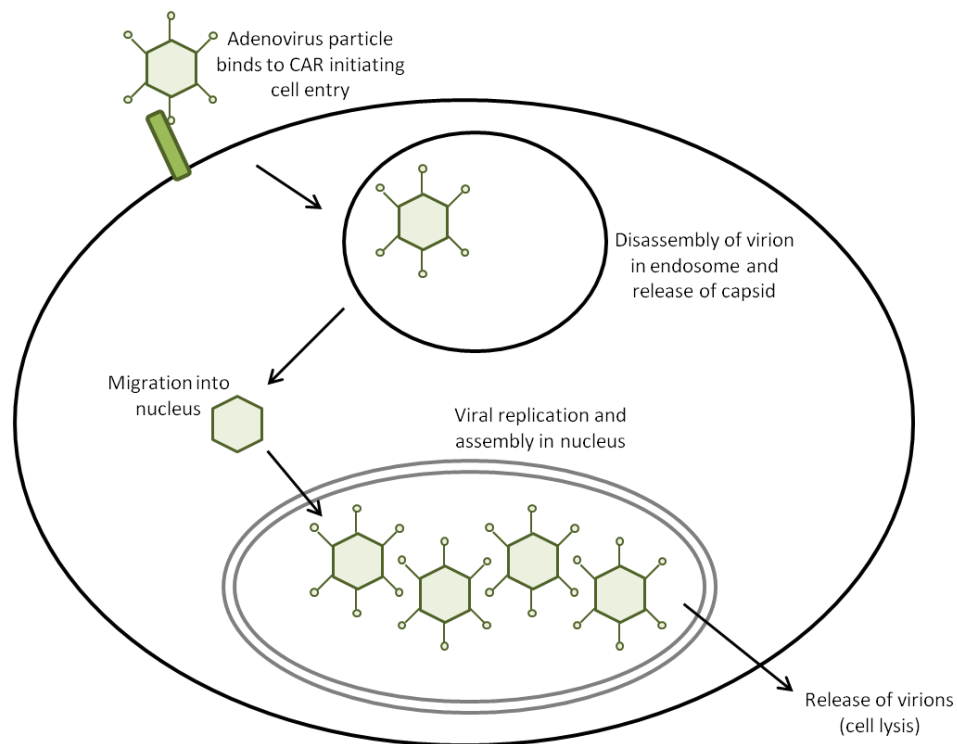


Figure 1.9 | Life cycle of adenovirus.

Adenovirus binds to CAR (coxsackie virus and adenovirus receptor) on the cell surface. Interaction with integrins leads to endocytosis of the adenoviral particle into an endosome. Acidification of the endosome results in disassembly of the viral particle, and the release of the virus capsid into the surrounding cytoplasm. The virus is further disassembled leading to the release of viral DNA, which enters the nucleus through nuclear pores. Virus replication takes place, characterised by the early phase (non-structural proteins) and late phase (structural proteins). New virion particles are assembled, which are subsequently released by virally-induced cell lysis. Figure adapted from Gomez-Navarro et al 2000.

1.5 Choice of Tumour Antigens

1.5.1 5T4 – an oncofoetal glycoprotein

5T4 (also known as TPBG, trophoblast glycoprotein) is a glycoprotein that is normally expressed in the specialised trophoblast cells of the foetal placenta during the early stages of pregnancy¹⁰⁰. It is a type I membrane protein of 72 kDa, and is thought to assist in the implantation of the foetus into the endometrium of the womb¹⁰¹. 5T4 presents itself as an ideal choice for cancer vaccination as under normal conditions it is only present in high levels in the placenta (foetal trophoblast and amnion), but has been implicated in a wide range of cancers such as colorectal, ovarian and lung¹⁰². As 5T4 may be involved in epithelial-mesenchymal transition (associated with cell migration and adhesion), it is used as an indicator of poor prognosis in cancer¹⁰³; hence, 5T4 is an attractive target for cancer immunotherapy.

Despite its potential as a cancer vaccine, few therapies have been generated using 5T4 to date. In mice, a heterologous prime-boost strategy using both replication defective adenovirus and transduced DCs carrying human 5T4 showed efficacy when given as a prophylactic vaccination, but failed to show any therapeutic benefits in mice with established tumours¹⁰⁴. More promisingly, human 5T4 has been encoded in modified vaccinia Ankara (MVA) viral vector, a highly attenuated strain of vaccinia, known as TroVax. Prophylactic vaccination with TroVax confers protection towards tumour challenge in a syngeneic model for BALB/c mice, while administration of the vaccine in a therapeutic setting results in a significant reduction of tumour burden¹⁰⁵. Further investigation of the immune mechanisms of TroVax found that the anti-tumour activity was due to antibody and CD4+ T cell responses, as depletion of CD4+ T cells abolishes any anti-tumour activity, while CD8+ T cell depletion had little effect on therapeutic vaccination of TroVax. This highlights the importance of CD4+ T cells in anti-tumour immune mechanisms, and possibly indicates that any response is unlikely to be wholly T cell mediated.

TroVax was administered to patients with metastatic renal cancer along with IL-2 in a phase II clinical study. Out of a total of 25 patients, 21 showed specific anti-5T4 responses, peaking after the third administration of TroVax; however all 25 patients showed anti-MVA antibodies at higher magnitudes than for 5T4¹⁰⁶. In a phase II clinical trial treating patients with prostate cancer, all 24 patients showed significant anti-5T4 immune responses, with seven patients

developing high numbers of 5T4-specific T cells and ten patients displaying high anti-5T4 antibody titres¹⁰⁷. Out of the 21 patients with pre-existing MVA immunity, ten patients showed a two fold increase in anti-MVA responses after vaccination. Patients receiving first-line standard of care (SOC) treatment for metastatic renal cancer were also administered TroVax in a phase III placebo-controlled trial. A total of 733 patients were recruited and 365 were given 13 TroVax injections in combination with sunitinib (a receptor tyrosine kinase inhibitor, IL-2 or interferon- α , while the remaining patients only received the normal SOC treatment. Antibody responses were analysed after the third and fourth vaccinations, where 96% of patients receiving TroVax showed positive MVA antibody responses while only 56% showed anti-5T4 antibodies¹⁰⁸. No statistically significant difference was seen in overall survival rates between the TroVax vaccinated and placebo group, with the median rates being 20.1 months and 19.2 months respectively. Despite the progression of TroVax through many clinical trials, an effective 5T4 cancer vaccine remains elusive.

1.5.2 HER2 receptor tyrosine kinase

The human epidermal growth receptor (HER) family are a group of transmembrane tyrosine kinases that are important in many cellular processes, such as survival, proliferation, adhesion and migration¹⁰⁹. The family is made up of four distinct receptors – HER1 or EGFR (epidermal growth factor receptor), HER2, HER3 and HER4. Each receptor, excluding HER2, binds a variety of ligands including EGF (epidermal growth factor), TGF- α (tumour growth factor) and neuregulins. These ligands bind to the EC domains of HER1, 3 or 4, resulting in the formation of homodimers or heterodimers with other receptors in the same family.

Once activated, tyrosine residues on the cytoplasmic portion of each receptor in the dimer pair are cross-phosphorylated, forming docking sites for various signalling complexes. This leads to the activation of several signal transduction pathways, such as the MAPK (mitogen activated protein kinase) pathway. The endpoint of these pathways leads to the activation of transcription factors that act on many genes involved in cell proliferation and survival. This is summarised in Figure 1.10.

HER2 is an important dimer partner for the other members of the HER family, and activated HER2 heterodimers are often more stable than other pairings, leading to sustained receptor signalling. As HER3 lacks a tyrosine kinase domain, it must depend on its dimer pair for phosphorylation; HER2 tyrosine kinase has the strongest catalytic activity, and when paired

with HER3 can lead to strong signalling. Once internalised, HER2 heterodimers are often recycled and returned to the cell surface for another cycle of activation, while other HER homodimers are internalised and targeted for degradation instead. HER2 can also pair with other membrane receptors, such as insulin-like growth receptor I¹¹⁰. As a result, the number of HER2 molecules available on the cell surface for heterodimerisation can play an important role in cell signalling, despite its lack of a ligand binding domain.

HER2 overexpression is seen in approximately 20% to 40% of breast cancer patients, and correlates with poor prognosis and an aggressive phenotype¹¹¹. Breast cancers can have up to 50 copies of the HER2 gene, leading to a 40 – 100 fold increase in HER2 receptors at the cell surface. Clinical HER2 expression can be analysed for by immunohistochemistry, fluorescence in situ hybridisation (FISH) for gene amplification, or through the detection of HER2 in serum, as overexpression is associated with increasing shedding of the EC domain of HER2 into the circulation¹¹². Normal tissues show relatively low HER2 expression; hence this differential expression between normal and cancerous tissues makes HER2 an ideal target for cancer therapy.

Trastuzumab (or Herceptin) is a mAb licensed for patients identified as HER2-positive. The humanised IgG1 antibody binds to the membrane proximal region of the EC portion of HER2, and works by several mechanisms. Firstly, trastuzumab binding blocks HER2 dimerisation, and can therefore promote increased internalisation of HER2, lowering the potential for HER2 signalling from the cell surface. Trastuzumab also recruits immune cells via its human Fc portion, initiating antibody-dependent cell mediated cytotoxicity and tumour apoptosis¹². However despite its success in treating both early and advanced stage HER2-breast cancer, most patients eventually develop resistance to trastuzumab, with relapse and disease progression in less than 6 months. Although the mechanisms behind this resistance remain unclear, it is thought that treatment with trastuzumab may select for HER2-negative cells, which survive and continue to proliferate, or that HER2 can avoid interaction with trastuzumab by shedding its EC domain, leaving behind a phosphorylated truncated protein (p95) which is still able to recruit signalling complexes¹¹³. Up to 90% of HER2-positive breast cancers can also become resistant to trastuzumab by upregulating expression of HER2 splice variant Delta16, which has an altered EC domain leading to the formation of constitutively active homodimers on the cell surface¹¹⁴. Other mechanisms that resistant tumours may have developed include the use of alternative signalling pathways, alternative dimer partners, or constitutive signalling of the pathways downstream to the HER family, such as the PI3K-Akt pathway

Due to the risk of developing trastuzumab-resistant tumours, and the association of trastuzumab treatment with cardiac dysfunction¹¹⁵, new treatments for HER2 positive cancers are urgently needed. Alternative HER2 targeted therapies include arming trastuzumab with various molecules to increase cytotoxicity, although some combinations have resulted in hepatotoxicity. The most successful to date has been trastuzumab conjugated to microtubule polymerisation inhibitor (DM1). Patients with trastuzumab and lapatinib (a small molecule inhibitor of tyrosine kinase)-pretreated metastatic cancers and showing disease progression were treated with trastuzumab-DM1 in a phase II study, producing response rates of up to 41%¹¹⁶.

Pertuzumab is a mAb approved in 2012 by the FDA (Federal Drug Administration) for treatment of HER2 positive breast cancer¹¹⁷. It binds to a different site of HER2 than trastuzumab, and was seen to block ligand induced dimerisation of HER2 and its partners more effectively than trastuzumab. Preclinical studies indicated that the combination of both trastuzumab and pertuzumab, each targeting distinct epitopes and working by different mechanisms would complement one another and result in enhanced cytotoxicity¹¹⁸. HER2-positive patients whose disease had progressed despite trastuzumab therapy were recruited for a phase II study and treated with a combination of pertuzumab and trastuzumab. Out of a total of 66 patients, an overall response rate of 24.2% was achieved, and the observed median time to progression-free survival was 5.5 months¹¹⁹. However as with trastuzumab, the main limitation of treating with a single agent is that resistance can easily arise as only a single site of HER2 can be targeted at a time.

It would be preferable to treat HER2-positive cancers with active immunotherapy, as the generation of polyclonal antibodies would slow the development of resistant tumours. Polyclonal antibodies would be able to target multiple epitopes and adapt to epitope changes through somatic hypermutation. HER2 peptide vaccines were initially trialled with moderate success; however, despite being easy to manufacture and eliciting antigen-specific immune responses, the HLA restricted peptides studied only activated CD8+ T cells and contained too few epitopes to stimulate a strong and sustained immune response against HER2¹²⁰. It was found that by using either the full-length HER2 protein or a truncated form (either the EC or IC domain), the immune response against HER2 could encompass both class I and class II epitopes, thus fully activating the entire immune system against HER2. A phase I dose-escalation study in breast cancer patients found that four immunisations with a protein-based

vaccine made up of the EC domain of HER2 and a portion of the IC domain resulted in antibodies specific to the whole HER2 protein¹²¹. Hence, active immune therapy using HER2 as a target may achieve better efficacy if full length domains are used, rather than single peptides.

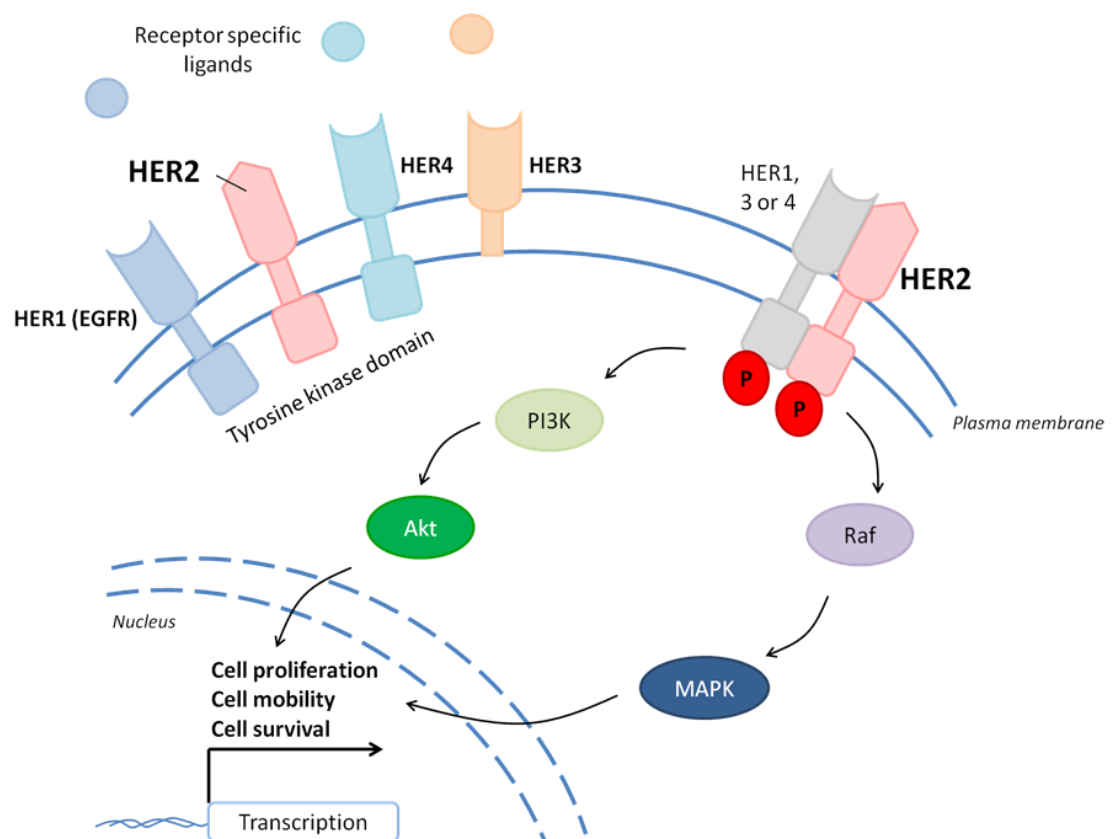


Figure 1.10 | Summary of signalling by HER family.

Phosphorylation through homodimerisation or heterodimerisation between members of the HER family leads to increased cell proliferation and survival. HER2 is the major partner for heterodimerisation. Phosphorylated tyrosines lead to the induction of the PI3K/Akt or MAPK pathway. Transcription factors are activated downstream of these signalling pathways, targeted important genes for cell mobility, survival and proliferation. Figure adapted from Hudis 2007.

1.6 Thesis Objectives

1.6.1 Hypothesis

The aim of this thesis is to induce T cell-dependent activation of B cells encoding BCRs against weak antigens by linking these to hidden strong viral antigens. Our hypothesis is that by obtaining this required T cell help, B cells specific for weak antigens can be activated and undergo proliferation and antibody production.

The hypothesis will be tested *in vivo* by “priming” T cells first with viral antigens, followed by a “boost” vaccination containing both the viral antigens and the weak antigens. This initial prime vaccine is to allow for the expansion of virus-specific CD4⁺ T cells that are available to provide intermolecular help to B cells after administration of the boost vaccine. This method of “homologous” prime-boosting differs from conventional heterologous prime-boost strategies, where different viral vectors encode the same weak antigen; instead, the same viral vector will be used but only the boost vaccination will contain the weak antigen, as the purpose of the prime vaccine is to stimulate virus-specific T cells only.

1.6.2 Outline of thesis

To produce an effective antibody response against weak antigens by:

- Genetically engineering amphotrophic Moloney MLV to express tumour antigens 5T4 and HER2 without viral Env
- Determining if these antigens can be expressed on the surface of the virus particle as a fusion protein
- Optimising virus production and purification to achieve high viral titres
- Development of a vaccination protocol *in vivo* using MLV vaccine and its ability to generate an antibody response
- Testing the hypothesis *in vivo* of intermolecular help (whether primed virus-specific T cells can activate antigen-specific B cells)

2 Materials and Methods

2.1 Tissue Culture

2.1.1 Cell lines and culture

Cell lines were cultured using 10% foetal calf serum (FCS, PAA Laboratories) and 1% penicillin (104 units/ml)-streptomycin (10 mg/ml) solution (Sigma-Aldrich) in various growth media (Table 2.1). Cells were cultured in a humidified incubator at 37°C and 5% CO₂.

2.1.2 Cell passage

When confluent, cells were sub-cultured at ratios of 1:10, 1:5, 1:2 depending on the growth rate of each cell line. Culture medium was removed and cells were washed with phosphate buffered saline (PBS, PAA Laboratories). PBS was removed and 0.05% trypsin/0.2% EDTA (ethylenedinitrilotetraacetic acid, Sigma-Aldrich) solution was added (2 ml for a standard T75 flask) and incubated for several minutes. Once cells had lifted from the surface, fresh medium containing serum (at four times the volume of trypsin) was added to deactivate the proteolytic action of trypsin. Cell suspensions and fresh medium were added to sterile tissue culture flasks, labelled and returned to the incubator.

Table 2.1 | Cell lines and growth media

Cell line	Origin	Use	Growth Medium
BHK-21 (BHK)	Baby Syrian hamster kidney fibroblasts	HER2 and 5T4 negative cell line, transfections	DMEM, 10% FCS and antibiotics.
293A	Subclone from 293 cell line (primary embryonal human endothelial kidney transformed with sheared human adenovirus type 5 DNA).	Transfections, VSV-G pseudotyping and stable recombinant retrovirus production, non-replicating adenovirus production	DMEM, 10% FCS and antibiotics.
SKBr3	Human mammary gland adenocarcinoma	HER2 positive cell line	DMEM, 10% FCS and antibiotics
MCF-7	Human epithelial mammary gland adenocarcinoma	5T4 positive cell line	MEM Alpha with Glutamax, 0.01 mg/ml bovine insulin, 10% FCS and antibiotics
CT26	Mouse colon carcinoma fibroblast (obtained from Oxford BioMedica)	Production of WT retrovirus	RPMI-1640 Medium, 10% FCS and antibiotics.
NIH 3T3	Mouse embryonic fibroblasts	Production of WT retrovirus	DMEM, 10% FCS and antibiotics
MLV HER2 293A	293A cell lines created from VSV-pseudotyped MLV HER2 infection	Production of MLV HER2 vaccine particles	DMEM, 10% FCS and antibiotics
MLV 5T4 293A	293A cell lines created from VSV-pseudotyped MLV 5T4 infection	Production of MLV 5T4 vaccine particles	DMEM, 10% FCS and antibiotics
MLV EGFP 293A	293A cell lines created from VSV-pseudotyped MLV EGFP infection	Production of MLV GFP vaccine particles	DMEM, 10% FCS and antibiotics

DMEM: Dulbecco's Modified Eagle's Medium (PAA Laboratories) – 4 mM L-glutamine, 4500 mg/l glucose, 1 mM sodium pyruvate, 1500 mg/l sodium bicarbonate; MEM: Minimum Essential Medium (Invitrogen) – Alpha medium: Earles balanced salt solution, Glutamax, sodium pyruvate (1 mM), non-essential amino acids, 2.2 g/l sodium bicarbonate; RPMI-1640: Roswell Park Memorial Institute (PAA Laboratories); contains 300mg/l L-glutamine.

2.2 Molecular Biology Protocols

Please see Section 2.14 for Buffers and Solutions.

2.2.1 Bacterial media

Bacterial media Luria-Bertani (LB) broth base (20g/l, Sigma-Aldrich) and LB broth agar (32g/l, Sigma-Aldrich) were made up with deionised water (dH₂O) and sterilised by autoclaving. Sterile agar was cooled before the addition of antibiotics (ampicillin or kanamycin at 100 µg/ml, Sigma Aldrich) and poured into 10 cm dishes before storing at 4°C.

2.2.2 Preparation of competent bacterial cells

DNA plasmids were grown in XL-10 Gold Ultracompetent or JM110 *E. coli* cells (Agilent Technologies) prepared in advance. Briefly, bacterial cells were grown in 3 ml sterile 2xYT medium (see section 2.14) and incubated at 37°C until an optical density of OD₅₅₀ = 0.300 was reached. Cells were transferred to a liquid culture of 200 ml 2xYT medium and grown until OD₅₅₀ = 0.480, after which cells were kept on ice for 5 minutes. Cells were spun at 3000 rpm at 4°C for 20 minutes before re-suspension in 80 ml sterile transformation buffer I (TFBI) total. Tubes were kept on ice for 5 minutes before spinning again. Pellets were re-suspended in 8 ml sterile transformation buffer II (TFBII) and left on ice for 15 minutes. Cells in TFBII were aliquoted in 100 µl volumes into eppendorfs before storage at -80°C for future use.

2.2.3 Transformation by heat shock

Competent cells were thawed on ice before 5 – 100 ng DNA was added and mixed by pipetting. Cells were left on ice for 20 minutes before heat shock at 37°C for 3 minutes, after which sterile LB broth was added and cells were incubated at 37°C for a further 15 minutes. Transformed cells were added to antibiotic-selective agar plates by pouring or streaking to allow individual colony isolation and incubated at 37°C overnight.

2.2.4 Plasmid amplification

Positive colonies were screened by polymerase chain reaction (see section 2.2.9), where primers were available. Correctly transformed clones were selected and amplified in selective liquid culture, either 5 ml or 250 ml LB broth with the appropriate antibiotic. Bacteria cultures

were incubated overnight at 37°C with agitation. Isolation of plasmids was carried out by miniprep or maxiprep (QIAGEN). Amplified plasmids were verified by restriction digest and DNA sequencing (Source Bioscience). Purified DNA samples were quantified by NanoDrop spectrophotometer (Thermo Scientific).

2.2.5 Construction of plasmid DNA vectors

Sub-cloning is a technique used to move a particular gene or fragment of DNA from a donor plasmid into a recipient vector. Both donor and the recipient plasmids are cut with the appropriate restriction nucleases. The recipient plasmid is dephosphorylated with CIAP (calf intestinal alkaline phosphatase) to avoid re-ligation to itself without the inserted fragment, and to minimise background colonies after transformation. Both DNA plasmids are purified and ligated together form a new plasmid. New plasmids were analysed by restriction digestion and sequenced by the DNA sequencing facility at Source BioScience (Oxford).

2.2.6 Restriction digest

Plasmid DNA was digested using restriction endonucleases with their appropriate buffers (New England Biolabs). A typical reaction for screening purposes included DNA (1 µl or 0.5 µg), 10X buffer (NEB 1 – 4, 1 µl) 10X BSA (if required, 1 µl) and the appropriate restriction enzyme (0.3 µl) made up to 10 µl with DNase free water. This was incubated at 37°C for 1 hour before screening by gel electrophoresis.

2.2.7 Agarose gel electrophoresis

Agarose gels were used to estimate the size of fractionated DNA samples. They consisted of 1% agarose (Sigma-Aldrich) and ethidium bromide (0.5 µg/ml, Sigma Aldrich) in TAE buffer (see section 2.14). DNA Hyperladder I (Bioline) was run alongside samples to allow estimation of size. Samples were electrophoresed at 120 volts for 60 minutes in electrophoresis tanks (BioRad Laboratories), and DNA bands were visualised by UV transillumination (Alpha Imager, Alpha Innotech, USA). When required, DNA was extracted from gel by cutting the band out with a clean scalpel and using QIAquick Gel Extraction Kit (QIAGEN).

2.2.8 DNA ligation

After the appropriate restriction enzyme digest, the recipient vector was dephosphorylated with 2 μ l CIAP for 1 hour at 37°C. Enzymes were removed using miniprep kit buffers. The donor fragment was usually purified by gel extraction. The ligation reaction contained 10X ligation buffer (2 μ l), donor DNA (6 μ l) and recipient DNA (3 μ l), with T4 DNA ligase (1 μ l) and DNase free water up to 20 μ l, and incubated at room temperature. Control ligations were also set up, excluding donor fragment and/or DNA ligase. These were then transformed into competent cells.

2.2.9 Polymerase chain reaction (PCR)

PCR can be used to amplify a region of DNA using specific primer pairs (short 20 nucleotide sequences) flanking the desired sequence of interest. PCRs were carried out on PTC-225 DNA Engine Tetrad thermal cycler (MJ Research, Massachusetts). For amplifying specific sequences of DNA and/or adding restriction enzyme sites, AccuPrime Pfx DNA polymerase (Invitrogen), Expand HiFi PCR System (Roche) or Phusion Master Mix DNA polymerase (NEB) were used for high fidelity; for PCR screening (to confirm whether a sequence was correctly sub-cloned into a vector), PCR SuperMix (Invitrogen) or Platinum Taq DNA Polymerase (Invitrogen) was used.

Standard PCR cycles followed the same steps (Table 2.2). Temperatures and time apply to all polymerases unless otherwise stated in specific protocols. PCR products were analysed by agarose gel electrophoresis.

Table 2.2 | Standard PCR cycles.

Denaturing, annealing and extension steps were repeated in a cycle 30 times.

Step	Temperature	Time
Denature template	95°C	2 minutes
	94°C (Platinum)	30 seconds (Platinum)
Denaturing	95°C	15 seconds (AccuPrime)
	94°C (Platinum)	30 seconds
Annealing	Dependent on T _m of primers	30 seconds
Extension	68°C (AccuPrime)	1 minute per kb
	72°C	

2.3 Detailed Cloning Procedures

Table 2.3 | DNA plasmids used.

Plasmid Name	Description	Origin	Purpose
MLV EMD (pAC3 – EMD)	Full WT MLV genome with reporter GFP	Prof. Nori Kasahara (UCLA)	5T4 and HER2 cloned into MLV EMD
MLV YCD (pAC3 – YCD)	Full WT MLV genome with yeast cytosine deaminase	Prof. Nori Kasahara (UCLA)	EGFP cloned into MLV YCD
5T4 EC - Env/Shuttle Vector	Contains 5T4-Env fusion sequence	Mr Gene (Regensburg, Germany now Invitrogen)	Cloning 5T4 into MLV EMD; intermediary/shuttle vector for HER2 and EGFP cloning
pSV2-c-erbB2	Contains HER2 gene	Addgene (University of Texas MD Anderson Cancer Centre)	Cloning HER2 into MLV EMD (after PCR amplification)
pEGFP-C1	Contains EGFP gene	Clontech (USA)	Cloning EGFP into MLV YCD (after PCR amplification)
pMD2.G	Contains VSV-G gene	Dr. Aliaa Alamoudi (University of Oxford)	VSV-G pseudotyping
pSF-CMV-MLV-GAG-His	MLV gag sequence fused to His tag	Oxford Genetics (UK)	Production of recombinant Gag
OG313	Cloning plasmid with C-terminal His tag	Oxford Genetics (UK)	Production of recombinant 5T4
AdZ5	Replication defective Ad5, encoded in bacteria	Prof. Gavin Wilkinson (Cardiff University), Dr. Alison Tedcastle (University of Oxford)	Production of recombinant adenoviruses

Table 2.4 | Viruses generated from DNA plasmids.

Virus	Description	Origin	Purpose
MLV EMD (WT MLV)	Wild type virus with reporter GFP gene	MLV EMD DNA plasmid (Prof. Nori Kasahara, UCLA)	Comparison to recombinant viruses; heat inactivated virus used as a prime for <i>in vivo</i> studies.
MLV HER2	Recombinant MLV expressing HER2 on the virus surface.	MLV HER2 DNA plasmid, modified from MLV EMD	For use <i>in vivo</i> to generate anti-HER2 antibodies
MLV 5T4	Recombinant MLV expressing 5T4 on the virus surface	MLV 5T4 DNA plasmid, modified from MLV EMD	For use <i>in vivo</i> to generate anti-5T4 antibodies
MLV EGFP	Recombinant MLV expressing EGFP on the virus surface	MLV EGFP DNA plasmid, modified from MLV YCD	Prime for <i>in vivo</i> studies and a control against MLV HER2 and MLV 5T4
AdHER2	Replication defective Ad expressing HER2 transgene	AdHER2 DNA plasmid, modified from AdZ5	For use <i>in vivo</i> as a comparison to MLV HER2
Ad5T4	Replication defective Ad expressing 5T4 transgene	Ad5T4 DNA plasmid, modified from AdZ5	For use <i>in vivo</i> as a comparison to MLV 5T4

2.3.1 MLV plasmid and cloning of MLV 5T4

Murine leukaemia virus (MLV) plasmid containing replication competent MLV genome with either GFP (MLV EMD) or yeast cytosine deaminase (MLV YCD), a kind gift from Professor Nori Kasahara (UCLA), was cut with *NdeI* and re-circularised to increase the number of unique restriction sites available for sub-cloning (known as MLV *NdeI*). A synthetic gene was ordered from Mr Gene (Invitrogen) containing the corresponding MLV signal peptide, the extracellular domain (EC) of 5T4 followed by the viral transmembrane (TM) domains and cytoplasmic (CP) domains, flanked by *AgeI* and *Clal* restriction sites (Figure 2.1).

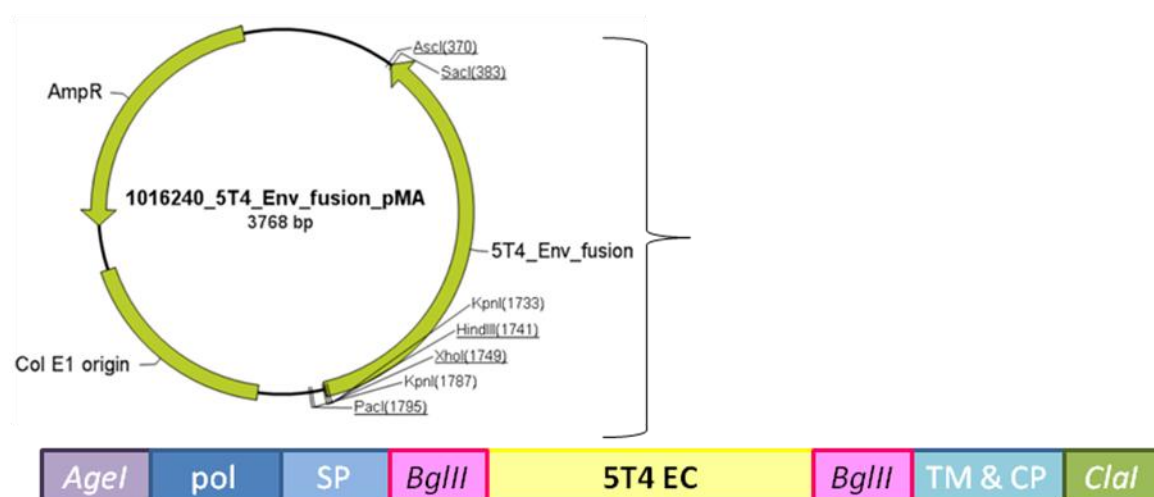


Figure 2.1 | 5T4 fusion gene.

DNA plasmid containing synthetic gene of 5T4 EC domain fused with IC viral domains, flanked by *BglIII* sites.

The 5T4 fusion gene sequence was missing a cytosine base, resulting in a frame shift and a truncated protein. To correct this, site directed insertion was carried out using PCR and the Mr Gene plasmid as a template and using phosphorylated primers: Fwd – CCTTCGCAACCTGACACAT, Rev – ACACGTAGGTCAGGCTCACC, where the underlined base marks the inserted cytosine. PCR was carried out using Phusion High Fidelity PCR Master Mix (New England Biolabs) and PCR cycles were as follows: 98°C 30 seconds then 30 cycles of 98°C 15 seconds, 54°C – 65°C 30 seconds, 72°C 2 mins followed by a final extension of 72°C 10 mins. The PCR product was purified by agarose gel extraction and ligated to itself to form a new vector. The corrected 5T4 fusion gene was sub-cloned into MLV *NdeI* by a double digest with

AgeI and *Clal*, and the subsequent MLV NdeI 5T4 was sub-cloned back into the original MLV EMD plasmid by *NdeI* digest. The plasmid was sequenced by Source BioScience (Oxford) to confirm correct, and amplified by maxiprep (QIAGEN).

2.3.2 Cloning of MLV HER2

HER2/neu plasmid (pSV2-c-erbB2) was obtained from Addgene (plasmid 16250, University of Texas MD Anderson Cancer Centre), and first cut with *BglIII* and re-ligated to remove an internal *BamHI* site (found in 0.6kb region of EC domain). HER2 EC domain was PCR amplified using AccuPrime Pfx DNA Polymerase (Invitrogen) and primers were: Fwd – ATA TGG ATC CAC CCA AGT GTG C, Rev – ATA TGG ATC CCG TCA GAG GGC T (Sigma-Aldrich), where underlined sites denote *BamHI* restriction sites introduced into the 5' and 3' termini of HER2 EC domain. PCR cycles were as follows: 95°C 2 minutes, then 30 cycles at 95°C 15 seconds, 55°C – 70°C 30 seconds, 68°C 5 minutes. The purified PCR product was digested with *BamHI* and cloned into shuttle vector (Mr Gene), replacing the 5T4 EC domain (*BglIII* and *BamHI* cut ends are compatible). Once the remaining 0.6 kb fragment of HER2 (containing the internal *BamHI* site) was sub-cloned into this shuttle vector, the plasmid was cut with *AgeI* and *Clal* and the complete HER2-viral fusion gene was cloned into MLV NdeI, then MLV EMD as before. The resulting plasmid was amplified by maxiprep (Qiagen) and verified by sequencing and restriction digest.

2.3.3 Cloning of MLV EGFP

EGFP (enhanced green fluorescent protein) encoded on pEGFP-C1 plasmid (Clontech) was amplified using AccuPrime Pfx DNA polymerase (Invitrogen) with primers: Fwd – TAT AGA TCT ATG GTG AGC AAG, Rev – **CTC** AAG ATC TGA GTC CGG ACT (Sigma-Aldrich) where underlined sites denote introduced *BglIII* restriction sites and in bold, an inserted stop codon. PCR cycles were as follows: 95°C 2 minutes, then 30 cycles at 95°C 15 minutes, 46°C – 65°C 30 seconds, 68°C for 1 minute. Purified PCR product was digested with *BglIII* and ligated into the shuttle vector (Mr Gene), replacing the 5T4 EC domain. This plasmid was then cut with *AgeI* and *Clal* to clone EGFP-viral fusion gene into MLV YCD NdeI and subsequently into MLV YCD. The final plasmid was amplified using a maxiprep kit (QIAGEN) and verified by sequencing and restriction digest.

2.3.4 PCR on cellular DNA for presence of *env*

PCR was carried out on DNA from virus producer cell lines to detect the presence of viral *env* gene in MLV genome. Briefly, DNA was extracted from cells using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's protocol. DNA concentrations were determined by nanodrop, and diluted to match the lowest concentration (52 ng/μl). PCR was carried out using Phusion Master Mix DNA Polymerase (NEB) and cycles were as follows: 98°C 30 seconds then 35 cycles at 98°C 10 seconds 60°C 30 seconds, 72°C 15 secs, followed by 72°C 10 mins. Primer sequences for targeting MLV *env* were: Fwd (pol region) – CTC ACT TAC AGG CTC TCT ACT TAG TCC, Rev (viral signal peptide) – TTA ATC TTA TCT TGA GGG GGT TTT G, Rev (viral envelope) – CAC ATA GAT CAA AAT ATA ATT TTG GGA A. PCR products were analysed by gel electrophoresis.

2.4 DNA Transfections

2.4.1 MLV transfection

Cells were seeded at 1 or 2.5×10^5 cells/well (depending on growth rate of cell) in 6 well plates and incubated overnight. DNA plasmids (5 μg/well) were diluted in Opti-MEM Reduced Serum Medium (Invitrogen) to 5 μg/well and mixed with Lipofectamine LTX and Plus Reagents (Invitrogen) modified from manufacturer's protocol; using 10 μl lipofectamine LTX and 3 μl Plus reagent for 5 μg DNA. This was made up to 500 μl and left for 30 minutes at room temperature before pipetting directly onto wells with 1 ml 10% FCS media. After incubation at 37°C for 4 hours, media was removed from wells and washed once with PBS, before replacing with 2 ml fresh media. Analysis of transfected cells and supernatant was carried out 24 or 48 hours after transfection.

Additional transfections were carried out using GeneJuice (Novagen). Cells were seeded in 6 well plates depending on growth rate of cell, and after incubating at 37°C for 30 mins – 3 hours in 10% FCS media, were transfected with 1 μg of DNA and 1.5 μl of GeneJuice per well (modified from manufacturer's protocol). Analysis of transfected cells were carried out 48 – 72 hours post-transfection. For larger scale transfections, cells were seeded in 10 cm plates and

left for a period of time for cells to adhere to plates, then transfected with 10 µg of DNA and 15 µl of GeneJuice, and analysed 72 hours later.

2.4.2 VSV-G pseudotyping

VSV-G plasmid (pMD2.G) and MLV HER2/EGFP/5T4 plasmid were co-transfected together in 1:4 ratio (40 µg total DNA) into 293A cells seeded 2×10^6 /10 cm plate. After 72 hours, supernatant containing pseudotyped MLV was collected and passed through a 0.22 µm filter and immediately added to a 6 well plate seeded with 293A cells (1×10^5 cells/well). Plates were centrifuged for 90 minutes at 1500 *g*. Cells were checked for successful VSV-G infection by FACS (see section 2.7).

2.4.3 Production of wild-type virus

MLV EMD DNA plasmid was transfected into 10 cm plates seeded with 1×10^6 293A cells. After 72 hours, supernatant was removed and filtered, and added to a 6 well plate of CT26 or NIH3T3 cells (2 ml/well). Plates were spun at 1250 rpm at 37°C for 1 hour before incubating at 37°C. Fluorescent cells were checked 72 – 96 hours post-infection. Successfully infected CT26 or NIH 3T3 cells were subsequently passaged.

2.4.4 Production of MLV HER2 and MLV 5T4 from BHK cells

BHK cells were seeded in 10 cm plates at 2.5×10^6 cells/plate in 10% FCS media. After 24 hours, cells were transfected with 30 µg DNA plasmid per plate with GeneJuice (1.5 µl per 1µg DNA) in 0% FCS media. After 72 hours, supernatant was collected and concentrated in 10 kDa Amicon tubes (Millipore). RNA was extracted from aliquots of supernatant to determine viral titre by RT-QPCR (see section 2.5).

2.5 Viral RNA Analysis

2.5.1 RNA extraction

RNA was extracted from cells and supernatant using SV Total RNA Isolation System (Promega). Cellular RNA was obtained following manufacturer's protocol. Supernatant was treated by adding 175 µl RNA lysis buffer to 175 µl supernatant, then 350 µl RNA dilution buffer before continuing with protocol. When extracting RNA from purified virus, 50 µl RNA lysis buffer was added to 50 µl virus, and mixed with 100 µl RNA dilution buffer before proceeding with the protocol as before. RNA concentrations were calculated by NanoDrop Spectrophotometer (Thermo Scientific).

2.5.2 Real time reverse transcriptase quantitative PCR (RT-QPCR)

RT-QPCR was carried out to quantify viral genomes, using Taqman RNA-to-CT 1-Step RT-PCR kit (Applied Biosystems). Reactions were set up in 96 well plates (Applied Biosystems) with volumes as shown in Table 2.5. PCR cycles were as follows: 48°C 15 minutes, 95°C 10 minutes, then 40 cycles at 95°C 15 seconds, 60°C 1 minute. Primer sequences for targeting MLV *gag* were: Fwd – CTT GAA CCT CCT CGT TCG AC, Rev – TTA GGT TTG GCG CCT AGA GA and the Taqman probe – GCC TCG ATC CTC CCT TTA, dual labelled at the 5' end with 6-carboxyfluorescein and at the 3' end with 6-carboxytetramethylrhodamine. Results were analysed using software for StepOne Plus QPCR (Applied Biosystems). Standard curves were made by using MLV HER2 DNA plasmid ranging from 10 ng to 1x10⁻⁶ ng.

Table 2.5 | Reaction mixture for RT-QPCR.

A master mix was first made up and 18 μ l pipetted into each well. 2 μ l of DNA standard or purified RNA was pipetted on the side of each well, and plates were spun down at 1000 rpm for 1 minute then sealed with adhesive film (Fisher Scientific).

Reaction component	Volume per well
Taqman RT-PCR Mix (40X)	10 μ l
Forward primer	0.18 μ l
Reverse primer	0.18 μ l
Probe	0.05 μ l
Taqman Enzyme Mix	0.5 μ l
Nuclease-free water	7.09 μ l
TOTAL	18 μ l

2.6 General Protein Protocols

2.6.1 Bicinchoninic acid (BCA) protein assay

The total protein content of whole cell lysates were quantified using QuantiPro BCA assay (Sigma-Aldrich). Proteins interact with Cu^{2+} through peptide bonds, and cysteine, tryptophan and tyrosine side chains, leading to the formation of protein- Cu^{2+} complexes. This interaction also results in the reduction of Cu^{2+} to Cu^+ , which is proportional to the amount of protein present. Under alkaline conditions, BCA forms a blue-purple complex with Cu^+ which can be used to measure Cu^{2+} reduction by proteins. Protein quantification was carried out in a 96 well plate by adding 50 μl BCA mixture (made up according to manufacturer's protocol) to 50 μl cell lysate diluted with PBS. A standard curve of BSA (bovine serum albumin, NEB) was also set up, ranging from 10 $\mu\text{g/ml}$ – 1000 $\mu\text{g/ml}$ (50 $\mu\text{l/well}$). Plates were left for colour to develop for 1 hour at 37°C or 30 minutes at 60°C before reading at 595 nm using a Wallac 1420 Victor² multi-label plate reader (PerkinElmer Life and Analytical Sciences). To determine protein content, absorbances of unknown samples were extrapolated from the absorbance of the BSA standard curve.

2.6.2 Native and SDS polyacrylamide gel electrophoresis (PAGE)

To analyse the intracellular and extracellular protein content of cells, lysed cells were run on a polyacrylamide gel and subsequently transferred onto a nitrocellulose membrane for probing with a specific antibody. Native PAGE was carried out for proteins where the antibodies could only recognise their target if non-denatured (HER2 and 5T4).

Cells were lysed with Reporter Lysis Buffer (Promega) or MACS Lysis Buffer (Miltenyi Biotec) if purifying His tagged proteins, and frozen at -20°C overnight or -80°C for one hour. Samples were thawed and total protein concentration was calculated using QuantiPro BCA assay (Sigma-Aldrich). Protein samples were mixed with sample buffer and loaded onto a native or SDS polyacrylamide gel (Table 2.6). Protein samples to be loaded on SDS gels were first heated to 95°C to denature proteins. Gels were run at 100 V for 2 hours (native) or 160 V – 200 V for 1 – 2 hours (SDS) alongside a protein ladder (Page Ruler Plus prestained protein ladder, Fermentas).

2.6.3 Western blotting

Polyacrylamide gels were transferred onto nitrocellulose membranes (BioRad) for 90 minutes – 2 hours at 300 mA – 400 mA at 4°C. Total protein was visualised by staining with Ponceau S Solution (Sigma-Aldrich), to ensure all lanes were evenly loaded. The membrane was blocked with 5% milk powder in PBS (Fluka, Sigma-Aldrich) for 1 hour at room temperature. For detection of His-tagged proteins, blots were blocked overnight at 4°C with 5% milk and 5% BSA (Sigma-Aldrich) in PBS to reduce background staining further. Primary antibodies (Table 2.7) were added to the blocking solution at 1:500 dilution and incubated for 1 hour or overnight at 4°C. After washing three times with PBS/0.1% Tween, the membrane was incubated with secondary goat anti-mouse HRP-conjugated antibody (Santa Cruz) for 1 hour (1:10000 dilution in blocking solution) before washing again. ECL (enhanced chemiluminescence) Western Blotting Detection Reagents (Amersham, GE Healthcare) were added and the blot was visualised using Alpha Innotech gel documentation system by chemi-luminescence detection or by exposure to X-ray film (Fujifilm).

2.6.4 Dot blots

This method was used to confirm the presence of a specific protein in samples without the need to separate the total proteins first on a gel (for example for purified viruses). Small volumes of samples (2 µl, with all samples diluted appropriately to the same concentration) were pipetted directly onto nitrocellulose membrane and left to dry at room temperature. The membrane was then blocked with 5% milk powder, and probed with antibodies as with Western blotting.

Table 2.6 | Polyacrylamide gel components

Reagent	SDS-PAGE 10% Running Gel	SDS-PAGE Stacking Gel	Native PAGE 8% Running Gel	Native PAGE Stacking Gel
Deionised water	5.9 ml	4.1 ml	7.1 ml	4.14 ml
1.5M Tris HCl pH8.8	3.8 ml	N/A	3.75 ml	N/A
1.5M Tris HCl pH 6.8	N/A	0.75 ml	N/A	0.75 ml
Acrylamide/Bis	5 ml	1 ml	4 ml	1.02 ml
10% SDS	150 µl	60 µl	N/A	N/A
Ammonium persulphate	150 µl	60 µl	150 µl	60 µl
TEMED (neat)	20 µl	15 µl	20 µl	15 µl

2.6.5 Production of recombinant Gag protein

A DNA plasmid containing MLV Gag sequence and a His tag (Oxford Genetics) was used to produce recombinant Gag-His fusion protein (Figure 2.2).

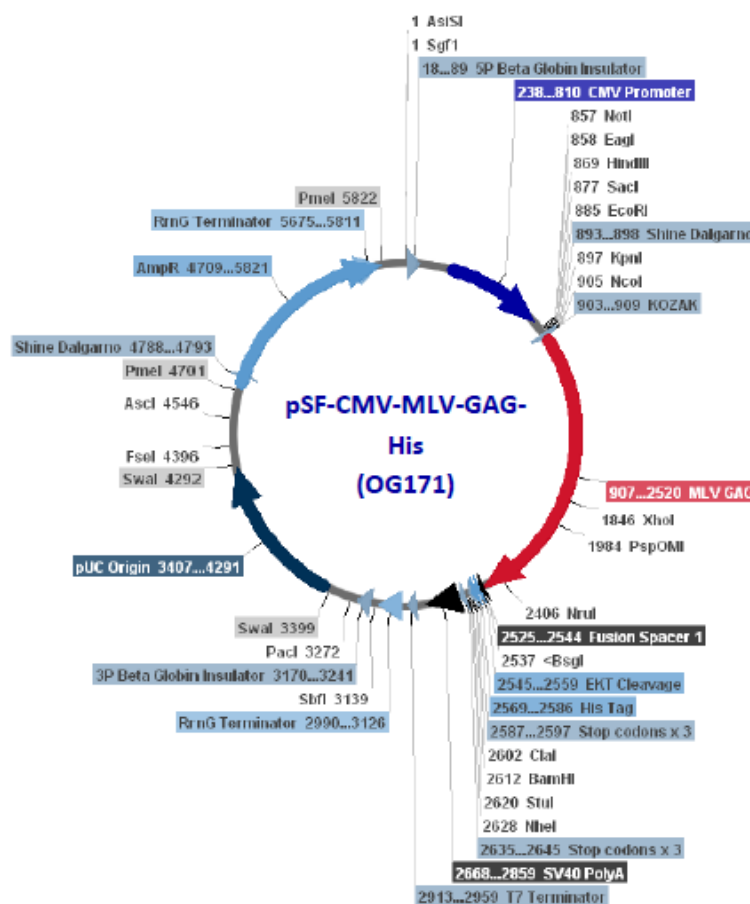


Figure 2.2 | Plasmid map of MLV Gag gene with hexa-His tag fused.

Image from Oxford Genetics.

10 cm plates were seeded with 2.5×10^6 BHK cells/plate and transfected later the same day with 15 μ g of MLV Gag-His DNA plasmid and GeneJuice. Cells were incubated for 72 hours before harvesting for protein purification, using μ MACS His Isolation kit (Miltenyi Biotec). Lysis buffer was added to each plate and cells were scraped and pooled together. Cell lysate was incubated for 30 minutes on ice before undergoing one freeze/thaw cycle to release further protein from cell membranes. Lysed cells were spun at 10 000 g for 10 minutes, after which supernatant was removed and incubated with 150 μ l anti-His microbeads per 2 ml cell supernatant for 30 minutes on ice. The supernatant and microbead mixture was then run

through an M column (Miltenyi Biotec) attached to a MACS separator, 2 ml per column. The manufacturer's protocol was followed, with several changes. Columns were rinsed five times with Wash Buffer 1 to reduce background binding, and Gag-His protein was eluted by pH shift resulting in native protein. Briefly, 60 µl of 0.1 M triethylamine (pH 11.8 with 0.1% Triton X-100) was applied to each column and incubated for 5 minutes. After this incubation period, 150 µl of 0.1 M triethylamine was added and collected in a tube containing 9 µl 1 M MES (pH 3) for neutralisation. Elutions from columns were pooled together. Subsequent elutions carried out using the same columns were found not to contain any Gag-His protein. To determine protein concentration, BCA assay was carried out and protein samples were analysed for purity by SDS-PAGE and Western blotting.

2.6.6 Production of recombinant 5T4-His protein

The EC domain of 5T4 was cloned into a plasmid containing a C-terminal His tag (plasmid OG313, Oxford Genetics). MLV 5T4 was used as a template for PCR to amplify 5T4 flanked by *NcoI* and *XhoI* restriction sites using Phusion Master Mix (NEB). PCR cycles were as follows: 98°C 30 seconds, then 35 cycles at 98°C 10 seconds, 55°C 30 seconds, 72°C 30 seconds and finally 72°C 10 minutes. Primer sequences for amplifying the 5T4 EC were: Fwd – ATA TAT ATC CAT GGC GCG TTC A , Rev – TAT CTC GAG AAG ATC TGG TTT G, where underlined sequences indicate *NcoI* and *XhoI* sites respectively. PCR products were pooled together and extracted from the gel using QIAGEN gel extraction kit. Both the PCR fragment and plasmid containing C-terminal His tag were digested with *NcoI* and *XhoI* before ligating and transformation. Correct colonies were selected and the correct plasmid was maxiprep (QIAGEN).

The plasmid was transfected into a 6 well plate seeded with BHK cells to test for correct expression of 5T4-His fusion protein. Cells were seeded in 10% FCS media and transfected 24 hours later in 0% FCS media. Cell lysate and supernatant were harvested 72 hours post-transfection. Cells were lysed with Promega lysis buffer and supernatant was concentrated in 3 kDa Amicon tubes before loading onto protein gels. Samples were run both on native PAGE (for 5T4 antibody probing) and SDS PAGE (for His antibody probing) gels before Western blotting. Purification of 5T4-His was then scaled up into 10 cm dishes. Supernatant was collected 72 hours post-transfection and concentrated in 10 kDa Amicon tubes, after which purification by MACS took place in the same manner as recombinant Gag-His protein. Correct production of 5T4-His fusion protein was verified by Western blotting.

2.7 Flow Cytometry and Fluorescent Activated Cell Sorting (FACS)

2.7.1 Flow cytometry

Cells were detached from plates by adding 300 µl enzyme-free cell dissociation buffer (Invitrogen) to each well. After incubation at 37°C for 10 – 20 minutes, cells were transferred into tubes containing 1 ml 10% FCS media. After spinning at 1500 – 2000 rpm for 5 minutes, the resulting pellet was resuspended in 200 µl PBS/0.05% sodium azide and incubated for 1 hour on ice with primary antibody (Table 2.7) or an isotype control antibody, at 1 µg/million cells. Cells were washed twice with PBS/azide and labelled with secondary antibody (donkey anti mouse IgG conjugated to Alexa 647, Invitrogen) in 200 µl PBS/azide on ice for 1 hour. Cells were washed five times before resuspension in 400 µl and analysed by CyAn ADP Analyzer (Beckman Coulter). Flow cytometry for GFP fluorescence only (no labelling) was carried out same as before without antibody labelling or washing steps, using FACS Calibur (BD Bioscience).

2.7.2 Selection of stable transfected cells by FACS

Infected cells were sorted after 48 hours by GFP fluorescence using FACS MoFlow, carried out by Andrew Worth (Jenner Institute, University of Oxford). Selected cells were sorted into a single well in a 96 well plate for optimal growth.

2.7.3 Flow cytometry for virus particles

Anti-HER2 antibody (10 µg, Millipore) was incubated with 8.5 µm Protein G beads (Bangs Laboratories) overnight at 4°C. After washing twice at 5000 rpm for 5 minutes with filtered PBS, virus particles produced from BHK cells (see section 2.4.4) and diluted with PBS to 1×10^8 vp total were subsequently incubated with the bead-antibody complex overnight at 4°C. The virus-bead complexes were washed twice with filtered PBS then once with filtered PBS/0.05% sodium azide/0.5% BSA before resuspending in 200 µl filtered PBS/0.05% sodium azide and labelled with anti-HER2 FITC-conjugated antibody (BD Biosciences). After 1 hour incubation on ice, the virus-bead complexes were washed twice at 1500 rpm for 5 minutes with filtered PBS then resuspended in 400 µl PBS/azide for flow cytometric analysis.

2.7.4 Flow cytometry for magnetically sorted splenocytes

Aliquots of splenocytes were taken from magnetic sorting (pre-sort, non-CD8⁺ cells and CD8⁺ cells), added to fresh 20% FCS RPMI media in 12 well plates, and left overnight at 37°C. Supernatant was removed from each well and divided into 2 separate FACS tubes (for antibody labelled cells and non-antibody labelled cells) with 2 ml PBS/azide in each. Wells were washed with 500 µl PBS/azide, then 120 µl cell dissociation buffer (Invitrogen) was added to each well, and incubated at 37°C for 10 – 20 minutes. Cells were removed from wells and added to eppendorfs (1 per well) with 400 µl media and mixed thoroughly to inactivate the dissociation buffer. The cell/media mixture for each eppendorf was divided between 2 FACS tubes containing supernatant and PBS/azide. Tubes were spun at 1500 rpm for 5 minutes and cell pellets were resuspended in 200 µl PBS/azide, and incubated with 0.5 µg anti-mouse CD8 antibody conjugated to Alexa 488 (BD Biosciences) for 1 hour on ice with shaking. After this, tubes were washed twice with 3 ml PBS/azide, and resuspended in 400 µl PBS/azide for flow cytometric analysis.

2.7.5 Live cell imaging

Pictures were taken under fluorescence microscopy using a Nikon 80i microscope and NIS Elements Imaging Software.

2.7.6 Flow cytometry analysis

Analyses were carried out using FlowJo (Treestar).

Table 2.7 | Antibodies used for Western blotting, flow cytometry, ELISA and electron microscopy.

Specificity	Type	Company	Purpose
Human 5T4	Mouse IgG ₁	Oxford BioMedica, UK	Binding to 5T4 (primary antibody) <i>WB & FC</i>
Human HER2, EC domain	Mouse IgG ₁	Santa Cruz (sc-08)	Binding to HER2 (primary antibody) <i>WB & FC</i>
Human HER2, EC domain	Mouse IgG ₁	Millipore (MABE320)	Binding to HER2 (primary antibody) <i>FC</i>
Human HER2, EC domain	Mouse IgG ₁ conjugated to FITC	Becton Dickinson (340553)	Binding to HER2 (primary antibody) <i>FC</i>
Green Fluorescent Protein	Mouse IgG _{2a}	Santa Cruz (sc-9996)	Binding to GFP (primary antibody) <i>WB</i>
Keyhole limpet haemocyanin	Mouse IgG ₁	BD Biosciences (349040)	Isotype control <i>FC</i>
Mouse IgG	Donkey conjugated to Alexa 647 fluorophore	Invitrogen (A31571)	Secondary antibody <i>FC</i>
Mouse IgG	Goat conjugated to horseradish peroxidase	Santa Cruz (sc-2031)	Secondary antibody <i>WB, ELISA</i>
Penta-His tag	Mouse IgG ₁	Qiagen (34660)	Binding to His-tagged Gag protein <i>WB</i>
Adenovirus 5	Polyclonal human serum	Sera Laboratories Intl (E412239)	Binding to adenovirus <i>ELISA</i>
Human IgG	Goat conjugated to horseradish peroxidase	Santa Cruz (sc-2453)	Secondary antibody <i>ELISA</i>
Mouse CD8	Rat IgG _{2a} conjugated to Alexa 488 fluorophore	BD Biosciences (557668)	Binding to mouse CD8 <i>FC</i>
Isotype	Mouse IgG ₁ conjugated to FITC	Chemicon Intl (CBL600F)	Isotype control <i>FC</i>
Mouse IgG	Goat Fab fragment conjugated to FITC	Sigma-Aldrich (F2772-5ML)	Secondary antibody <i>FC</i>

WB – Western blot; **FC** – flow cytometry; **EM** – electron microscopy; **ELISA** – enzyme-linked immunosorbent assay

2.8 *MLV Purification*

Stable cells (Table 2.1) producing modified MLV were grown in T175 flasks with 25 – 30 ml 2% FCS media. After a period of 4 days, supernatant was collected and spun down to remove cell debris. Virus-containing supernatant was either used immediately or frozen at -80°C for later use. For purification, 3 ml supernatant was transferred into UltraClear tubes (Becton Dickinson). UltraPure sucrose powder (Invitrogen) was made up into a 50% solution with sterile PBS and filtered. This was further diluted to 10% and 20% solutions. First, a 1 ml 10% sucrose cushion was carefully pipetted beneath the supernatant using a 50 mm 21G needle and syringe. A 1 ml 20% sucrose cushion was then underlayered, and the tube was filled to the top with viral supernatant. This was repeated for 5 more tubes, before loading onto a SW40Ti rotor and spun in Beckmann Dickinson L-70 Ultracentrifuge at 25 000 rpm at 4°C for 2 hours. After centrifugation, tubes were carefully decanted and washed gently with PBS, without disturbing the pellet at the bottom. The pellets were resuspended in sterile PBS (1 – 2 ml total for all 6 tubes) and pooled together, before inserting into a dialysis cassette (Slide-a-lyzer 10kDa, Fisher Scientific) using a needle and syringe. The purified virus was dialysed against PBS at 4°C, with PBS changed twice after one hour intervals before being left overnight. Dialysed virus was removed from the cassette using a needle and syringe, before being aliquotted and frozen.

The same procedure was carried out for purifying wild-type (WT) MLV from infected CT26 cells except after one round of centrifugation, the tubes were emptied and filled with 10% and 20% sucrose cushions, and supernatant as before. The same tubes were spun again at 25 000 rpm at 4°C for 2 hours, before decanting supernatant and refilling with more supernatant and sucrose cushions for one final spin (3 rounds of centrifugation in total), after which the supernatant was removed and virus pellets were resuspended in PBS as before. Multiple rounds of centrifugation were carried out to increase pellet size, as CT26 cells were not as permissive to infection by WT MLV as 293A cells, leading to lower titres if only one round of centrifugation was carried out.

2.9 Adenovirus Protocols

This protocol was taken from “Re-engineering adenovirus vector systems to enable high-throughput analyses of gene function”, Stanton et al 2008¹²².

2.9.1 PCR of transgene for insertion into adenovirus genome

PCR was used to add regions of homology to MLV-HER2 and MLV-5T4 to enable insertion into the AdZ5 genome by recombination (HER2 and 5T4 fused to MLV transmembrane and cytoplasmic domains). Primers used were: Fwd AAC CGT CAG ATC GCC TGG AGA CGC CAT CCA CGC TGT TTT GAC CTC CAT AGA AGA CAC CGG GAC CGA TCC AGC CTG GAT CCA TGG CGC GTT CAA CGC TCT C (sequence underlined corresponds to viral signal peptide), Rev GGC GTG ACA CGT TTA TTG AGT AGG ATT ACA GAG TAT AAC ATA GAG TAT AAT ATA GAG TAT ACA ATA GTG ACG TGG GAT CCT CAT GGC TCG TAC TCT ATG G (sequence underlined corresponds to viral cytoplasmic domain) from Sigma-Aldrich, 0.05 μ mol desalt purification. PCR was carried out using Roche Expand High Fidelity PCR System (Table 2.8).

Table 2.8 | PCR mix for amplification of transgene flanked by regions of homology.

DNA plasmids were first diluted 100x, and the total volume per reaction equalled 400 μ l.

	1 x 50 μ l reaction	MLV HER2	MLV 5T4
15 ng plasmid DNA		21.92 μ l	26.8 μ l
10 μ M Fwd primer	1.5 μ l	12 μ l	12 μ l
10 μ M Rev primer	1.5 μ l	12 μ l	12 μ l
10X buffer + MgCl ₂	5 μ l	40 μ l	40 μ l
dNTPs	1 μ l	8 μ l	8 μ l
Enzyme	0.5 μ l	4 μ l	4 μ l
dH ₂ O	Up to 50 μ l	302.08 μ l	297.2 μ l

PCR cycles were as follows: 95°C 2 minutes, 35 cycles of 95°C 30 seconds, 55°C 30 seconds, 68°C 1 minute 30 seconds; 68°C 15 minutes. PCR products were run on an agarose gel for verification, then purified further by ethanol precipitation. Briefly, 0.1 volumes of 3M sodium acetate pH 5.2 were added to the PCR products, followed by 2.5 volumes 100% ethanol. This was mixed and left at -20°C overnight. The mixture was spun in a bench top centrifuge at maximum speed at 4°C for 30 minutes, and the supernatant was removed. The pellet was

carefully washed with room temperature 70% ethanol, and spun again for 15 minutes at 4°C. The supernatant was removed and the pellet was air dried, and later resuspended in nuclease-free water (final volume 50 µl).

2.9.2 Making competent cells (SW102)

SW102 cells contain both the adenovirus genome and relevant recombination components in a bacterial artificial chromosome. Briefly, cells were inoculated into 5 ml LB containing 50 µg/ml ampicillin and 12.5 µg/ml chloramphenicol. Cells were incubated overnight at 32°C, and 0.5 ml of this culture was transferred into 25 ml LB (with ampicillin and chloramphenicol) and incubated at 32°C until $OD_{600nm} = 0.6$ was reached. The culture was incubated at 42°C for 15 minutes to induce recombination, and then cooled on ice for 15 – 20 minutes. Cells were centrifuged at 4000 rpm 0°C for 5 minutes, and the resulting pellet was resuspended in 1 ml ice cold water by gentle swirling. Once resuspended, 25 ml water was added and cells were spun again, repeating the wash step. Supernatant was decanted carefully and resuspended in remaining water (approximately 400 µl). The cells were transferred to 0.5 ml eppendorfs in 25 µl aliquots, and 3 µl purified PCR products were added. Aliquots were transferred to pre-cooled 0.2 cm cuvettes and left on ice for 5 minutes before electroporating at 2.5 kV. Bacteria were recovered in 5 ml LB for 4 hours at 32°C before plating out on agar plates containing 5% sucrose, chloramphenicol and X-galactose. Colonies were left to grow for 30 – 48 hours at 32°C.

2.9.3 Selecting correct colonies and minipreps

Non-opaque white colonies were picked from plates and cultured in 6 ml LB and chloramphenicol overnight at 32°C. The liquid culture was spun down and DNA was extracted from the pellet using a modified protocol of QIAGEN Miniprep kit. Briefly, the pellets were resuspended in 250 µl Buffer P1 and to this 250 µl Buffer P2 was added and left to incubate for 5 minutes. 250 µl Buffer N3 was then added, and spun for 10 minutes. The supernatant was removed, and 750 µl isopropanol was added and spun again for 10 minutes. At this point the supernatant was discarded, leaving behind a small pellet. 500 µl 70% ethanol was added, and spun for 10 minutes, after which the pellet was air dried and redissolved in 20 µl Elution Buffer. Miniprep DNA was digested with *Bam*HI restriction enzyme and run on a 1% agarose gel (made with TBE buffer) at 120V for 90 minutes to assess whether recombination into the adenovirus genome had been successful. Correct colonies were selected and grown in

250 ml LB with chloramphenicol and left overnight at 32°C with agitation. The culture was spun at 3750 rpm for 15 minutes, after which the resulting pellet was resuspended in Buffer P1 and P2, then incubated for 5 minutes before adding Buffer P3 and leaving for 10 minutes. The cell lysate was filtered into equilibrated QIAGEN tip/columns and washed twice with 30 ml Buffer QC and eluted in 15 ml Buffer QF. DNA was precipitated by adding 10.5 ml isopropanol in 0.7 ml volumes then spun at 15 000 g for 30 minutes at 4°C. The pellet was washed with 5 ml 70% ethanol and spun again at 15 000 g for 10 minutes before leaving the pellet to air dry. Once dry the pellet was redissolved in 100 µl Elution Buffer.

2.9.4 Clonal selection of AdHER2 and Ad5T4

DNA maxipreps were transfected into T25 flasks seeded with 293A cells, using 8 µg of DNA per flask with lipofectamine. The AdZ5 vector is self-excising and encodes *I-SceI* endonuclease; upon transfection, expression of *I-SceI* which removes the viral genome from the prokaryotic backbone to undergo its infectious life cycle. 293A cells contain components of the adenovirus genome required for replication, so that non-replicating adenoviruses can grow in these cells by complementation. Plaques were seen after 7 – 14 days, after which supernatant was harvested from flasks. Virus transfection and supernatant harvesting was carried out by Dr Alison Tedcastle (University of Oxford).

In a 96 well plate, 1×10^4 293A cells were seeded per well and previously harvested adenovirus supernatant was added to the wells in serially 10 fold dilutions. After one week, the plate was assessed and from wells where only a few cells displayed CPE (cytopathic effects), supernatant was taken and transferred to a 6 well plate seeded with 2.5×10^5 293A (1 well per clone). After 3 days, CPE was seen and supernatant from each well was taken and frozen in 1 ml aliquots at -80°C.

2.9.5 Determining Ad clones viral titre to assess transgene production

200 µl of thawed supernatant was used for DNA extraction, using GenElute Mammalian genomic DNA miniprep kit (Sigma). To create a standard curve, supernatant from non-infected cells were also harvested at the same time point and spiked with wild type adenovirus, and run through the DNA extraction kit alongside. Protocol followed for extraction was same as manufacturer's protocol.

Viral titre was calculated by QPCR (quantitative polymerase chain reaction) using Quantace Sensimix (Bioline). Reactions were set up as shown in Table 2.9.

Table 2.9 | Reaction mixture per well for QPCR.

A master mix was set up for the plate and 20 μ l was pipetted per well. 5 μ l of extracted DNA was then pipetted in each well, before spinning the plate down at 1000 rpm for 1 minute and sealed with adhesive film (Fisher Scientific).

Reagent	Volume
Mastermix	12.5 μ l
Forward primer (5 μ M)	0.25 μ l
Reverse primer (5 μ M)	0.25 μ l
Rox (reference dye – undiluted 25 μ M)	0.5 μ l
Probe (5 μ M)	0.25 μ l
Nuclease-free water	6.25 μ l
Extracted DNA	5 μ l

PCR cycles were as follows: 40 cycles of 50°C 2 minutes, 95°C 10 minutes, 95°C 15 seconds, 60°C 1 minute. Primer sequences to KT region of Ad genome were as follows: Fwd – TGG CTG TTA AAG GCA GTT TGG, Rev – GCA CTC CAT TTT CGT CAA ATC TT and the probe – TCC AAT ATC TGG AAC AGT TCA AAG TGC TCA TCT. Data was converted to viral particles/ml.

To assess which adenovirus clone gave the strongest transgene expression (and subsequently would be chosen for use in *in vivo* experiments), supernatant containing virus clones was used to infect 2.5×10^5 293A cells seeded in 6 well plates, diluted to 50 virus particles/cell (calculated using viral titres). After 24 hours, cells were lysed and cell lysates were run on a native PAGE and probed using the appropriate antibodies for Western blotting. From these blots, clones with the highest transgene expression were selected for further purification.

2.9.6 Growth of AdHER2 and Ad5T4 clones for purification

Supernatant from virus clones were used to infect T75 flasks containing approximately 5×10^6 293A cells. After 96 hours, flasks were tapped to remove any remaining adherent cells and total flask contents were transferred to tubes and spun at 1500 rpm for 5 minutes. Supernatant was removed from the tubes, leaving behind 2 ml which was used to resuspend the cell pellet which was transferred to 2 ml eppendorfs. This was frozen in dry ice and thawed in a 37°C water bath, and repeated twice to lyse cells fully. Once thawed, the tubes were spun

down briefly to pellet any cell debris, and the resulting supernatant was aliquotted and frozen at -80°C for later use.

2.9.7 AdHER2 and Ad5T4 growth in hyperflasks for purification

1×10^7 293A cells were seeded in hyperflasks in 10% FCS media (Corning CellBIND Surface hyperflasks, Sigma-Aldrich), using 2 hyperflasks per virus to be purified. When cells were 90% confluent (approximately after 4 days) 200 µl virus cell lysate from previous step was added to each flask, and media was replaced with 2% FCS media for infection. Infected cells were ready for harvesting when just rounded up and before cell lysis. For harvesting infected cells, each hyperflask was tapped to remove remaining adherent cells and emptied into 2 x 250 ml tubes per hyperflask. These were spun at 2000 rpm for 10 minutes at 4°C. PBS was used to wash the empty hyperflasks to ensure there were no remaining cells, and used to fill the same tubes and spun again. This was repeated with the second hyperflask (for the same virus) and used to fill the same 2 x 250 ml tubes as before. Once the washing and centrifugation steps were complete, each pellet was resuspended in 10 ml 2% FCS media and transferred to separate 15 ml tubes (2 tubes per virus). These were frozen at -80°C and thawed in a 37°C water bath a total of 3 times to fully lyse cells.

2.9.8 Purification of AdHER2 and Ad5T4 by caesium chloride banding

Buffers for purification were made up the day before purification (see section 2.14). To prepare the virus supernatant for purification, thawed virus lysate was spun at 2000 rpm for 10 minutes at 4°C. N-butanol was added to the supernatant in a 1:100 ratio and left on ice for 1 hour. Tubes were spun again at 2000 rpm for 10 minutes at 4°C and supernatant was loaded onto caesium chloride gradients in Beckman tubes. To prepare the gradients, 3 ml of $\rho = 1.32$ CsCl was added to the tube using a 5 ml stripette. This was underlayered with 2 ml $\rho = 1.45$ CsCl using a 50 mm 21G needle and syringe. On top of the CsCl gradients, 40% glycerol was added again using a needle and syringe. This was repeated for a further 3 tubes (4 in total per virus). Virus supernatant was added to each tube (6 -7 ml) and topped up with PBS if necessary. Tubes were spun as before (see section 2.8) at 25 000 rpm for 2.5 hrs at 4°C. After centrifugation, 3 white bands were present in each tube – the top band containing cell debris and protein, the second band empty capsid, and the final band virus. Using a 5 ml syringe and 40 mm 21G needle, each tube was carefully punctured and withdrawn to extract virus band. All virus bands were pooled together and injected into a 10kDa MW cassette (maximum

volume 12 – 15ml, Thermo Scientific) and dialysed against dialysis buffer 1 overnight at 4°C, with 2 buffer changes. Virus was removed from the dialysis cassette using a needle and syringe, and incubated with 6 µl benzonase per 1 ml virus for 30 minutes at room temperature. Caesium chloride gradients were set up as before, but into 2 tubes per virus. 1.5 ml virus was added to each tube, topped up with PBS to ensure tubes were balanced, and spun at 25 000 rpm for 2.5 hours at 4°C as before. After centrifugation, virus bands were extracted as before, inserted into a 20kDa MW dialysis cassette (maximum volume 3 ml, Thermo Scientific) and dialysed against dialysis buffer 2 overnight at 4°C. Virus was removed from dialysis cassette and aliquotted into eppendorfs, and stored at -80°C. Viral titres were determined by picogreen assay.

2.9.9 Picogreen assay

Picogreen is a fluorescent dye that binds to double-stranded DNA, giving a measurable fluorescent signal¹²³. The amount of double-stranded DNA in a virus preparation can be calculated by extrapolating from a standard curve of fluorescent intensities of known amounts of double-stranded DNA, and hence the number of virus particles can be approximated. Free DNA was removed by benzonase (part of purification step) to allow calculation of virus particles, assuming that 1 µg/ml of DNA is equivalent to 2.7×10^{10} vp. Using the Quant-iT PicoGreen Assay Kit (Invitrogen), purified virus was diluted in TE (Tris base and EDTA buffer)/0.5% SDS and incubated in a 56°C water bath for 30 minutes to allow lysis of viral capsids. A standard curve was made by diluting known amounts of bacteriophage λ DNA in TE/SDS by ten fold each time. 50 µl of either standard curve or sample were pipetted into a black 96 half-well microplate, along with 50 µl diluted PicoGreen reagent (Molecular Probes, Invitrogen). After incubation at room temperature for 2 minutes, the plate was read in a Wallac 1420 Victor² multi-label counter (PerkinElmer) using an excitation wavelength of 485 nm and emission of 535 nm. The amount of DNA in the samples was extrapolated from the signals of the bacteriophage λ DNA standard curve.

2.10 Immunological Assays

2.10.1 Enzyme-linked immunosorbent assay (ELISA)

High protein binding 96 well plates (Nunc) were coated with diluted recombinant peptide or virus (Table 2.10) and incubated at 4°C overnight. Wells were washed six times with PBS/0.05% Tween, then blocked with 5% BSA diluted in PBS. After 1 hour, blocking solution was removed from plates. Diluted serum samples and primary antibodies (see Table 2.7) were added to the plate (50 µl/well) and incubated for 2 hours at room temperature. After washing six times with PBS/Tween, secondary antibody (anti-mouse conjugated to HRP) was added to the plate in a 1:5000 dilution, and incubated for 1 hour at room temperature, followed by a final six washes in PBS/Tween. HRP substrate 1-Step UltraTMB (Fisher Scientific) was added to the wells (50 µl/well) and colour change was stopped after an appropriate time with acid stop solution (Alpha Diagnostics). Plates were read at 450 nm using a Wallac 1420 Victor² multilabel counter (PerkinElmer). For data analysis, background values (calculated from wells with secondary antibody only) were subtracted from raw data, to give net OD.

Table 2.10 | Antigen coated on ELISA plates.

Peptide/virus	Amount per well	Origin
Recombinant HER2 fused to human Fc	50 ng diluted in PBS	R & D Systems
Recombinant EGFP	50 ng diluted in PBS	Santa Cruz
Recombinant 5T4	50 ng diluted in PBS	Own protein stock
Non-replicating AdZ5 or non-replicating Ad5T4/AdHER2	1 x 10 ⁹ virus particles diluted in neutral pH coating buffer (Alpha Laboratories)	Dr Alison Tedcastle (AdZ5), own virus stocks (Ad5T4/HER2)
Heat inactivated WT MLV	1 x 10 ⁷ virus particles diluted in neutral pH coating buffer	Own virus stocks
Recombinant Gag	50 ng diluted in PBS	Dr. Siddharta Datta and Dr. Alan Rein (NIH/NCI)

2.10.2 Preparation of mouse splenocytes

Spleens were crushed with a syringe plunger or with a pestle and filtered through a 70 μ M cell strainer (Becton Dickinson) on top of a 50 ml Falcon tube. 10 ml 20% FCS RPMI media was added and cells were spun at 1350 rpm for 5 minutes. Red blood cells were lysed in ACK lysis buffer (see section 2.14) and incubated at room temperature for 5 minutes. 25 ml PBS was added and mixed with cells by inversion, then poured through a fresh 70 μ M cell strainer into a new 50 ml Falcon tube, before being spun again at 1350 rpm for 5 minutes. The cell pellet was resuspended in 10 ml media for cell counting.

For negative selection of CD8⁺ T cells, splenocytes were spun down in 15 ml Falcon tubes at 300 g for 10 minutes. Supernatant was removed, and cells were resuspended in cold MACS buffer, 90 μ l/ 10^7 cells. CD8a microbeads (Miltenyi Biotec) was added to the cell suspension, 10 μ l/ 10^7 cells. This was mixed well and incubated at 4°C for 15 minutes. After this incubation period, 10 ml MACS buffer was added and cells were spun down at 300 g for 10 minutes. The resulting cell pellet was resuspended in 500 μ l MACS buffer before being passed through pre-rinsed LS magnetic columns (Miltenyi Biotec). Fractions of non-magnetically labelled cells and magnetically labelled cells were retained for flow cytometry (see section 2.7.4). The flow through was retained (non-CD8⁺ cell population) and counted.

2.10.3 Enzyme-linked immunosorbent spot (ELISPOT) for IFN γ

IFN- γ ELISPOT ALP kit (Mabtech) was used to detect interferon gamma (IFN- γ) secretion from activated mouse CD4⁺ T cells. High protein binding PVDF 96 well plates (Millipore) were activated with 35% ethanol per well. 15 μ l of ethanol and sterile water was added to each well and left for a maximum of 1 minute, before washing three times with sterile PBS. Plates were coated with 50 μ l diluted capture antibody (AN18, 5 μ g/ml in PBS) and incubated overnight at 4°C.

Plates were washed three times with sterile PBS, and blocked with RPMI media containing 20% FCS for at least 1 hour at 37°C. When ready to seed cells, media was removed from wells and replaced with 1×10^5 – 2.5×10^5 splenocytes per well in 50 μ l media. An additional 50 μ l media was added to negative control wells (cells only). Concanavalin A Type VI (Sigma-Aldrich, 1 mg/ml stock in 0.9% sodium chloride solution) was used as a positive control, and used at 1 μ g/well. Recombinant peptides were added at varying concentrations in 50 μ l, and plates were

incubated at 37°C for 20 hours. Plates were washed five times with PBS then 50 µl biotinylated detection antibody (R4-642, 1 µg/ml) was added to each well. The detection antibody was diluted in PBS and filtered to reduce unspecific background due to protein aggregates. The plates were covered in tin foil and left for 2 hours at room temperature, after which plates were washed five times with PBS and incubated with 50 µl streptavidin-ALP (1 µg/ml in PBS) per well for 45 minutes. After washing plates five times with PBS, 50 µl of BCIP/NBT colour development buffer (Sigma-Aldrich) was added per well. Plates were left at room temperature until spots were seen developing (approximately 30 minutes – 1 hour) at which point wells were washed extensively with tap water to stop colour development. Plates were dried thoroughly and left to dry further overnight before reading in a plate reader (Jenner Institute).

For analysis, spot values from raw data was scaled up to a million cells. The background was taken to be the wells containing cells only for each spleen and subtracted. Positive values were those where the final value was more than 10 spot forming colonies per million cells.

2.11 Animal studies

2.11.1 Housing conditions and diet

Mice were housed in temperature controlled conditions at Biomedical Services, John Radcliffe Hospital, Oxford. Mice had access to sterile water and formulated diets, in individually ventilated cages (IVCs).

2.11.2 General animal techniques

Female BALB/c mice aged 4 – 6 weeks old were used in subsequent animal studies. All mice were anaesthetised for immunisation by inhalation of oxygen and isoflurane (2% - 4%). Vaccinations were carried out with 25G needle and 1 ml syringe sub-cutaneously on the back of each animal. For tail bleeds, mice were placed in a restrainer and the tip of the tail was cut with a clean sharp scalpel. Blood was pipetted into eppendorf tubes before centrifuging and removal of serum into fresh tubes. Serum was frozen at -20°C or -80°C. Cardiac punctures were carried out under anaesthesia using a 23G needle and blood was spun down as before. Mice

were sacrificed by a Schedule 1 approved method, and spleens were removed and placed into tubes containing 2 ml of 20% FCS RPMI media before transporting back to the laboratory.

2.11.3 Investigating prime-boost strategy using MLV HER2 and MLV EGFP

Mice were vaccinated three times in week 1 and three times in week 4 with 1×10^8 vp per mouse (30 μ l volumes), and tail bled once a week until week 6. For tail bleeds, 30 μ l of blood was collected and spun down at 13 000 rpm for 10 – 15 minutes and 10 μ l of serum was retained, or 20 μ l of blood was taken and added to 50 μ l PBS, centrifuged and 50 μ l diluted serum was retained. At week 9, mice were sacrificed by cardiac puncture. Diluted sera from various time points were tested for anti-HER2 or anti-GFP antibodies by ELISA.

2.11.4 Improving antibody responses *in vivo* with adjuvants

Table 2.11 | Groups for testing different adjuvants on antibody responses.

An additional mouse was used as a control and received no treatment. CFA: complete Freund's adjuvant; IFA: incomplete Freund's adjuvant. Numbers in brackets indicate number of mice.

	Antigen only (x 3)	Titermax only (x 1)	Titermax + virus (x 3)	Freund's only (x 1)	Freund's + virus (x 3)	Alhydrolgel only (x 1)	Alhydrolgel + virus (x 3)
Week 1 + 3	50 μ l MLV EGFP	50 μ l PBS + Titermax	50 μ l MLV EGFP + Titermax	100 μ l PBS + CFA	100 μ l MLV EGFP + CFA	50 μ l PBS + Alhydrolgel	50 μ l MLV EGFP + Alhydrolgel
Week 5, 7 + 9	50 μ l MLV HER2	50 μ l PBS + Titermax	50 μ l MLV HER2 + Titermax	100 μ l PBS + IFA	100 μ l MLV HER2 + IFA	50 μ l PBS + Alhydrolgel	50 μ l MLV HER2 + Alhydrolgel

Mice were injected sub-cutaneously in the back in 2 sites, following the dosing and schedule as shown in Table 2.11. All antigen and adjuvant dilutions were made up with low phosphate PBS (Gibco Invitrogen) to minimise phosphate interference with aluminium. Titermax Gold, Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA) were obtained from Sigma-Aldrich, 2% Alhydrolgel from Brenntag Biosector. All virus doses were 2×10^9 vp per mouse. Virus/PBS-adjuvant mixtures were prepared in a 1:1 ratio, except with Alhydrolgel where virus was mixed with 85 μ g per mouse. Titermax Gold-antigen formulations were emulsified by vortexing, Freund's adjuvant by using a hand-held homogeniser, and stable emulsions were tested by pipetting a droplet onto distilled water¹²⁴. All formulations were kept on ice and injected within 2 hours. Mice were bled at weeks 0, 4 and 6 by tail bleed where 20

μ l of blood was taken and added to 50 μ l PBS in an eppendorf. This was spun down at 13 000 rpm for 10 – 15 minutes, and 50 μ l diluted serum was transferred to fresh tubes and placed on dry ice. Mice were sacrificed at week 11 by cardiac puncture, and spleens were also removed for ELISPOT. Diluted sera from mice was tested by ELISAs.

2.11.5 Comparison between adenovirus and MLV vectors, and prime-boosting

Table 2.12 | Groups for comparison between Ad and MLV, and prime-boosting.

An additional group of three mice were used as controls, and vaccinated at the same time points as the other groups but with PBS and Titermax Gold only. Numbers in brackets indicate number of mice.

	MLV HER2 prime and boost (x 5)	MLV HER2 boost only (x 5)	MLV 5T4 prime and boost (x 5)	MLV 5T4 no prime (x 5)
Week 1	Inactivated WT MLV 5 x 10 ⁸ vp + Titermax in 100 μ l	PBS + Titermax in 50 μ l	Inactivated WT MLV 5 x 10 ⁸ vp + Titermax in 100 μ l	PBS + Titermax in 50 μ l
Week 3 + 4	MLV HER2 1 x 10 ⁹ + Titermax in 50 μ l	MLV HER2 1 x 10 ⁹ + Titermax in 50 μ l	MLV 5T4 1 x 10 ⁹ + Titermax in 50 μ l	MLV 5T4 1 x 10 ⁹ + Titermax in 50 μ l
Week 7	MLV HER2 2 x 10 ⁹ + Titermax in 50 μ l	MLV HER2 2 x 10 ⁹ + Titermax in 50 μ l	MLV 5T4 1 x 10 ⁹ + Titermax in 50 μ l	MLV 5T4 1 x 10 ⁹ + Titermax in 50 μ l

	AdHER2 prime and boost (x 5)	AdHER2 no prime (x 5)	Ad5T4 prime and boost (x 5)	Ad5T4 no prime (x 5)
Week 1	Inactivated non-replicating Ad5 5 x 10 ⁸ vp + Titermax in 100 μ l	PBS + Titermax in 50 μ l	Inactivated non-replicating Ad5 5 x 10 ⁸ vp + Titermax in 100 μ l	PBS + Titermax in 50 μ l
Week 3 + 4	AdHER2 1 x 10 ⁹ + Titermax in 50 μ l	AdHER2 1 x 10 ⁹ + Titermax in 50 μ l	Ad5T4 1 x 10 ⁹ + Titermax in 50 μ l	Ad5T4 1 x 10 ⁹ + Titermax in 50 μ l
Week 7	AdHER2 2 x 10 ⁹ + Titermax in 50 μ l	AdHER2 2 x 10 ⁹ + Titermax in 50 μ l	Ad5T4 1 x 10 ⁹ + Titermax in 50 μ l	Ad5T4 1 x 10 ⁹ + Titermax in 50 μ l

Mice were vaccinated subcutaneously in 2 sites on the back following the dosing and scheduled as detailed in Table 2.12. Mice were bled from the tail in weeks 0, 2, 5, 6 and 7. Briefly, the tip of the tail was cut off with a sharp scalpel and 20 μ l of blood was taken and added to 80 μ l PBS in an eppendorf. This was spun down at 13 000 rpm for 10 – 15 minutes, and 80 μ l diluted serum was transferred to fresh tubes and placed on dry ice. Mice were sacrificed at week 9 by cardiac puncture. Diluted sera from all time points were analysed for antibody responses by ELISA

2.11.6 Analysis of Gag prime vaccination

Mice were vaccinated sub-cutaneously with 25 µg of recombinant Gag protein (Dr. Siddharta Datta and Dr. Alan Rein, NIH/NCI) with Titermax Gold in 100 µl total volume per mouse. Different groups received either 1, 2 or 3 doses of Gag. Mice were also bled each week as described previously. At week 4 mice were sacrificed by cardiac puncture and spleens were removed for ELISPOT analysis. ELISAs were carried out on serum samples.

Table 2.13 | Groups for analysis of Gag priming.

Numbers in brackets indicate number of mice.

	Group 1 (x 4)	Group 2 (x 4)	Group 3 (x 4)	Group 4 (x 4)
Week 0	Tail bleed	Tail bleed	Tail bleed	Tail bleed
Week 1	Vaccinate 25 µg Gag	Vaccinate 25 µg Gag	Vaccinate 25 µg Gag	Vaccinate PBS
Week 2	Tail bleed	Tail bleed Vaccinate 25 µg Gag	Tail bleed Vaccinate 25 µg Gag	Tail bleed Vaccinate PBS
Week 3	Tail bleed	Tail bleed	Tail bleed Vaccinate 25 µg Gag	Tail bleed Vaccinate PBS

2.11.7 Prime-boost vaccination with Gag and MLV HER2

Mice were primed with 25 µg Gag recombinant protein plus Titermax Gold three times at 1 week intervals, and boosted three times at 2 week intervals with 2×10^9 vp MLV HER2, with no adjuvant (Table 2.14). Mice were bled after the prime vaccinations and between each boost vaccination. At week 10, mice were sacrificed by cardiac puncture and spleens were removed for ELISPOT analysis. ELISAs were carried out on serum samples.

Table 2.14 | Groups for analysis of prime-boost vaccination

Numbers in brackets indicate number of mice.

	Group 1 (x 5) Prime-boost	Group 2 (x 5) Boost only	Group 3 (x 5) Control
Week 1, 2 + 3	Prime 25 µg Gag + Titermax	Vaccinate PBS + Titermax	Vaccinate PBS + Titermax
Week 5, 7 + 9	Boost 2×10^9 vp MLV HER2	Boost 2×10^9 vp MLV HER2	Vaccination PBS

2.11.8 Prime-boost vaccination with Gag and MLV HER2 or MLV 5T4

Mice were primed once with 25 µg recombinant Gag and Titermax Gold, and boosted several times with either MLV HER2 and Titermax Gold or MLV 5T4 and Titermax Gold. Blood was taken after each vaccination and analysed by ELISA. Mice were sacrificed in week 8 by cardiac puncture and spleens were removed for ELISPOT analysis.

Table 2.15 | Groups for analysis of prime-boost vaccination with MLV HER2 or MLV 5T4

Numbers in brackets indicate number of mice.

	Group 1 (x 5) Prime-boost MLV HER2	Group 2 (x 5) Boost only MLV HER2	Group 3 (x 5) Prime-boost MLV 5T4	Group 4 (x 5) Boost only MLV 5T4	Group 5 (x 5) Control
Week 1	Prime 25 µg Gag	Vaccinate PBS	Prime 25 µg Gag	Vaccinate PBS	Vaccinate PBS
Week 3, 5 + 6	Boost 5 x 10 ⁹ vp MLV HER2	Boost 5 x 10 ⁹ vp MLV HER2	Boost 5 x 10 ⁹ vp MLV 5T4	Boost 5 x 10 ⁹ vp MLV 5T4	Vaccination PBS

2.11.9 Animal experimentation ethics statement

All animal experiments were performed in accordance with the terms of the UK Home Office guidelines and with the approval of the Medical Sciences Animal Ethics Committee, University of Oxford. The Home Office Project Licence number under which these experiments were conducted were PPL 30/2816 (2011 – 2012) and PPL 30/2819 (2013). Studies were carried out under the personal licence PIL 30/8941.

2.12 Cryo-Electron Microscopy (EM)

Cryo-EM was carried out by Dr Robert Gilbert (Division of Structural Biology, University of Oxford). Approximately 1 x 10⁷ purified virus particles were loaded onto a pre-charged carbon/copper grid before blotting and freezing the grid in liquid ethane. The grid was transferred to liquid nitrogen for 10 minutes, then loaded onto the electron microscope and left to stabilise for a further 10 minutes before imaging.

2.13 Statistical Analyses

Data is expressed as the mean of replicates +/- standard deviation. For comparison between two groups, statistical analyses were carried out using two-tailed t-tests. For analysis of data in which more than 2 means were compared, significance was evaluated using one-way analysis of variance (ANOVA) method to limit the chances of observing statistical error. Significances are denoted on the graphs as * = $p < 0.05$, ** = $p < 0.01$ or *** = $p < 0.001$.

2.14 Solutions

2xYT Medium: 16 g Bacto-tryptone, 10 g Bacto-yeast extract, 5 g NaCl in 800 ml dH₂O. Adjusted to pH 7 with 5 M NaOH and made up to 1 litre with dH₂O, autoclaved and stored at 4°C.

TFBI: 0.589 g potassium acetate (30 mM), 2.418 g rubidium chloride (100 mM), 0.294 g calcium chloride dihydrate (10 mM), 1.979 g manganese chloride tetrahydrate (50 mM), 30 ml glycerol (15% v/v). Adjusted to pH 5.8 with 0.2 M acetic acid, made up to 200 ml with dH₂O. Autoclaved and stored at 4°C

TFBII: 0.419 g MOPS (10 mM), 0.242 g rubidium chloride (10 mM), 2.205 g calcium chloride dihydrate (10 mM), 30 ml glycerol (15% v/v). Adjusted to pH 6.6 with 5 M NaOH, made up to 200 ml with dH₂O. Autoclaved and stored at 4°C

100X TAE BUFFER: 4.84 g Tris base, 1.09 ml glacial acetic acid, 0.29 g EDTA. Made up to 1 litre in dH₂O. Diluted 1X with dH₂O before use.

1.5 M Tris HCl pH 8.8: 27.23 g Tris base, 80 ml deionised H₂O. Adjusted pH with 6M HCl, Made up to 150 ml with dH₂O.

1 M Tris HCl pH 6.8: 12.0 g Tris base, 60 ml H₂O, adjusted pH with 6M HCl and made up to 100 ml with dH₂O

0.5 M Tris HCl pH 6.8: 6.60 g Tris base, 60 ml dH₂O. Adjusted pH with 6M HCl, made up to 100 ml with dH₂O

10% SDS (sodium dodecyl sulphate): 10 g SDS, 90 ml dH₂O. Dissolved with gentle stirring and made up to 100ml with dH₂O

Ammonium Persulphate: 100 mg ammonium persulphate (Sigma-Aldrich), 1 ml dH₂O. Made up fresh each time

PBS + 0.1% Tween: 1 ml Tween, 1 litre PBS

Protein Loading/Sample Buffer + SDS: 2.4 ml 1 M Tris HCl pH 6.8, 3 ml glycerol, 3 ml 20% SDS (20 g SDS, 80 ml dH₂O), 1.6 ml β-mercaptoethanol, 6 mg bromophenol blue. Stored at 4°C

Protein Loading/Sample Buffer: 1 ml 0.5 M Tris HCl pH6.8, 2 ml glycerol, 1 ml 1% bromophenol blue (10 mg bromophenol blue, 1 ml dH₂O), 4.92 ml dH₂O. Stored at 4°C

10X Running Buffer + SDS: 19.8 g Tris base, 86.4 g glycine, 6 g SDS, 1200 ml dH₂O. Adjusted to pH 8.3 with HCl, diluted 1X with dH₂O.

10X Running Buffer: same as 10X Transfer Buffer, diluted with dH₂O

10X Transfer Buffer: 30.29 g Tris base, 144 g glycine. Made up to 1 litre with dH₂O. For use, diluted 1X with dH₂O and 100 ml methanol, adjusted pH to 8.3 with HCl/NaOH. Used on same day.

0.5 M Tris pH 7.9: 6.057 g Tris base, 100 ml dH₂O. Adjusted to pH 7.9, filtered into sterile bottles.

Caesium Chloride Gradient p = 1.45 CsCl: 20.5 g caesium chloride, 2.9 ml 0.5 M Tris pH 7.9, 25 ml dH₂O, filtered into sterile bottles.

Caesium Chloride Gradient p = 1.32 CsCl: 32.0 g caesium chloride, 6.8 ml 0.5 M Tris pH 7.9, 61.2 ml dH₂O, filtered into sterile bottles.

0.2 M EDTA: 2.9224 g EDTA, 50 ml dH₂O, filter sterilised.

40% Glycerol: 40 g glycerol, 2 ml 0.5 M Tris pH 7.9, 0.5 ml 0.2 M EDTA. Made up to 100 ml with sterile dH₂O

Dialysis Buffer 1: 100 ml HEPES (50 mM), PBS (10 tablets for 1X), 40 ml magnesium chloride (0.2 g/litre), 20 ml calcium chloride (0.1 g/litre), 200 ml glycerol (10%). Made up to 2 litres with dH₂O, filtered and stored at 4°C.

Dialysis Buffer 2: made up in the same way as Dialysis Buffer 1 but with 20 ml magnesium chloride instead.

ACK Lysis Buffer: 8.023 g ammonium chloride, 1 g potassium bicarbonate, 37.2 mg disodium EDTA. Made up to 1 litre with dH₂O, pH adjusted to 7.2 – 7.4 and autoclaved.

MACS Buffer: 500 ml sterile PBS, 0.5% BSA, 2 mM EDTA. Sterile filtered and stored at 4°C.

0.1M Triethylamine: 7 ml neat triethylamine (7.2 M), 0.1% Triton X-100. Made up to 500 ml with dH₂O, adjust pH to 11.8 and sterile filtered.

1M MES: 9.76 g MES hydrate, adjusted to pH 3, made up to 50 ml with dH₂O. Sterile filtered.

3 Molecular Engineering of Viruses to Present Tumour Antigens

3.1 Introduction

To test the overarching vaccine hypothesis using recombinant MLV particles, MLV was genetically modified to present weak antigens on its surface. This chapter details these modifications, and the subsequent production of vaccine particles. Membranous tumour antigens 5T4 and HER2 were sub-cloned into the MLV genome, replacing the gene encoding the EC domain of the viral Env protein and leading to the creation of a fusion protein consisting of the EC domains of 5T4 or HER2 fused to the TM and IC domain of MLV Env. Viral particle formation should remain unaffected, as the internal Env domains required for this process remain intact. This will lead to the production of recombinant viruses with only the EC domain of the tumour antigen displayed on the surface, as no other viral protein is present (Figure 3.1). Enhanced green fluorescent protein (EGFP) was also cloned into the viral genome to verify that a functional fusion protein could be produced, and to act as a control for later studies. Virus production was scaled up by the generation of stable producer cells. The purification of the recombinant viruses will also be detailed.

This chapter also describes the use of adenovirus as a vaccine vector compared to MLV. Adenoviruses differ greatly from retroviruses, being non-enveloped and containing a double stranded DNA genome¹²⁵. After infection of a cell, the adenovirus particle is internalised into endosomes, where acidification of the compartments releases viral components into the cytosol. These travel to the nucleus utilising cellular proteins, for transcription of the viral DNA genome and hence the production of virus progeny. Deletion of several of the early transcriptional units within the adenoviral genome renders the virus replication defective and allows for the insertion of transgenes. This has proven useful in generating safe recombinant adenovirus vectors encoding different transgenes. While MLV as a non-infectious vaccine particle presents antigens on the surface of the virus particle, recombinant adenovirus-infected cells will secrete the antigen encoded by the transgene instead. This will inevitably trigger different immune responses *in vivo* compared to MLV vaccine particles. Adenovirus vectors were initially used in gene therapy before being used for vaccination, as they exhibit high transgene expression in the absence of viral replication and display broad tissue tropism.

Non-replicating adenoviruses can be cultured in 293A cell lines to high titres due to complementation of the missing early transcriptional units. However, administration of replication incompetent adenoviruses is often associated with anti-viral immune responses, so the search continues to find alternative serotypes that can be used for human administration.

AdZ5 is a plasmid containing the adenovirus serotype-5 genome but is replication defective due to deletions in the E1 and E3 regions (Figure 3.8). The plasmid still allows for the production of an infectious virus in 293A cells, allowing infection of cells and transgene expression (driven by a separate cytomegalovirus (CMV) promoter), but no infectious virus progeny are produced. This section details the insertion of the EC domains of tumour antigens HER2 and 5T4 (fused to the internal components of MLV Env) into AdZ5, and the generation of replication defective recombinant adenoviruses expressing these weak antigens. These were used to compare to recombinant MLV for *in vivo* vaccination.

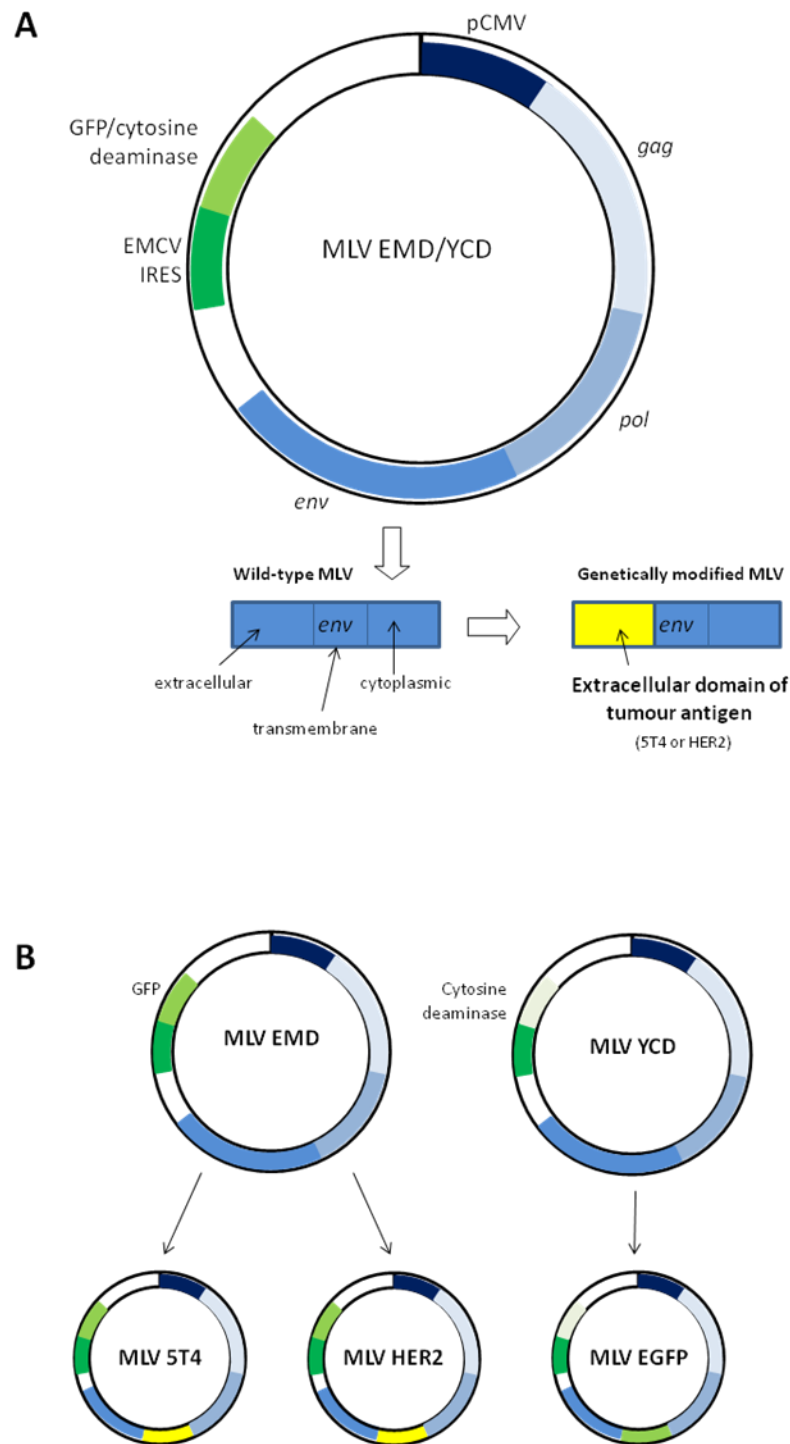


Figure 3.1 | Schematic diagram of MLV plasmids.

(A) Structure of MLV plasmid. MLV Env EC domain is replaced by antigen. pCMV – cytomegalovirus promoter; EMCV IRES - encephalomyocarditis virus internal ribosome entry site. (B) Parental plasmids and cloned plasmids with either HER2, 5T4 or EGFP.

3.2 Murine Leukaemia Virus – Modification, Production and Purification

3.2.1 Genetic modification of MLV

Two plasmids, MLV EMD and MLV YCD were used to generate three recombinant viral vectors. Both parental plasmids are identical, encoding the entire MLV genome but differ only by reporter gene – MLV EMD contains GFP, while MLV YCD possesses yeast cytosine deaminase (Figure 3.2). Viral genes are driven by a CMV promoter, allowing expression in most cell types, while the reporter gene is under a separate internal ribosome entry site (IRES) for translation of the transcribed RNA. The EC domains of 5T4 and HER2 were cloned into parental plasmid MLV EMD while EGFP was cloned into MLV YCD, to avoid any background fluorescence. Although at 10 kb the MLV genome is relatively small in comparison to other viral vectors, it was still desirable to work with a smaller plasmid in order to increase the number of unique restriction sites available for cloning. This was achieved by *NdeI* restriction digest of parental plasmid MLV EMD or MLV YCD and re-circularisation of the resulting 6.3 kb fragment, known as MLV (EMD/YCD) *NdeI*, which was used for all subsequent sub-cloning reactions (Figure 3.3).

A synthetic fusion gene was ordered from Mr Gene containing the corresponding sequences to the viral Env transmembrane (TM) and cytoplasmic (CP) domains from the wild type (WT) MLV virus, as well as the human 5T4 EC domain in place of the viral EC domain flanked by *BglII* sites. These restriction sites were inserted to allow the replacement of the 5T4 EC domain with any antigen by *BglII* restriction digest. Unfortunately the 5T4 sequence was missing a cytosine base, resulting in a frame shift and a truncated protein. To correct this, site directed insertion was carried out using PCR and the Mr Gene plasmid as a template (Figure 3.4) and the resulting plasmid was verified by sequencing. The PCR product was purified by agarose gel extraction and ligated to itself to form a new vector. The corrected 5T4 fusion gene was sub-cloned into MLV *NdeI* by a double digest with *AgeI* and *Clal*, and the subsequent MLV *NdeI* 5T4 was sub-cloned back into the original MLV EMD plasmid by *NdeI* digest (Figure 3.5).

To sub-clone human HER2 into the MLV genome, a plasmid containing full length human HER2/neu (Addgene) was first cut with *BglII* and re-ligated to remove an internal *BamHI* site. The EC truncated domain of HER2 was amplified by PCR with flanking *BamHI* restriction sites. The resulting PCR product was cut with *BamHI* and cloned into the Mr Gene vector to replace the EC domain of 5T4, as the cut ends of *BglII* and *BamHI* are compatible. The remaining HER2

fragment containing *Bam*HI was re-inserted, and the complete fusion HER2-virus gene was sub-cloned into MLV as before (Figure 3.6). This cloning strategy is summarised in Figure 3.7.

EGFP was cloned into MLV YCD instead of MLV EMD to avoid background reporter GFP expression. EGFP was amplified by PCR with flanking *Bgl*III sites on either end, and the resulting PCR product was cloned into the Mr Gene vector, acting as a shuttle vector again. After an additional digest, the EGFP-viral fusion gene was cloned into MLV YCD NdeI and finally into the full MLV YCD plasmid (Figure 3.8).

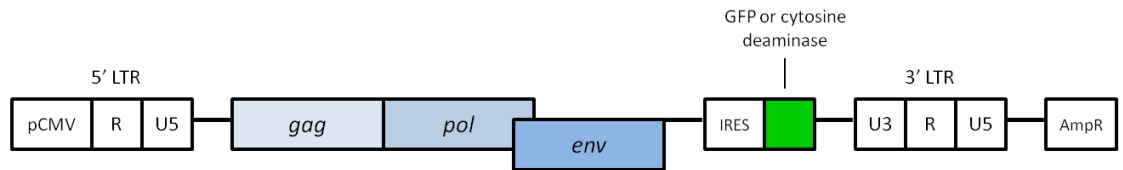
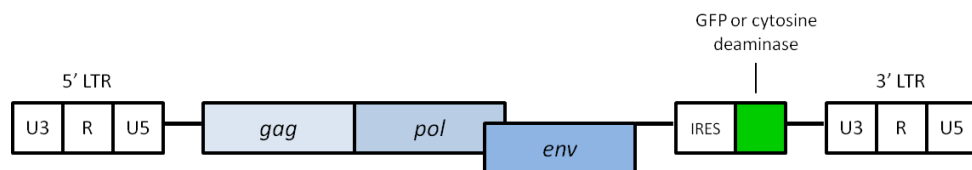
A**B**

Figure 3.2 | MLV EMD/YCD genome structure.

(A) Full length MLV EMD/YCD DNA plasmid, where LTR: long terminal repeat; pCMV: CMV promoter; IRES: internal ribosome entry site; AmpR: ampicillin resistance. (B) Proviral structure of MLV (after viral integration). Following reverse transcription, pCMV is replaced by U3 at the 5' LTR, and AmpR is removed.

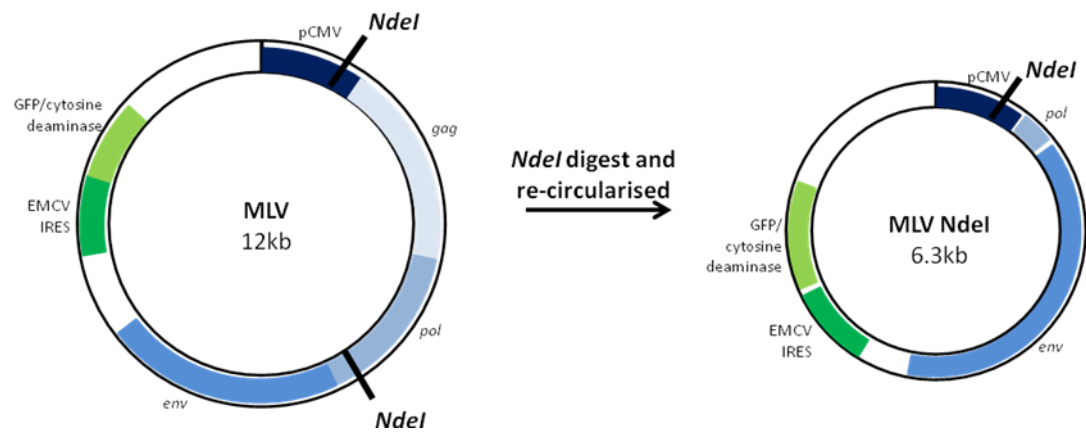


Figure 3.3 | Reduction of MLV DNA genome for sub-cloning.

The full MLV genome was halved by *NdeI* restriction digest and re-circularisation. This omitted most of the *gag/pol* regions, which would not be modified from WT in any way and hence were not required to be present during cloning. The resulting MLV NdeI plasmid was used for sub-cloning.

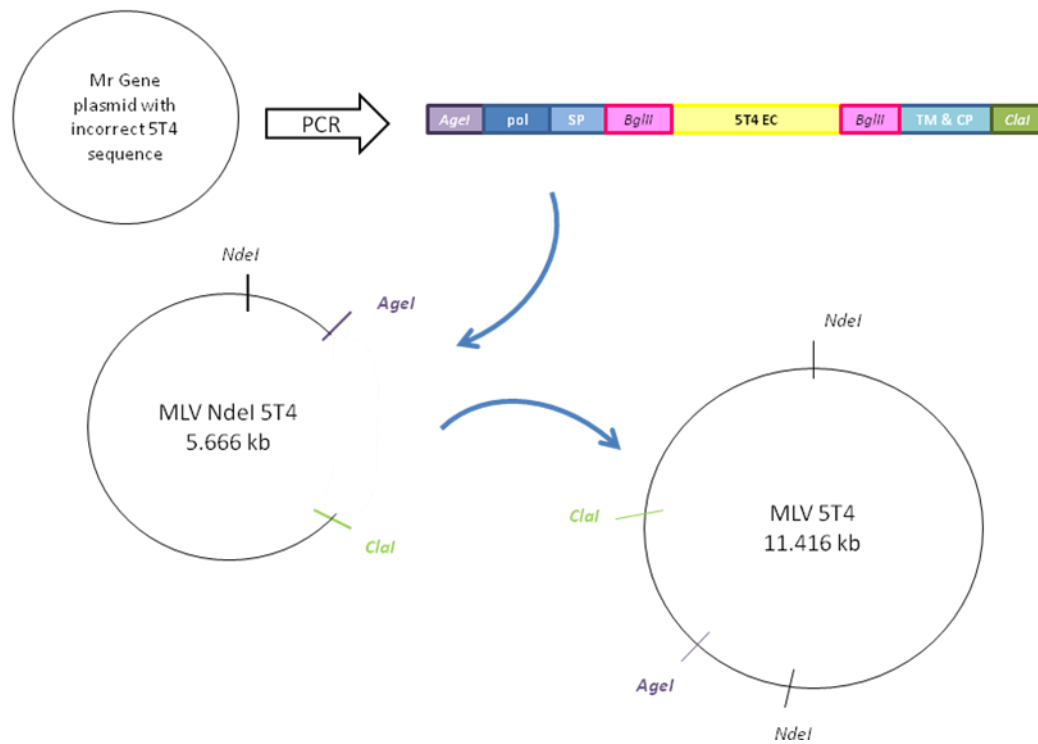


Figure 3.4 | Summary of 5T4 cloning.

DNA plasmid from Mr Gene containing the incorrect 5T4 sequence was corrected by PCR and re-ligated. This was sub-cloned into MLV NdeI (derived from MLV EMD) and subsequently into the full MLV plasmid to form MLV 5T4.

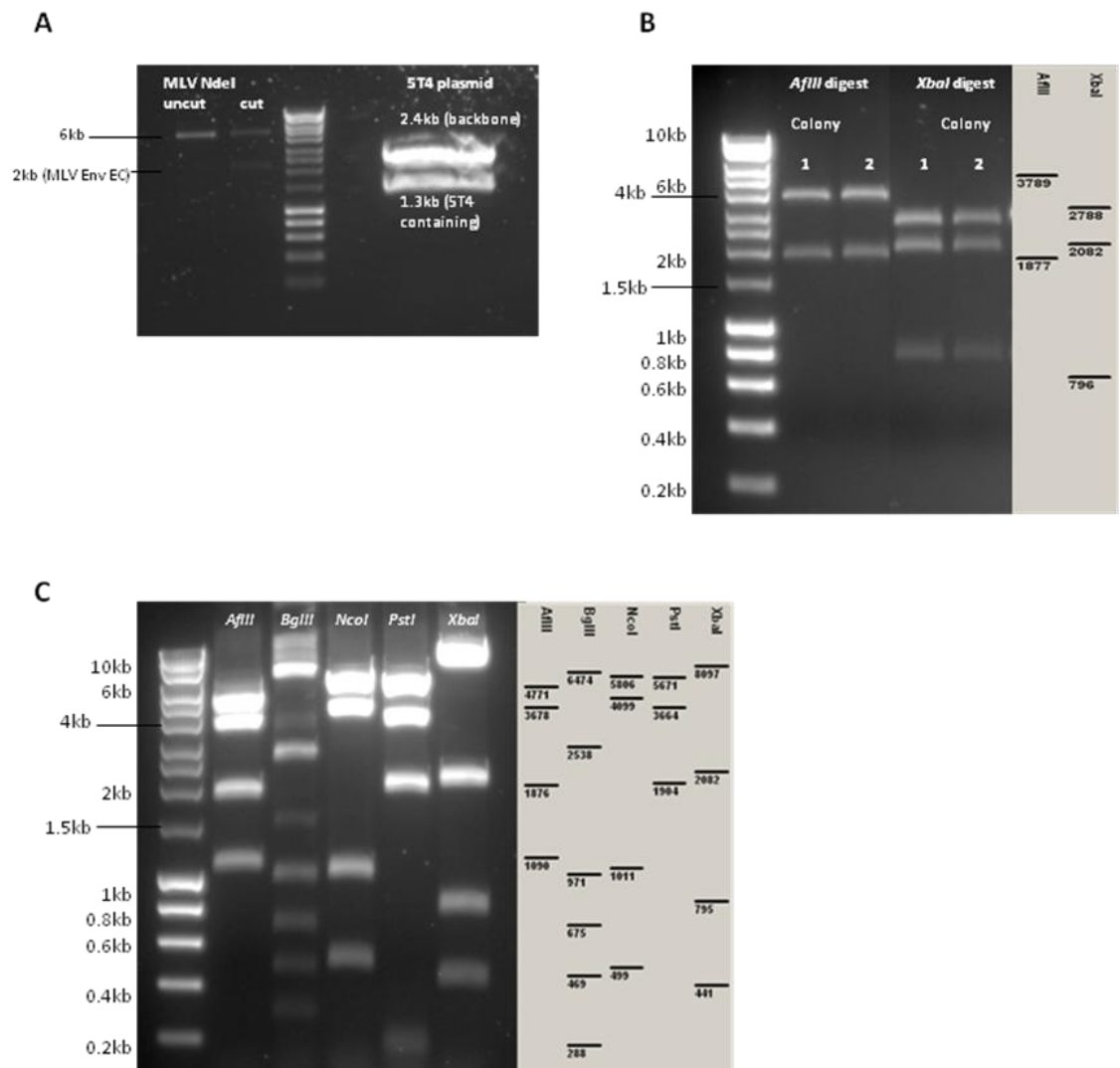


Figure 3.5 | Sub-cloning 5T4 gene into MLV DNA plasmid.

(A) 5T4 plasmid (from Mr Gene) was cut with *AgeI* and *Clal*. The 1.3 kb fragment containing the 5T4-viral fusion gene was excised. MLV Ndel was also cut with *AgeI* and *Clal* to open the vector. (B) Both fragment and vector (backbone) were ligated together, miniprep colonies were verified by *AflIII* and *XbaI* restriction digest, bands were compared to predicted sizes (shown on right). (C) MLV Ndel 5T4 was cloned back into MLV EMD to obtain complete genome. Finally, MLV 5T4 plasmid was screened by restriction digest and compared to the predicted digest results (shown to the right of the gel) to confirm the ligation was successful.

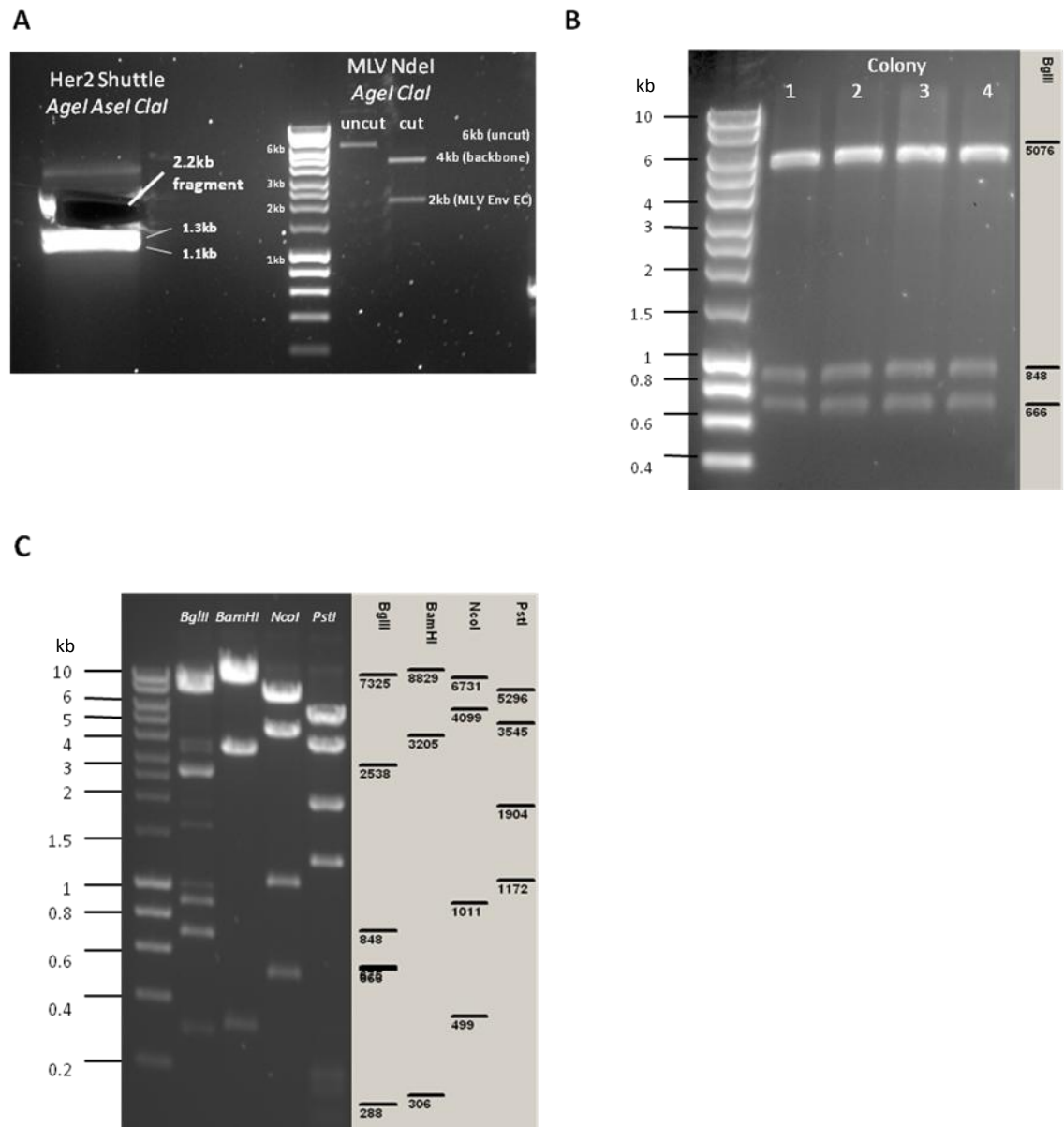


Figure 3.6 | Sub-cloning HER2 gene into MLV DNA plasmid.

(A) PCR amplified HER2 EC was cloned into shuttle vector (not shown). This vector was digested with *AgeI* and *Clal* to excise the HER2-viral fusion gene (2.2kb) and *Asel* to cut up the backbone (to distinguish the 2.2kb fragment better). This was ligated into the MLV Ndel vector (4kb backbone shown on the right of gel). (B) MLV Ndel HER2 miniprep colonies were verified correct by *Bgl*III digest and comparison to predicted bands (shown on right). (C) MLV Ndel HER2 was cloned into MLV EMD. MLV HER2 was screened by restriction digest and compared to the predicted digest results (shown to the right of the gel) to confirm the ligation was correct.

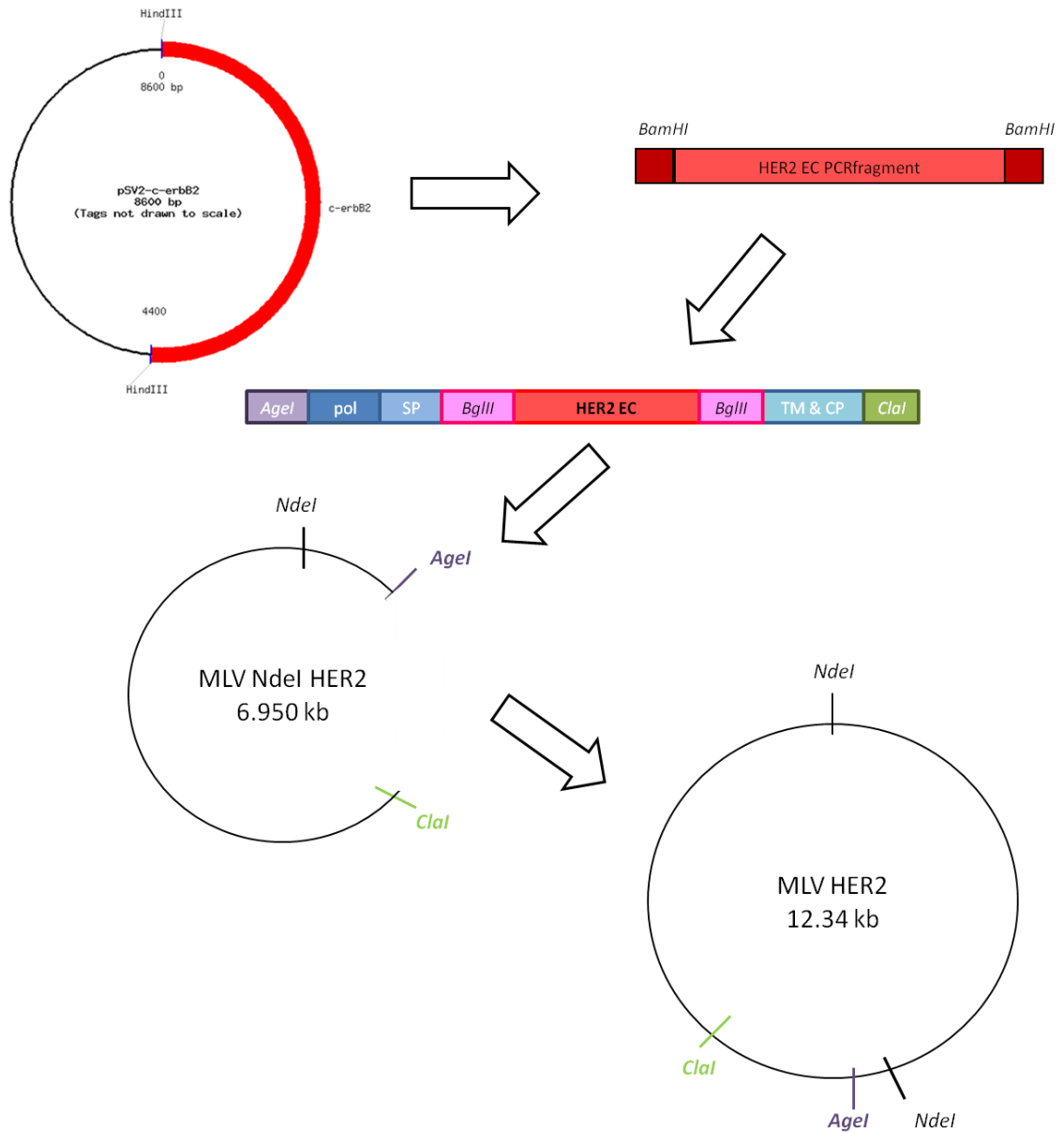


Figure 3.7 | Summary of HER2 cloning to produce MLV HER2 vaccine particle.

HER2 EC domain was amplified and *BamHI* restriction sites were added on the ends by PCR. The amplified PCR product was used to replace the 5T4 EC with the HER2 EC in the shuttle vector. As before, the HER2-viral fusion gene was cloned into MLV NdeI and then subsequently cloned into the MLV EMD.

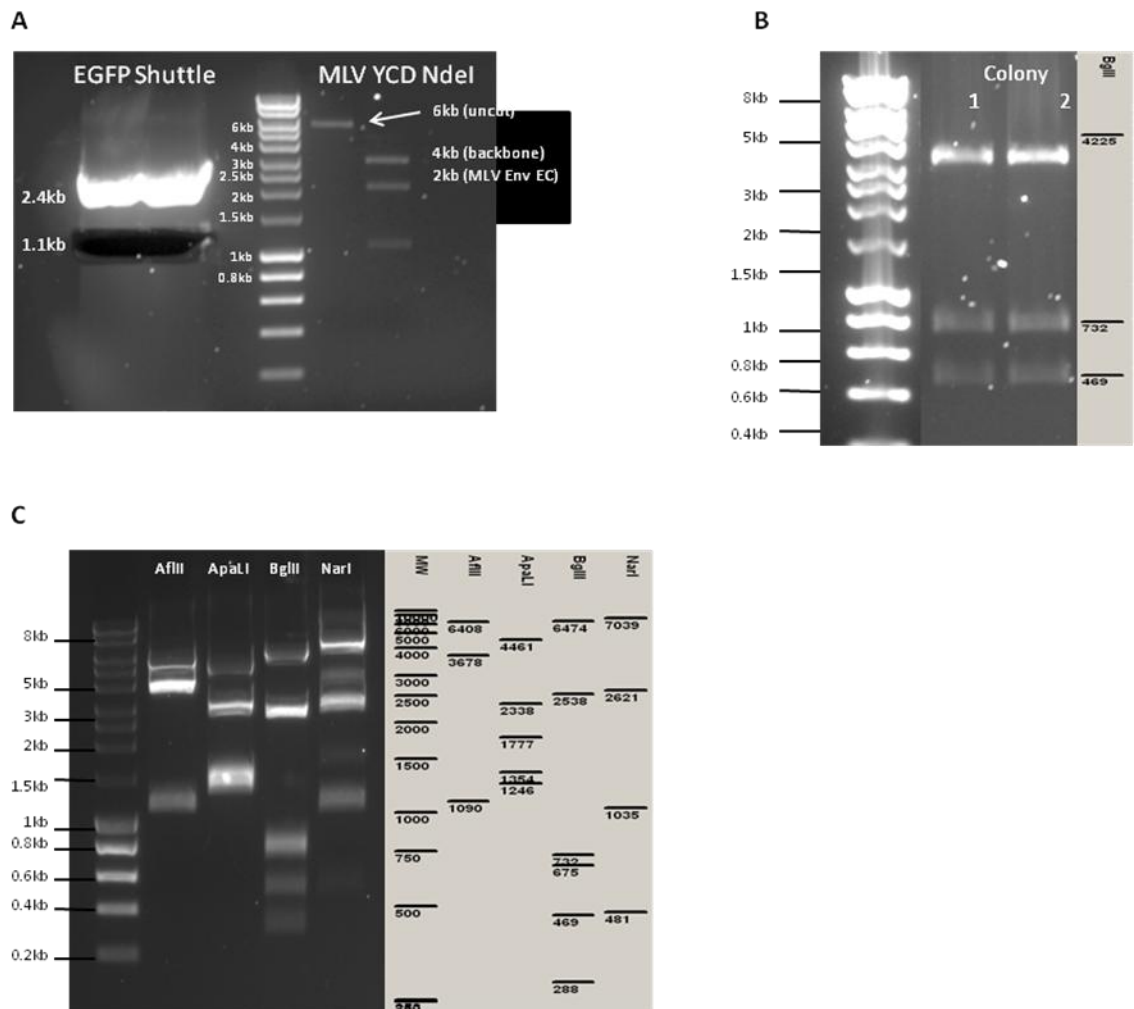


Figure 3.8 | Sub-cloning EGFP gene into MLV DNA plasmid.

(A) After amplifying EGFP by PCR and sub-cloning it into the shuttle vector, this was digested with *AgeI* and *ClaI* restriction enzymes, and the resulting 1.1kb fragment containing EGFP-viral fusion gene was extracted and cloned into the backbone of MLV NdeI (seen as a 4kb band on the gel). (B) MLV NdeI EGFP was screened by *BglIII* digest and compared to predicted bands (shown on right). (C) MLV NdeI EGFP was cloned into MLV and screened by restriction digest to confirm that the ligation was successful (compared with predicted digest results, shown to the right of the gel).

3.2.2 Transient and stable production of retrovirus vaccine particle

Transient transfection of the modified DNA viral plasmid into mammalian cells resulted in the production of retrovirus particles, due to the presence of a CMV promoter. Successful transfection could be gauged by reporter GFP expression for DNA plasmids MLV HER2 and MLV 5T4. This expression was monitored by microscopy (Figure 3.9) and showed that DNA transfection leads to expression of the modified plasmids. It is important to note that the presence of GFP only confirms that the transfection was successful (i.e. that the MLV DNA genome is present in the cell) but does not indicate itself that virus production is taking place correctly.

Transient transfection of cells led to inconsistent expression levels of GFP and hence varying efficiencies for transfection, which often could not be sustained for more than 72 hours. In order to reduce this variability and to scale up virus production, it was desirable to create stable cell lines producing the modified virus particles. 293A cells were chosen as producer cell lines as they are commonly used for retroviral production¹²⁶. As a retrovirus, wild type MLV can infect cells and incorporate its genome into cellular DNA, giving rise to the possibility of stable production of progeny viruses. Therefore it was desirable to produce a small quantity of virus by transient transfection, which could then be used to produce stable packaging cells. However as the modified MLV particles were rendered non-infectious, it was necessary to pseudotype the recombinant viruses with an envelope protein capable of infecting cells, to allow viral entry into the cell and insertion of the virus genome. Although non-infectious, the modified viruses still contain all the necessary viral machinery required for reverse transcription of the RNA and integration of DNA into the cellular genome.

293A cells were co-transfected with plasmids encoding glycoprotein G from vesicular stomatitis virus (VSV) for pseudotyping, and MLV HER2, 5T4 or EGFP plasmids in a 1:4 ratio (Figure 3.10). Supernatant containing VSV-G pseudotyped MLV viral particles was collected after 48 hours. This was used to infect fresh 293A cells by centrifugation. These infected cells contain the MLV genome integrated into the cellular genome but not VSV-G as this was encoded on a separate plasmid. Hence these cells will only produce recombinant MLV particles, without any VSV-G. Cells were monitored by GFP expression to ensure successful co-transfection and subsequent infection by retrovirus particles (Figure 3.11).

To obtain a pure population of stable cells, infected 293A cells were sorted by GFP expression in two separate rounds of FACS, carried out by Andrew Worth (Jenner Institute, University of Oxford). Cells were first selected on the basis that they displayed any GFP expression in comparison to uninfected cells (Figure 3.12). Selected cells were pooled together and passaged before undergoing a second round of FACS, where only the highest GFP expressing polyclonal cells were selected for future use.

Virus production was also monitored by viral RNA levels in the supernatant, as MLV leaves the cell by budding. Collection of supernatant and subsequent analysis would confirm whether the modified retroviral particles are able to follow the life cycle of the WT virus and bud from the cell correctly. Supernatant was collected from transfected and stable producer cells at specific time points and total RNA was extracted. For detection of viral RNA, reverse transcriptase-quantitative PCR (RT-QPCR) was carried out using specific primers to the viral *gag* gene (Figure 3.13). It can be seen that viral titres from transiently transfected cells are lower than those from stable producer cells at both time points, and decrease between three and six days, while the number of viral particles from stable cells increases.

As stated previously, modified virus particles from transient and stable cells are expected to be non-infectious as the recombinant virions do not contain the necessary viral envelope protein to bind to their cognate receptor for infection. PCR was carried out to confirm the absence of WT viral *env* from cellular DNA extracted from stable producer cells using primers directed to the viral *pol* sequence, viral signal peptide and viral *env*. Both *pol* and signal peptide sequences are present in both WT and modified DNA sequences, but the viral *env* is only present in the WT genome (Figure 3.14). PCR products were analysed by gel electrophoresis, indicating the absence of viral *env* in any of the cells producing the modified vaccine particles (Figure 3.15).

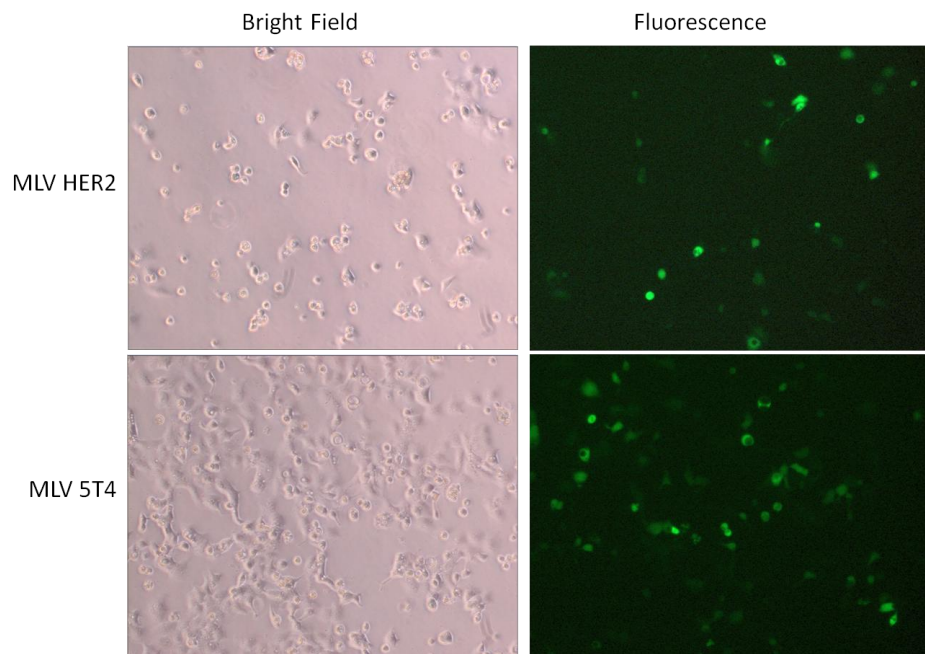


Figure 3.9 | Reporter GFP expression shows successful DNA transient transfection.

MLV HER2 and MLV 5T4 DNA plasmids were separately transfected into 293A cells using lipofectamine. GFP expression was visualised by fluorescence microscopy 48 hours after transfection.

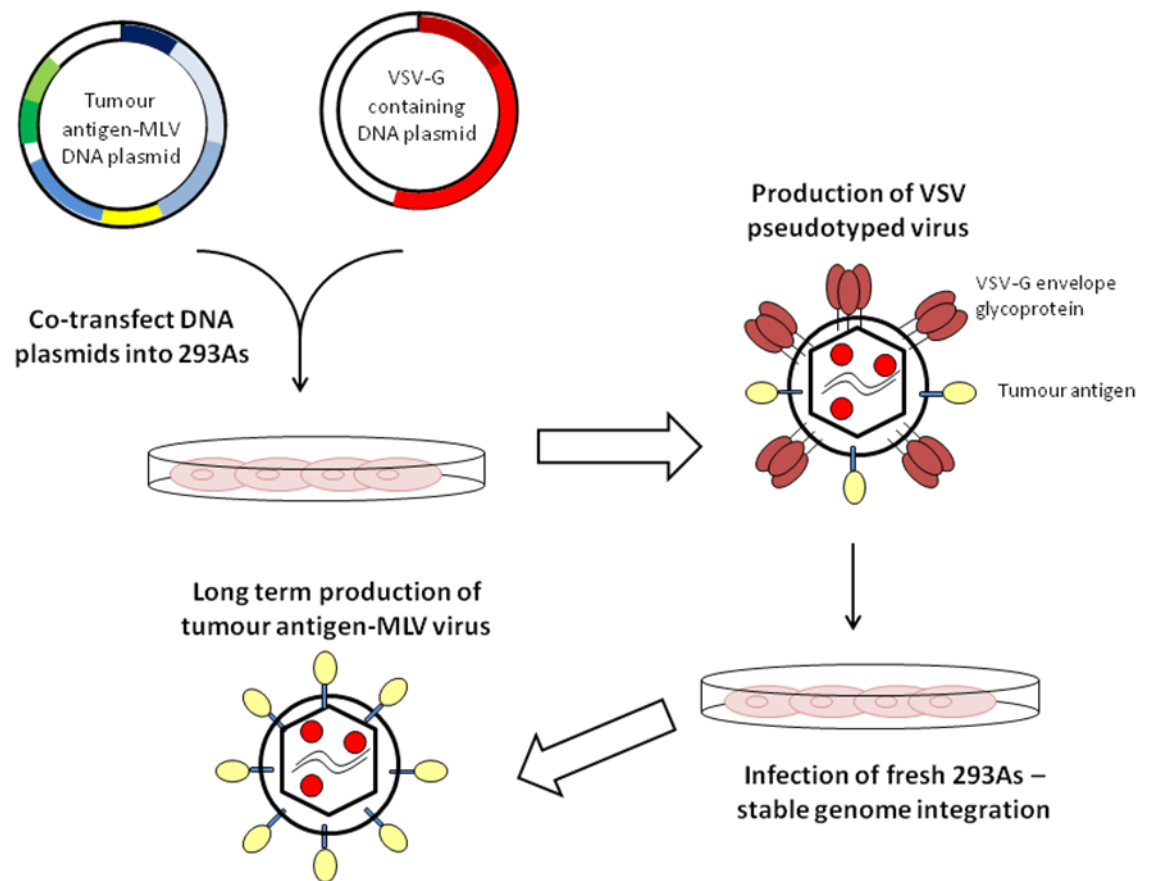


Figure 3.10 | Creation of stable cell lines by VSV-G pseudotyping to produce non-infectious vaccine virus particles.

Stable cell lines containing integrated retroviral genomes were produced by co-transfection with VSV-G, followed by infection of new cells to enable long term production of the modified viral vaccine.

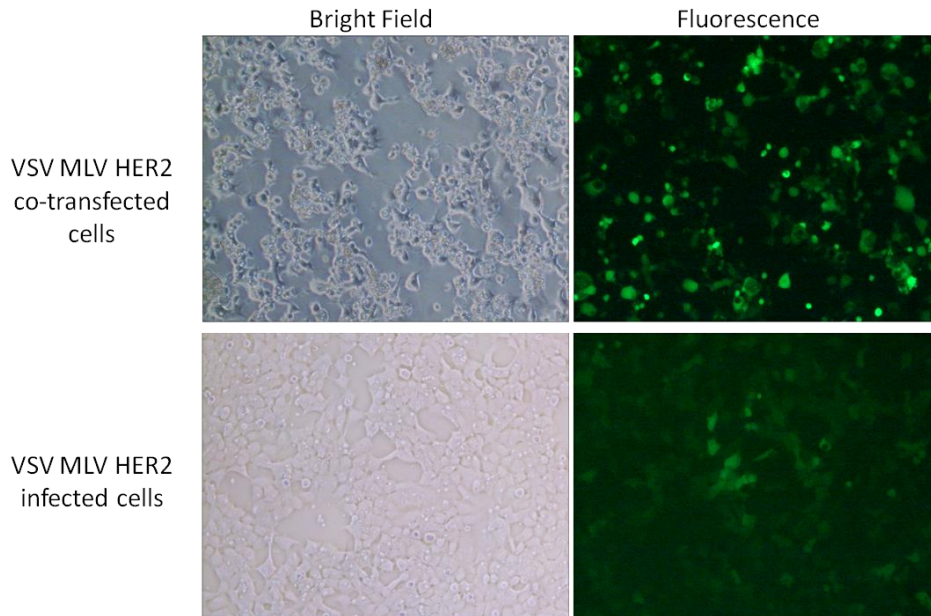


Figure 3.11 | Reporter GFP expression shows successful co-transfection, and subsequent infection with pseudotyped virus particles.

Cells were co-transfected with VSV-G and MLV HER2 DNA plasmids. After 48 hours, images were taken and supernatant was collected and used to infect a fresh batch of 293A cells. After 72 hours, images were taken to verify successful infection.

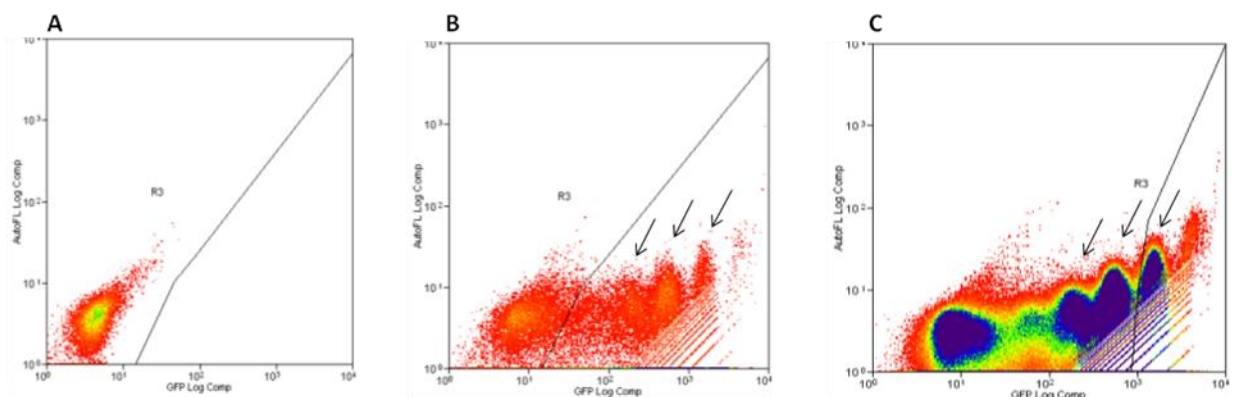


Figure 3.12 | 293A cells infected with pseudotyped viruses and sorted by GFP expression.

(A) Uninfected 293A cells used as a negative control for gating. (B) First round of sorting of VSV-G-MLV HER2 infected 293A cells. (C) Second round of sorting of MLV HER2 293A cells. Arrows indicate different cell populations, possibly due to different numbers of MLV genome integrations into the cellular genome. FACS plots show MLV HER2 293A cells but are representative of MLV 5T4 and MLV EGFP cell sorting.

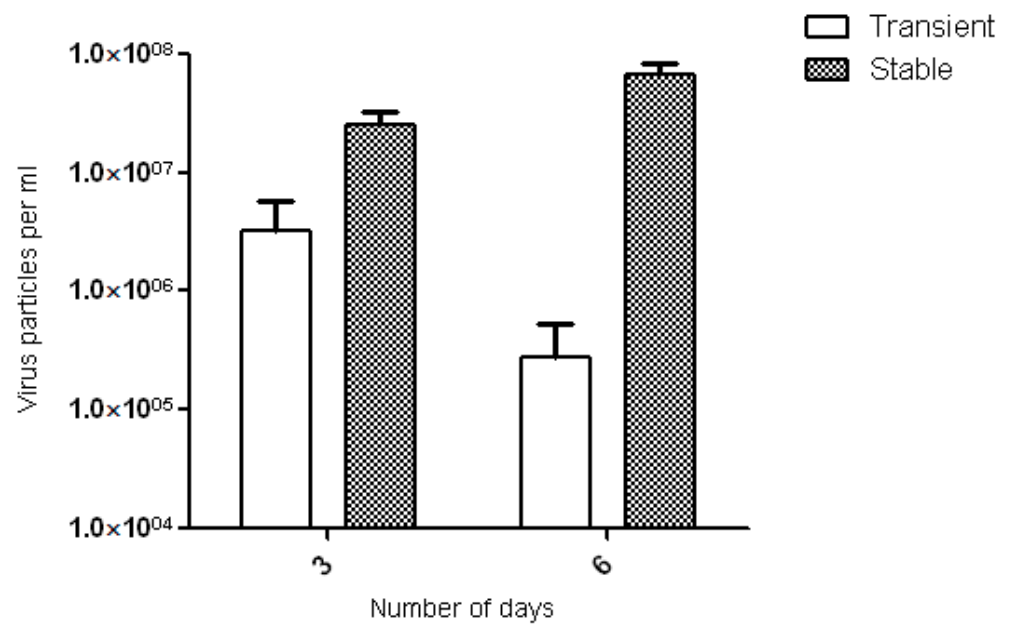


Figure 3.13 | Comparison of viral titres from transfected and stable cells.

MLV HER2 DNA plasmid was transiently transfected into 293A cells at the same time that MLV HER2 293A cells were seeded. Supernatant was collected from all cells 3 and 6 days after transfection/cell seeding and analysed for viral RNA content. Data represents geometric mean, error bars represent standard deviation (technical replicates).

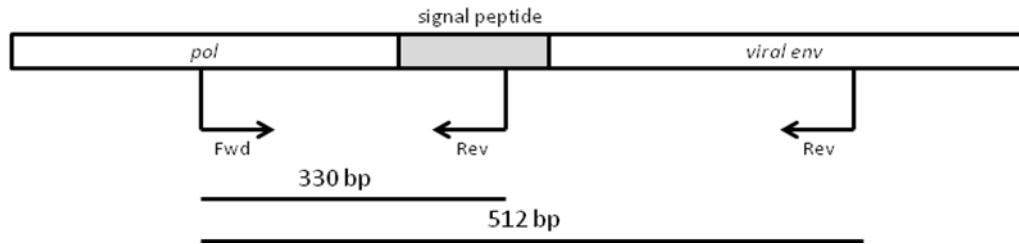


Figure 3.14 | Primer sequences used in PCR to detect viral *env*.

Cellular DNA containing both the viral signal peptide and viral *env* would result in 2 PCR products (330bp and 512bp); DNA containing only viral signal peptide and no viral *env* would result in only 1 PCR product (330bp). Both WT and modified viral DNA share the same viral *pol*.

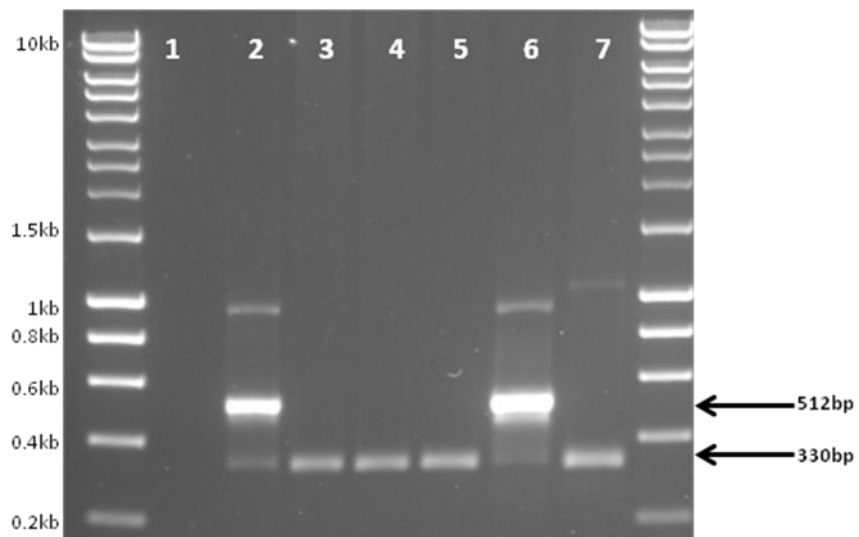


Figure 3.15 | Analysis of modified virus particles by gel electrophoresis.

PCR was carried out to confirm the lack of viral *env* in cell lines stably transduced with viral genomes. Lanes in gel – 1: 293A cells only; 2: WT MLV transfected 293A cells; 3: MLV HER2 293A; 4: MLV 5T4 293A; 5: MLV EGFP 293A; 6: MLV EMD DNA plasmid (WT); 7: MLV 5T4 DNA plasmid.

3.2.3 Purification of retroviral particles

WT MLV leaves the cell by gently budding from the cell membrane. Collection of supernatant is required to recover enveloped virions, but the titres of virus particles that can be found in supernatant is low (Figure 3.13) compared to that of non-budding viruses, as these produce infectious particles intracellularly and can be harvested by cell lysis. Concentration of supernatant from stable producer cells was carried out in order to increase viral titres. Briefly, MLV HER2, MLV 5T4 or MLV EGFP 293A stable cells were seeded in 2% FCS media at 80% - 90% confluency in T175 flasks, and supernatant was collected after a period of 4 days. Stable cells were still fluorescent up to 2 weeks after seeding in 2% FCS, indicating sustained virus production and retention of the virus genome incorporated into cellular DNA (Figure 3.16). This is because MLV is not a lytic virus; cells containing the integrated MLV genome can be passaged as normal, as the production of the virus does not kill the cell, and daughter cells will also contain the integrated viral DNA.

Collected supernatant containing the appropriate recombinant virus were pooled together and centrifuged at high speeds over a 10% and 20% sucrose cushion and dialysed overnight to remove any sucrose. Purification by this method led to an increase of titres by 100 fold as measured by RT-QPCR (Figure 3.17). Viral RNA titres were measured by this method as modified viruses are non-infectious and hence standard viral infectivity assays detecting cytopathic effects (CPE) are not applicable. Purified virus was stored at -80°C in aliquots, to avoid multiple freeze-thawing cycles.

MLV is an enveloped virus, containing both a lipid bilayer and a capsid to protect its RNA genome and contributing to the structural integrity of the particle. Purification by ultracentrifugation is unlikely to damage the viral vaccine particles. Verification of this was carried out by purifying infectious WT MLV expressing a GFP reporter gene (MLV EMD), and infecting cells with purified virus. As cells displayed GFP expression, purification did not affect the surface proteins on MLV particles required for infection (Figure 3.18).

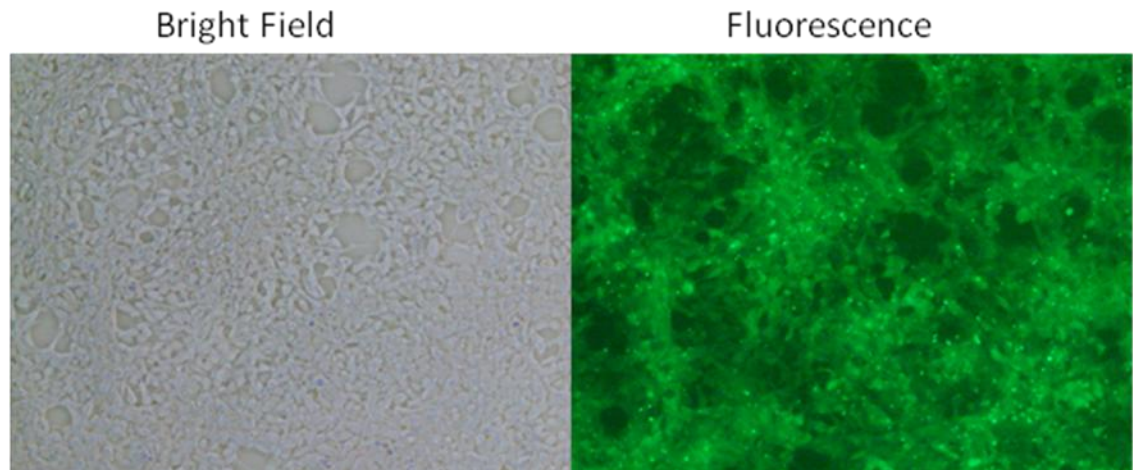


Figure 3.16 | Sustained integration of MLV HER2 genome in 293A producer cells

MLV HER2 stable cells were seeded in T175 flasks in 2% FCS media, and supernatant was collected periodically. Image taken after 2 weeks shows continued GFP fluorescence indicating the presence of the integrated viral genome in daughter cells and continued virus production.

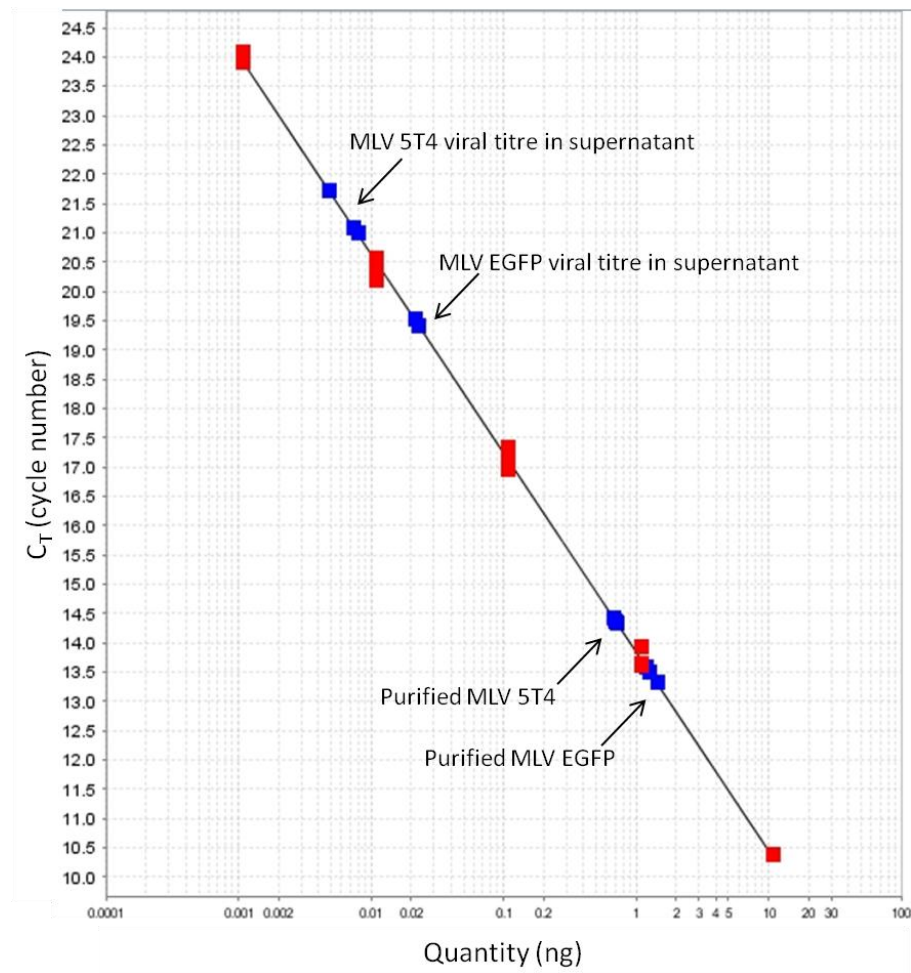


Figure 3.17 | Calculation of viral titres by RT-QPCR.

RNA was extracted from supernatant or purified samples and analysed by RT-QPCR. Viral titres in supernatant averaged 1×10^8 vp/ml, while purified virus titres ranged from 4×10^{10} to 1×10^{11} vp/ml. Red squares indicate standards, blue squares show titres of RNA samples.

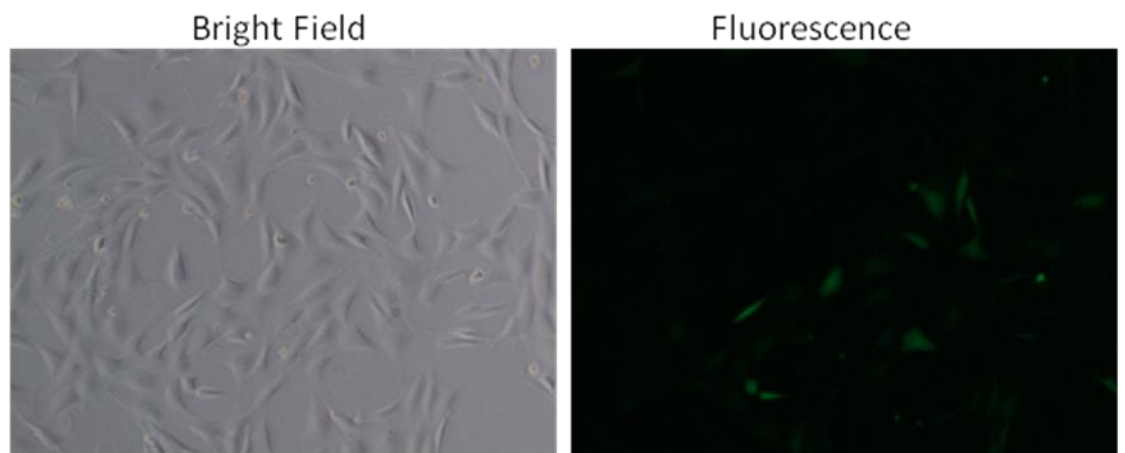


Figure 3.18 | MLV infection of NIH 3T3 cells.

Cells were infected with purified MLV expressing a reporter EGFP gene and visualised by fluorescence microscopy 4 days post-infection.

3.3 Adenovirus – Modification, Production and Purification

3.3.1 Genetic modification of adenovirus by recombination

The AdZ5 system allows for direct cloning of the adenovirus genome through recombination in *E.coli* and encodes the genome of adenovirus serotype 5, with deletions in the E1 and E3 sequences. (Figure 3.19). Insertion of transgenes was first carried out by electroporation of PCR sequences flanked by regions of homology into SW102 bacteria containing the appropriate machinery to mediate homologous recombination (Figure 3.21). The plasmid contains *Amp*, which results in resistance to ampicillin, *SacB*, which confers sensitivity to sucrose, and *LacZ*, encoding β -galactosidase which cleaves X-galactose to form a blue pigment. Bacteria containing the successfully recombined AdHER2 or Ad5T4 genome would have lost the *Amp-LacZ-SacB* cassette, as this would have been replaced by the transgene, thus giving rise to white colonies that are sucrose-insensitive and allowing for clonal selection and amplification of the correct plasmid (Figure 3.20). DNA was extracted from the appropriate colonies and after *BamHI* digest, analysed by gel electrophoresis, which showed that homologous recombination of HER2 or 5T4 PCR products into the Ad genome was successful (Figure 3.21).

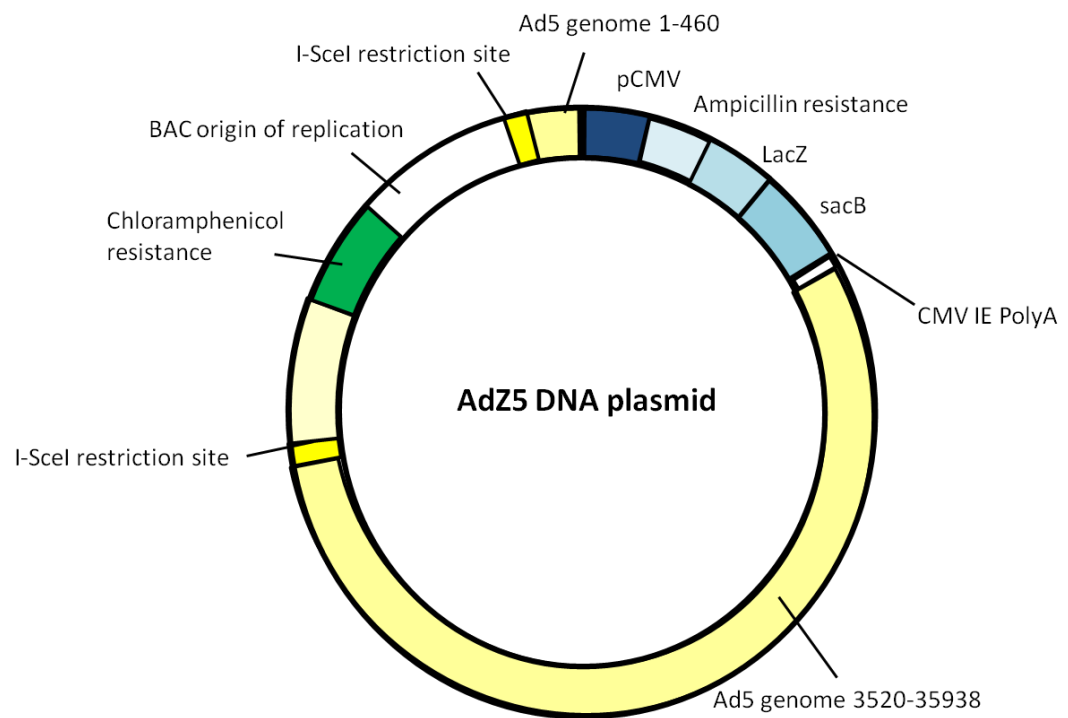


Figure 3.19 | AdZ5 DNA plasmid encoding replication defective adenovirus.

Insertion of transgene under CMV promoter (pCMV) replaces genes encoding ampicillin resistance, LacZ and sacB enabling selection of successfully recombined bacterial colonies. The plasmid is self-excising; upon transfection, expression of *I-SceI* excises bacterial genes and linearises plasmid for subsequent adenovirus production. CMV IE – immediate early CMV promoter. Image adapted from Stanton et al 2008.

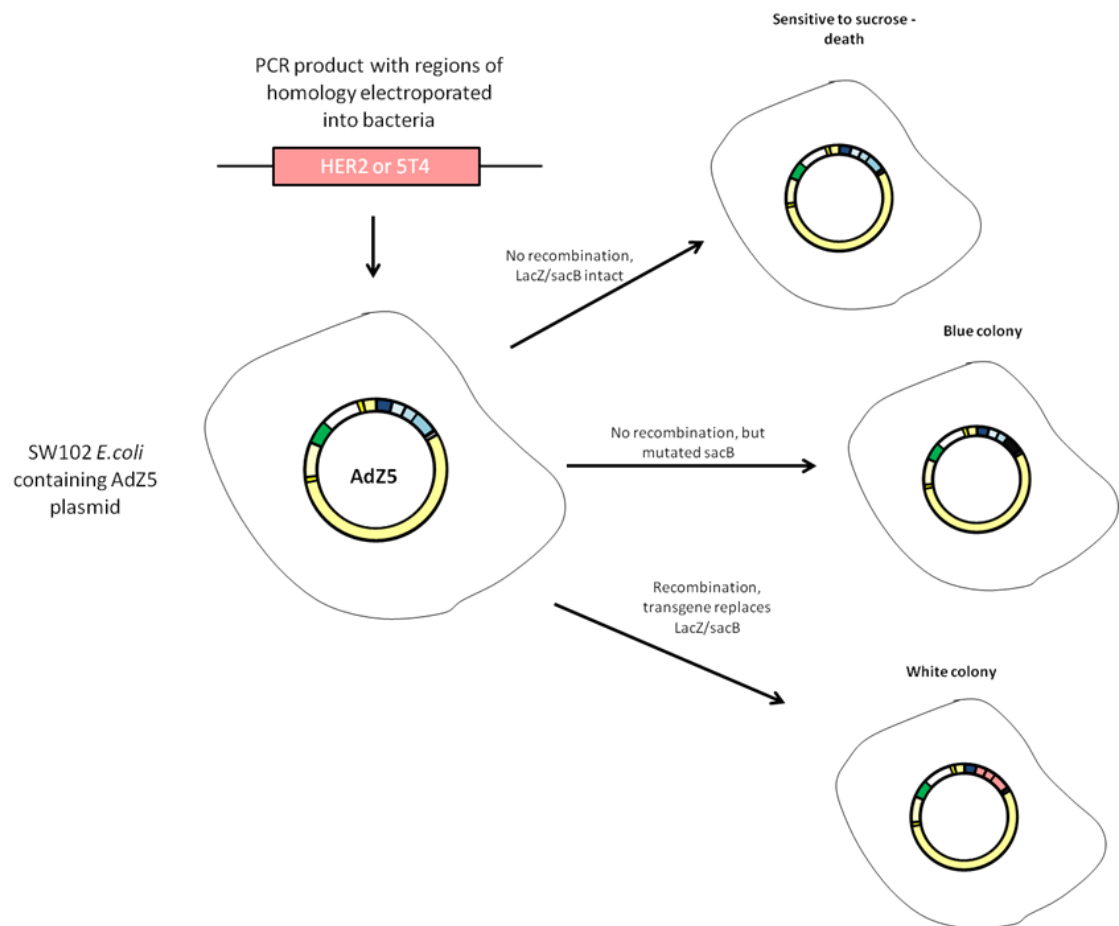


Figure 3.20 | Selection of transgene incorporated bacteria by growth and colony colour.

Replacement of the *Amp-LacZ-sacB* cassette allows selection of correct colonies containing the appropriate transgene. Image adapted from Stanton et al 2008.

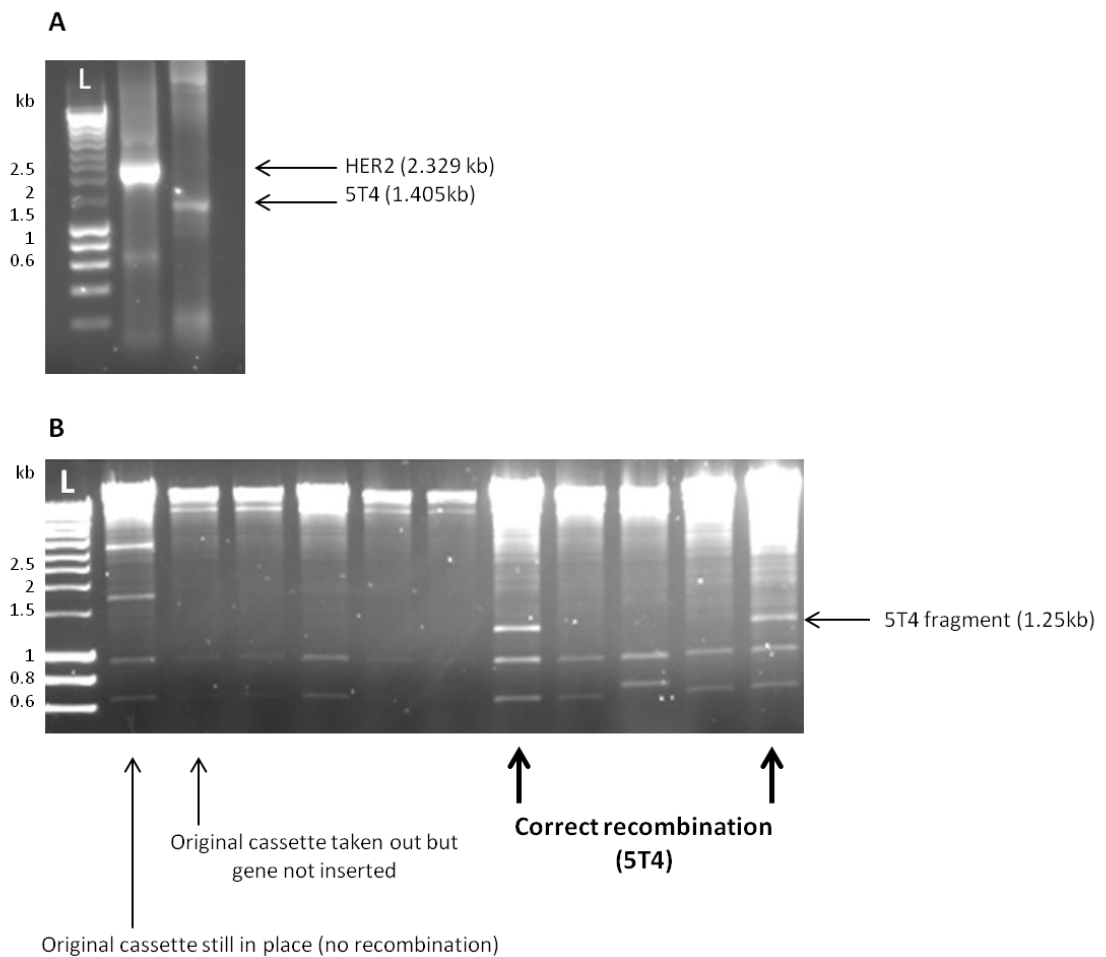


Figure 3.21 | Modification of AdZ5 DNA plasmid by homologous recombination.

(A) HER2 and 5T4 PCR products (EC domains fused to MLV Env transmembrane and cytoplasmic domains) flanked by homologous recombination sequences. (B) PCR products were electroporated into bacteria, after which DNA was extracted from bacterial colonies and digested by *Bam*HI. L denotes molecular ladder. Positive colonies were selected and amplified. Gel shows recombination of 5T4 only, but similar results were obtained for HER2.

3.3.2 AdHER2 and Ad5T4 production and purification

AdHER2 and Ad5T4 DNA plasmids were transfected into 293A cells and self-linearised by *Isce-I* restriction digest to remove bacterial genetic components. 293A cells contain the necessary adenoviral genes for complementation, thus allowing viral replication of replication-defective adenoviruses and production of viable particles. Supernatant from transfected 293A cells was removed and serially diluted to infect a 96 well plate. Once CPE was seen, supernatant was taken from the appropriate wells and DNA was extracted to allow for calculation of viral titre by quantitative PCR (QPCR). Fresh 293A cells were infected with the same number of virus particles to allow a fair comparison of viral clones. Virus clones were selected based on transgene expression (see Chapter 4) and purified by caesium chloride banding.

As adenovirus replicates by infecting a cell and then inducing cell lysis to facilitate viral spread, infected cell lysate was used for purification rather than supernatant. Higher viral yields are typically obtained from cell lysate rather than from supernatant. It was important to use infected virus-containing cells that were just ready to lyse, as opposed to cells that had already lysed and released virus into the supernatant in order to obtain high viral titres. Cells were infected in hyperflasks to maximise the number of cells that could be infected, and when ready, cells were detached by tapping flasks before collecting and centrifuging to form a pellet. Viral cell lysate was loaded onto caesium chloride gradients and ultracentrifuged, after which three bands could be seen in each tube, containing cell debris, empty viral capsids and complete intact viruses. This final band was extracted by piercing each tube with a needle and syringe. Viruses were left to dialyse overnight before incubation with benzonase to remove free DNA, and overlaid on a caesium chloride gradient and spun for a second time. After extraction from the centrifugation tubes again by needle and syringe, the virus was dialysed for a second time, after which viruses were stored at -80°C in aliquots to minimise multiple freeze-thaw cycles. Purified adenoviral titres were calculated by Picogreen assay. Viral titres as high as 5×10^{12} virus particles per ml can be obtained by this method of production and purification.

3.4 Discussion

To create vaccine particles presenting HER2 and 5T4 on the surface, WT MLV was genetically modified to produce recombinant viruses. Virus particles were produced through transient transfection of mammalian cells, and long-term production of vaccine particles was achieved through generating stable producer cell lines, in a similar method to lentiviral transduction. Viral titres were calculated by analysis of RNA genomes, due to the non-cytopathic nature of the virus which meant that conventional viral infectivity assays could not be used. A comparison between viral titres obtained from transiently transfected and stable cells indicated viral production is higher in stable cells and can be sustained for longer, due to retention of the MLV genome in the cellular DNA. Titres of MLV are often low compared to other viral vaccine vectors such as adenovirus, as the virus is not lytic, hence it was important to find a way of increasing viral titres. One such method was by selecting stable cells with the highest GFP expression levels, as this indicated that these cells would have the most number of viral genomes incorporated into the host cellular DNA, and hence the highest expression levels of virus. Another method was to optimise the purification of viruses which was achieved by ultracentrifugation over sucrose cushions, leading to a 100 fold increase in virus particles per ml from crude supernatant, enabling further assays to be carried out and for use in *in vivo* studies.

It was desirable to generate replication defective adenoviruses expressing HER2 or 5T4 to act as a control against MLV HER2 and MLV 5T4 for use *in vivo*. The hypothesis for such a comparison would be to compare the impact of pre-existing anti-vector immunity against anti-transgene antibody responses. As the modified MLV particles do not contain any virus proteins on the surface, there should be no neutralising anti-MLV antibodies targeted to the envelope protein. Although there may be anti-MLV antibodies to the internal viral proteins, these would not hamper the immune response to HER2 or 5T4. In contrast, the capsids of the modified adenoviruses are unchanged from the WT virus, and are hence exposed to neutralising antibodies. The effects that these different viral surfaces will have on antibody responses will be tested *in vivo*.

This chapter describes the modification of replication defective adenovirus to produce HER2 or 5T4 through recombination. Correctly recombined plasmids were selected to generate viruses, which were then purified using conventional caesium chloride gradients and ultracentrifugation. These methods allowed for the production of high viral titres for use *in*

vivo. Replication defective adenoviruses are often used as vaccine platforms, due to the relative ease of modification. Production in 293A cells and subsequent purification of these viral vectors is also well characterised for optimum titres. Their use in pre-clinical studies has become widespread; however the prevalence of pre-existing anti-adenovirus immunity has limited their potential in clinical use. Nonetheless recombinant adenoviruses vectors can be considered a gold standard in terms of achieving high anti-transgene immune responses for vaccination purposes, due in part to the long term episomal retention of the adenoviral genome long after infection. By comparing conventional adenoviral vaccine vectors to the novel MLV vaccine particles, new insights can be gained into the anti-vector immune response in vaccination.

4 Characterisation of Antigen in Producer Cells and on Virus Particles

4.1 Introduction

Three novel recombinant MLV-based virus particles - MLV HER2, MLV 5T4 and MLV EGFP, were successfully produced, as previously described in Chapter 3, each intended to present a specific antigen on the surface of the virus particle as a fusion protein. It was vital to characterise these viral vaccines thoroughly, to ensure that the genetic modifications were correct and that the antigen-virus fusion proteins were properly expressed in order to generate the required antibody response *in vivo*. This chapter describes the assessment of transgene expression in packaging cells, and subsequently the presence of the antigen-viral fusion protein in virus particles. A range of techniques was used to determine if the transgene products were presented correctly on the external surface of the virus particle, which was crucial to test the overarching vaccination hypothesis.

During the generation of recombinant adenoviruses (as outlined in Chapter 3) it was necessary to check that the modified viruses were contained the correct transgene and assess expression levels. It was important to confirm that the adenoviruses were expressing the correct transgene before and after purification for use in *in vivo* studies in order to elicit the appropriate antibody response and to act as a fair comparison to the modified MLV vaccine particles. In each case, the recombinant HER2 and 5T4 transgene products were not associated with the adenovirus particle but were produced in the infected cell independent of viral proteins; therefore it was not necessary to analyse the adenovirus capsid itself as this was unchanged from the WT AdZ5. Hence only the transgene expression of recombinant adenoviruses in complementing 293A cells was analysed.

4.2 Expression of Tumour Antigen-MLV Envelope Fusion Protein in Packaging Cells

4.2.1 Production of antigen on the surface of packaging cells

It was necessary to screen potential packaging cell lines to determine which cells were negative for human HER2 and/or human 5T4. This enabled accurate detection of the tumour antigen-viral envelope fusion protein with no competing endogenous cellular expression of human HER2 or 5T4. As both tumour antigens of interest were of human origin, non-human cell lines were selected for screening. Baby hamster kidney (BHK-21 or BHK) cells were found to have minimal levels of cross-reactivity with HER2 and 5T4 (Figure 4.1), and were subsequently used as packaging cells for transient transfections of MLV HER2 and MLV 5T4. In subsequent screens, human breast cancer cell lines SKBr3 and MCF-7 were found to be highly positive for HER2 and 5T4 respectively, and were used as positive controls in future assays.

To confirm the production of the fusion protein, flow cytometry was carried out to detect cell surface expression of HER2 or 5T4 after transient transfection of BHK cells. A secondary goat anti-mouse antibody conjugated to Alexa Fluor 647 (which fluoresces at red wavelengths) was used to bind to primary antibodies against HER2 or 5T4 (previously validated in cell line screening). Both MLV 5T4 and MLV HER2 DNA plasmids contain a reporter GFP gene, the cellular expression of which can be used as an indicator of a successful transient transfection. As cells were not permeabilised prior to staining, antibodies would only bind to the cell surface of transfected BHK cells, hence any red (Alexa647) fluorescence would represent the antigen-viral fusion protein on the cell membrane before virus budding.

MLV HER2 DNA plasmid was transiently transfected into BHK cells, and cells were analysed by flow cytometry 24 hours later (Figure 4.2). Both GFP and HER2 expression could be detected in over 25% of transfected cells, indicating that cloning of the transgene was successful. As the primary anti-HER2 antibody used for flow cytometry was validated on endogenous HER2 expressed on SKBr3 cells, the fact that the same antibody could be used to detect HER2 on transfected BHK cells indicates that the HER2-MLV fusion protein is properly presented on the cell surface for antibody binding. However there is a distinct subpopulation of cells that seem to only express HER2 and do not show any GFP expression. This could derive from the weak

strength of the internal promoter for GFP translation (EMCV IRES), or because GFP is produced in the cytoplasm as a soluble protein, and may be more diffuse and less amplified than the detection of membrane proteins using antibodies. Inorganic fluorescent dyes such as Alexa Fluor 647 are also much brighter and more stable than fluorescent proteins, hence leading to greater detection in the red fluorescence channel¹²⁷. Also, the cells were gated more harshly against the 488 channel due to autofluorescence, but not as harshly in the 647 channel; hence there would need to be much higher GFP expression to see a change in the 488 channel, but not as much for the 647 channel (although still remaining relative to one another).

MLV 5T4 DNA plasmid was also transiently transfected into BHK cells, and dual expression of GFP and 5T4 was detected by flow cytometry 24 hours later (Figure 4.3). There is a statistically significant population of dual expressing GFP and 5T4 cells (over 35%) and an additional population of 5T4 only expressing cells, as seen with MLV HER2 transfected cells. The primary anti-5T4 antibody used for this flow cytometric analysis was also validated on endogenous 5T4 expressed on MCF-7 cells, indicating that the 5T4-MLV fusion protein presented on the surface of transfected BHK cells is properly presented for antibody binding in a manner similar to endogenous native 5T4.

Stable producer cell lines were created as detailed in the previous chapter, and were denoted as follows: MLV HER2 293A (produce MLV HER2 particles), MLV 5T4 293A (MLV 5T4 particles) and MLV EGFP 293A (MLV EGFP particles). 293A cells were used to form producer cells as they are amenable to infection by retrovirus, and can produce higher titres of virus than other cell types. Flow cytometry was used to measure the presence of antigen on the cell surface of stable producer cell lines. Although 293A cells exhibit endogenous expression of both HER2 and 5T4, the presence of either HER2-viral fusion protein or 5T4-viral fusion protein resulted in a shift in fluorescence compared to normal 293A cells (Figure 4.4). A larger shift can be seen in the GFP expression of MLV EGFP 293A stable cells compared to untransfected 293A cells, which are negative for GFP.

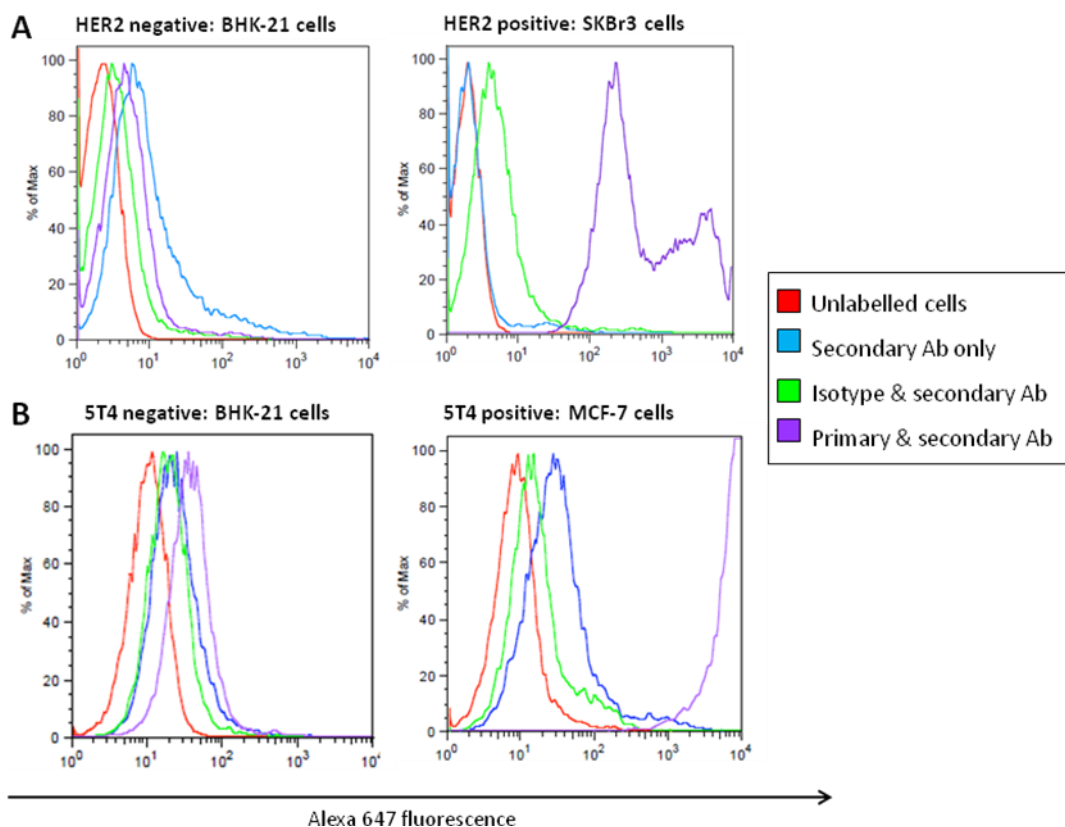


Figure 4.1 | Screen to find HER2/5T4 negative packaging cells and HER2/5T4 positive cells.

Flow cytometry was carried out to find negative and positive cells expressing HER2 or 5T4. Cells were labelled with primary anti-HER2 antibody, anti-5T4 antibody or isotype antibody, and a secondary antibody conjugated to Alexa 647. (A) HER2 screening. (B) 5T4 screening.

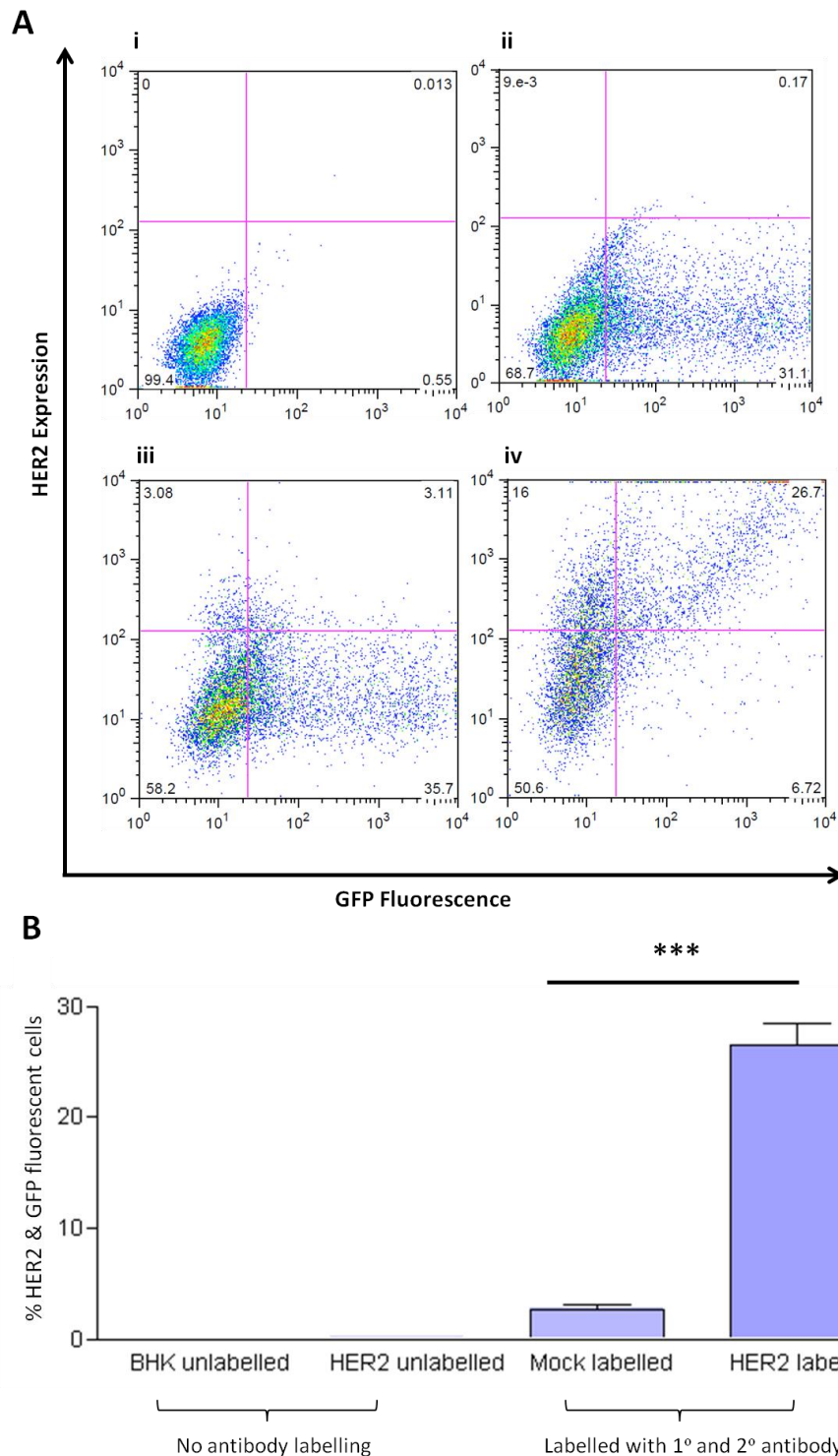


Figure 4.2 | Flow cytometry on MLV HER2 transiently transfected cells shows GFP expression and cell surface expression of HER2.

(A) Flow cytometry plots representative of $n = 3$. (i) Untransfected BHK cells with no antibody labelling as a negative control; (ii) MLV HER2 transfected cells with no antibody labelling; (iii) Mock transfected cells with antibody labelling, GFP positive but HER2 negative; (iv) MLV HER2 transfected cells with antibody labelling, both primary and secondary. (B) Percentages of double positive GFP and HER2 expressing cells from all replicates. Statistical analysis performed using one-way ANOVA (analysis of variance) where *** = $p < 0.001$. Error bars represent standard deviation from the mean.

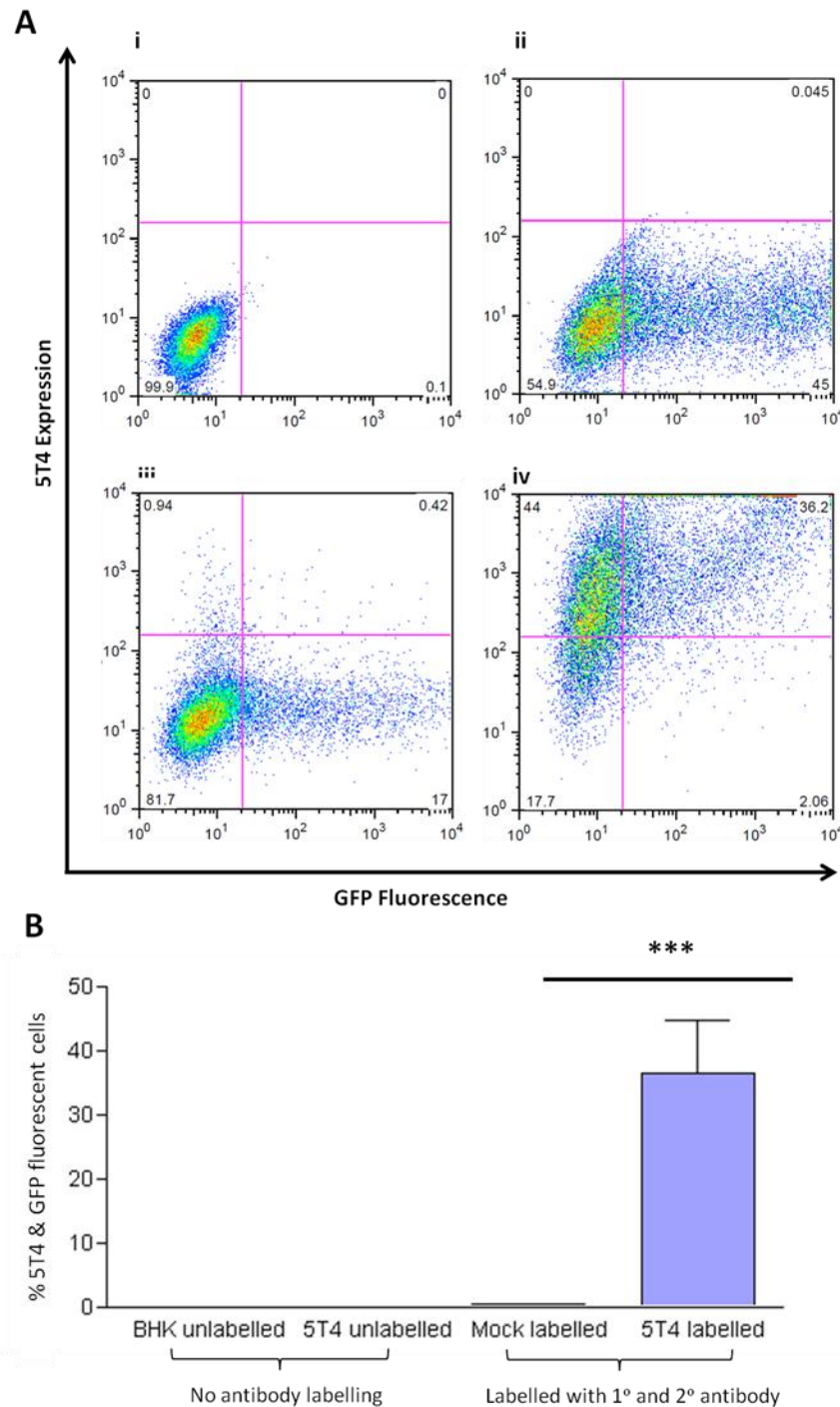


Figure 4.3 | Flow cytometry on MLV 5T4 transiently transfected cells shows GFP expression and cell surface expression of 5T4.

(A) Flow cytometry plots representative of $n = 3$. (i) Untransfected BHK cells with no antibody labelling as a negative control; (ii) MLV 5T4 transfected cells with no antibody labelling; (iii) Mock transfected cells with antibody labelling, GFP positive but 5T4 negative; (iv) MLV 5T4 transfected cells with antibody labelling, both primary and secondary. (B) Percentages of double positive GFP and 5T4 expressing cells from all replicates. Statistical analysis performed using one-way ANOVA where *** = $p < 0.001$. Error bars show standard deviation from the mean.

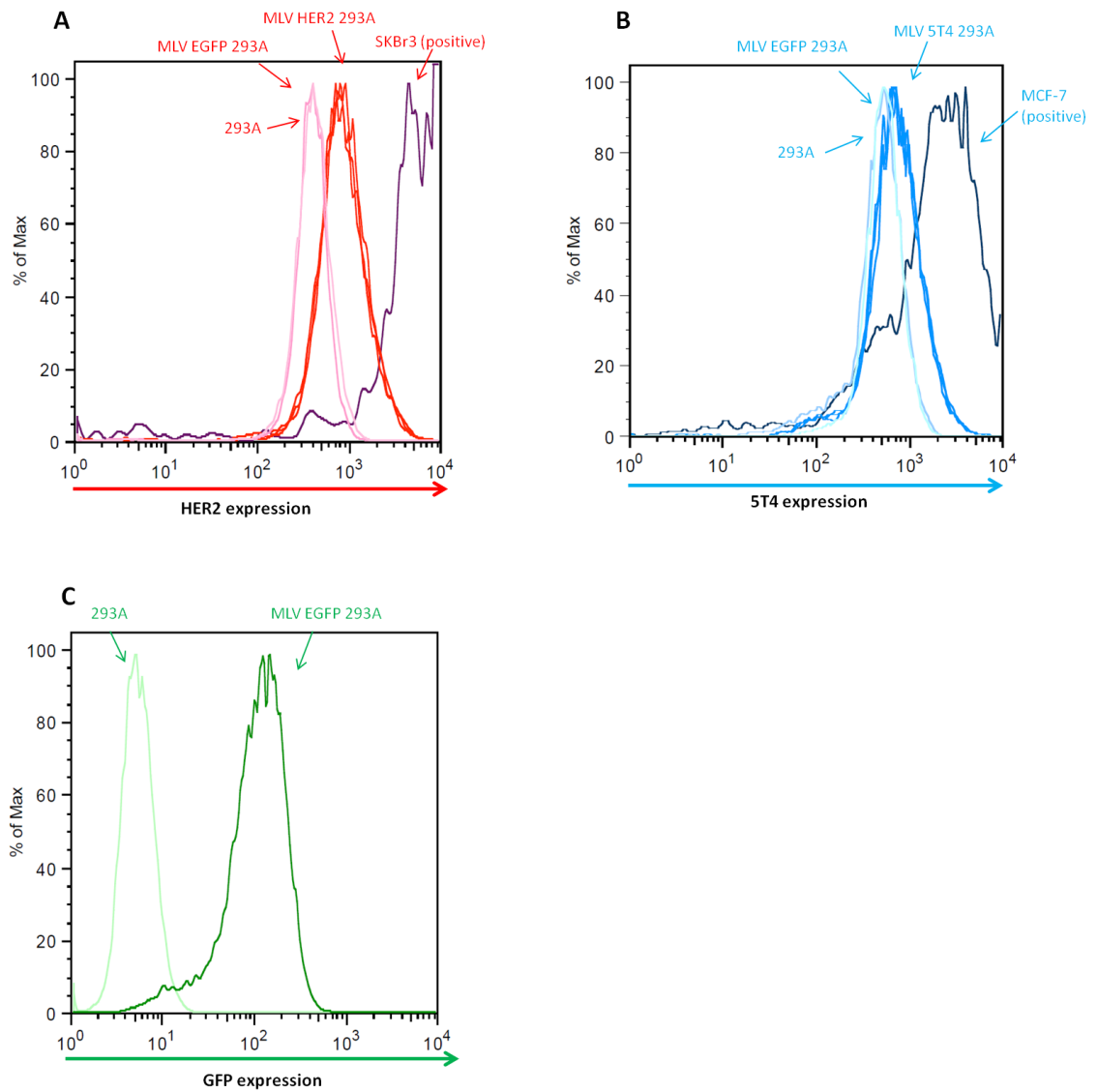


Figure 4.4 | Cell surface expression of antigen on stable producer cell lines.

(A) HER2 expression of MLV HER2 293A (3 replicates), negative controls 293A and MLV EGFP 293A, and SKBr3 (positive control). (B) 5T4 expression of MLV 5T4 293A (3 replicates), negative controls 293A and MLV EGFP 293A, and MCF-7 (positive control). (C) GFP expression of MLV EGFP 293A and negative control 293A cells.

4.2.2 Intracellular expression of tumour antigen

Previous flow cytometric data (section 4.2.1) only analysed the cell surface expression of the antigen-viral fusion proteins, highlighting one portion during the viral life cycle; specifically, the aggregation of envelope proteins at the cell surface to enable budding of virus particles from the membrane. In order to assess the total expression of the fusion protein in the cell as it is synthesised and trafficked to the cell membrane, polyacrylamide gel electrophoresis (PAGE) and Western blotting was carried out on packaging cell lysates.

Antibodies for HER2 and 5T4, previously validated and used in flow cytometry, were also used for Western blotting. However it was found that these antibodies were unable to recognise their cognate antigen when protein samples were first run in a denaturing and reducing gel. Therefore it was necessary to run and transfer proteins in their native form, for which a protocol for native PAGE was optimised. Typically, samples run on protein gels are heated first and β -mercaptoethanol is added to remove any secondary or tertiary structure, and SDS is added to impart a negative charge on the overall protein, enabling proteins to be separated by size. As there is no SDS present in native gels, and without heating or incubating protein samples with β -mercaptoethanol, proteins would retain their native structure and so would not be separated by size; however a protein ladder was still run alongside samples in the gel to verify that the gel was running properly. Proteins were transferred onto nitrocellulose membranes, which were first incubated with Ponceau stain for total protein content and to ensure even loading in all lanes, then probed with relevant antibodies. The blots show that MLV HER2 and MLV 5T4 transfected BHK cells express the appropriate antigen (Figure 4.5).

Stable cell lines MLV HER2 293A, MLV 5T4 293A and MLV EGFP 293A were analysed for expression of the appropriate antigen by native PAGE or SDS PAGE (for GFP samples) and Western blotting. It can be seen that all cell lines express the required antigen (Figure 4.6). All gels contain lanes with other stable cell lines as negative controls, with the exception of Figure 4.6 C where MLV HER2 293A lysate also contains GFP from expression of the reporter gene. This is stronger than the band for GFP in MLV EGFP 293A, possibly because GFP from MLV HER2 293A is produced in the cytoplasm and more accessible after cell lysis, whereas the EGFP-virus fusion protein still contains the virus signal peptide, and so will be associated with cellular membranes and not in solution after lysis. Also, more protein is expressed in the cytoplasm than at the membrane, thus contributing to the stronger GFP band seen in the MLV HER2 293A lysate.

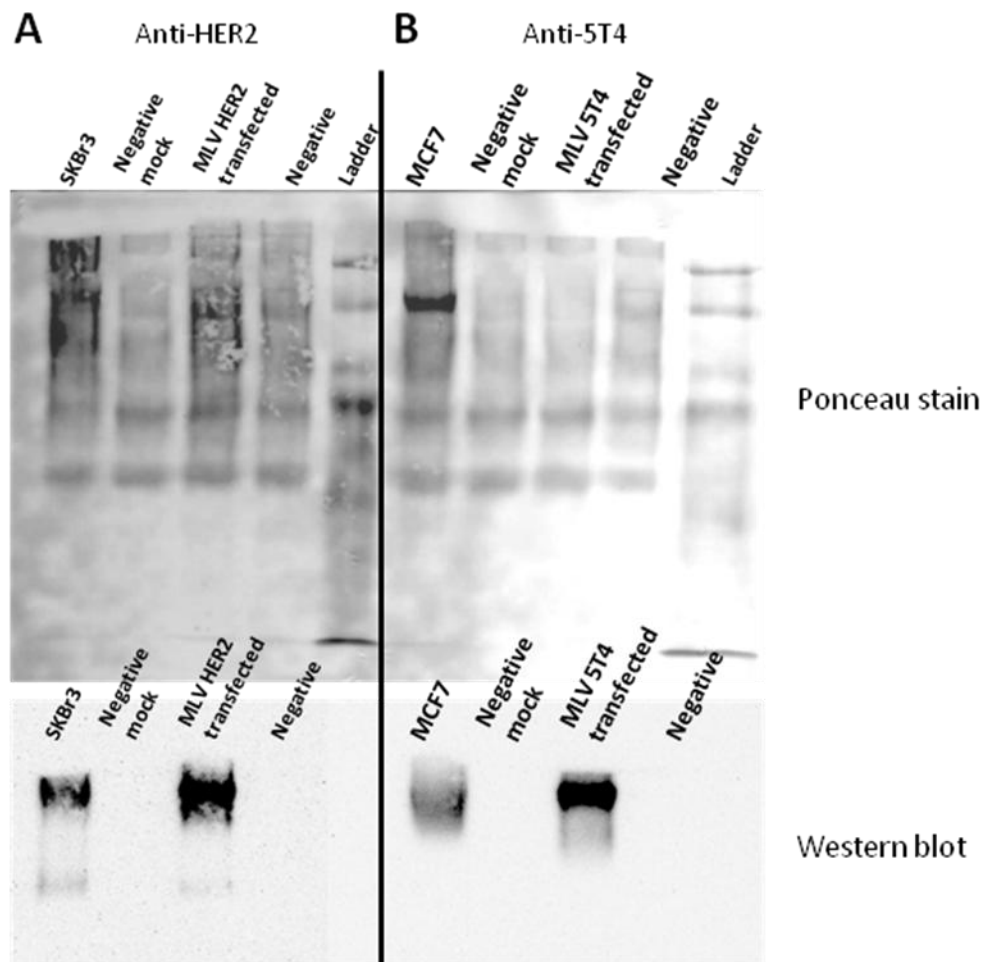


Figure 4.5 | Total expression of HER2 and 5T4 from transiently transfected BHK packaging cells on native gels.

(A) Blot probed with anti-HER2 antibody; SKBr3 cells were used as HER2 positive cells; negative mock indicates mock transfected cells that were HER2 negative, and negative cells were untransfected BHK cells. (B) Blot probed with anti-5T4 antibody; MCF-7 cells were used as 5T4 positive cells; negative mock indicates mock transfected cells that were 5T4 negative, and negative cells were untransfected BHK cells.

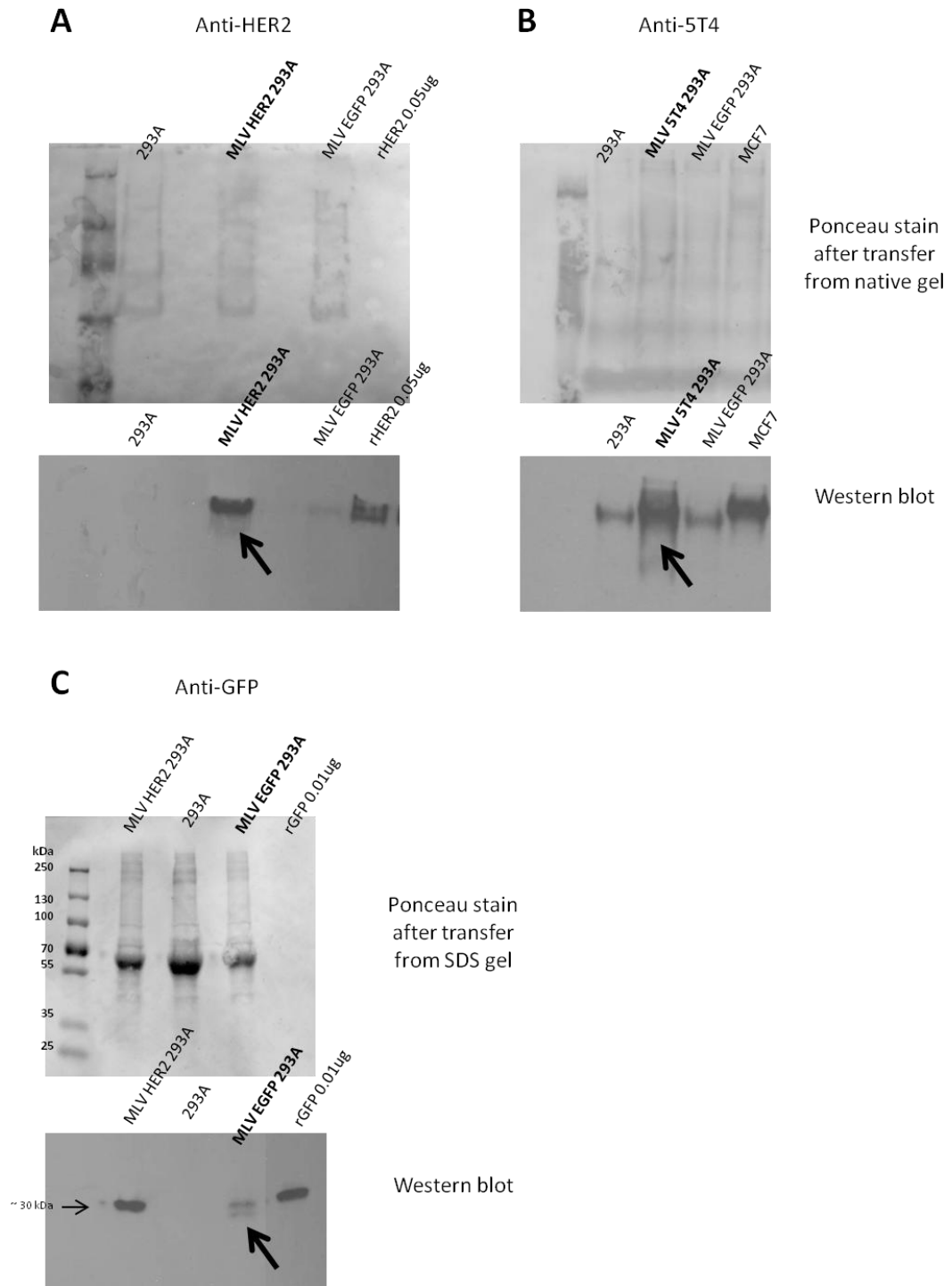


Figure 4.6 | Total expression of HER2, 5T4 and EGFP from stable cell lines.

(A) MLV HER2 293A Ponceau stain (top) and Western blot (bottom) for HER2. 293A and MLV EGFP 293A cell lysate were run as negative controls, recombinant HER2 was used as a positive control. Arrow indicates expression of HER2-virus fusion protein. (B) MLV 5T4 293A Ponceau stain and Western blot for 5T4. Negative controls used were 293A and MLV EGFP 293A lysates. MCF-7 cell lysate was used as a positive control. Arrow indicates expression of 5T4-virus fusion protein. (C) MLV EGFP 293A Ponceau stain and Western blot for GFP. 293A lysate was run as a negative control. Arrow indicates expression of EGFP-virus fusion protein. Recombinant GFP was used as a positive control.

4.3 Characterisation of Modified Retrovirus Particles

4.3.1 Visualisation of vaccine particle

Purified MLV HER2 was analysed by cryo-electron microscopy, carried out by Dr. Robert Gilbert (Wellcome Trust/Division of Structural Biology, University of Oxford). Intact retrovirus particles were seen after ultracentrifugation at high speeds prior to loading on the grid (Figure 4.7). The shape and diameter of MLV HER2 is unchanged from that of the WT virus (reported in literature to be approximately 100 nm⁷²). Therefore modification of the surface of MLV does not alter its physical shape or size, despite the change in external protein on the surface of the vaccine particle.

4.3.2 Detection of antigen on the virion surface by dot blot

Previous data have shown that packaging cells producing the modified retroviruses are positive for antigen expression. To assess whether the purified viruses themselves still contained the relevant tumour antigens, a dot blot was carried out 2.5 x 10⁸ virus particles were pipetted in each dot. The blots were probed using the relevant antibodies, and it was seen that each purified virus expressed the appropriate antigen (Figure 4.8).

However fainter spots can be detected in the dots containing viruses for the negative control; this could be because 293A expresses endogenous human HER2 and 5T4 (as seen previously in Figure 4.4), which might be incorporated into the membrane as the virus buds off; if this were the case then all viruses might contain some HER2 and 5T4 on the viral surface. To investigate this possibility, a dot blot on 293A cell lysate and other cell types was carried out. It can be seen that mouse cell lines CT26 and NIH 3T3 (used later to produce WT MLV, as BHK cells are not amenable to infection – see Chapter 6 for *in vivo* use of WT MLV) are negative for human HER2 and 5T4, while 293A cells express some HER2 but high amounts of 5T4, comparable to positive controls MCF-7 and CT26-h5T4 (stable cell line expressing human 5T4) (Figure 4.9). This would explain why HER2 and 5T4 can be found in purified viruses that are not genetically modified to express HER2 or 5T4, and supports the data seen in Figure 4.4.

4.3.3 Detecting HER2 on the surface of MLV HER2 virus particles

Although the dot blots show that each virus is associated with the appropriate antigen, a more sophisticated method was used to conclusively prove that the recombinant viruses displayed the correct antigen on the surface of the particle. As it has already been shown that 293A cells (used to stably produce the modified viruses) express low levels of HER2 on the cell surface, it was necessary to produce MLV HER2 from BHK cells, which are negative for HER2. In this way, any HER2 detected could be derived directly from the virus particle, rather than endogenously expressed by the producer cell.

Small amounts of MLV HER2 virus particles were produced transiently from BHK cells by transfection and collection of supernatant. This virus-containing supernatant was concentrated to reduce the total volume and increase the overall viral titre. Analysis of MLV HER2 particles was carried out by flow cytometry. However as the particles themselves are too small to be detected by conventional flow cytometry, it was necessary to bind the viruses to large beads or microspheres (Bangs Laboratories) to enhance any potential signal picked up by flow cytometry, as multiple particles would bind to a single bead. Protein G-microspheres were incubated with anti-HER2 antibodies, before incubation with MLV HER2 viruses. The bead-virus complex was subsequently incubated with an anti-HER2 antibody conjugated to FITC for analysis by flow cytometry. A schematic diagram for this complex can be seen in Figure 4.10.

Unconjugated anti-HER2 antibodies were first screened alongside an anti-HER2 antibody conjugated to FITC. It was important that both antibodies did not occupy the same epitope of HER2, as this would lead to decreased fluorescence based on antibody competition for the same HER2 site, as opposed to actual absence of HER2. SKBr3 cells were used as surrogates for HER2 antibody screening. It can be seen that combining two anti-HER2 antibodies does not decrease the fluorescence of the conjugated antibody, meaning that both antibodies are binding to different sites on HER2 (Figure 4.11). The antibodies both bind separately to HER2, as demonstrated by the controls used; firstly by pairing the unconjugated antibody with a secondary antibody bound to FITC, and secondly by testing the anti-HER2-FITC antibody alone. Isotype staining did not show any background fluorescence, indicating that both anti-HER2 antibodies bound to HER2 specifically.

Microspheres were incubated with the appropriate antibodies and MLV HER2, and analysed by flow cytometry. Compared to the negative control of MLV 5T4-bead complexes, MLV HER2-

beads display a shift in FITC fluorescence (Figure 4.12). This shows that HER2 is present on the surface of MLV HER2 virus particles, and can be detected by flow cytometry with microspheres.

Flow cytometry of MLV 5T4-bead complexes was not carried out as a fluorophore conjugated antibody against 5T4 that bound to a different epitope than the anti-5T4 antibody used for previous flow cytometry and Western blotting was not available.

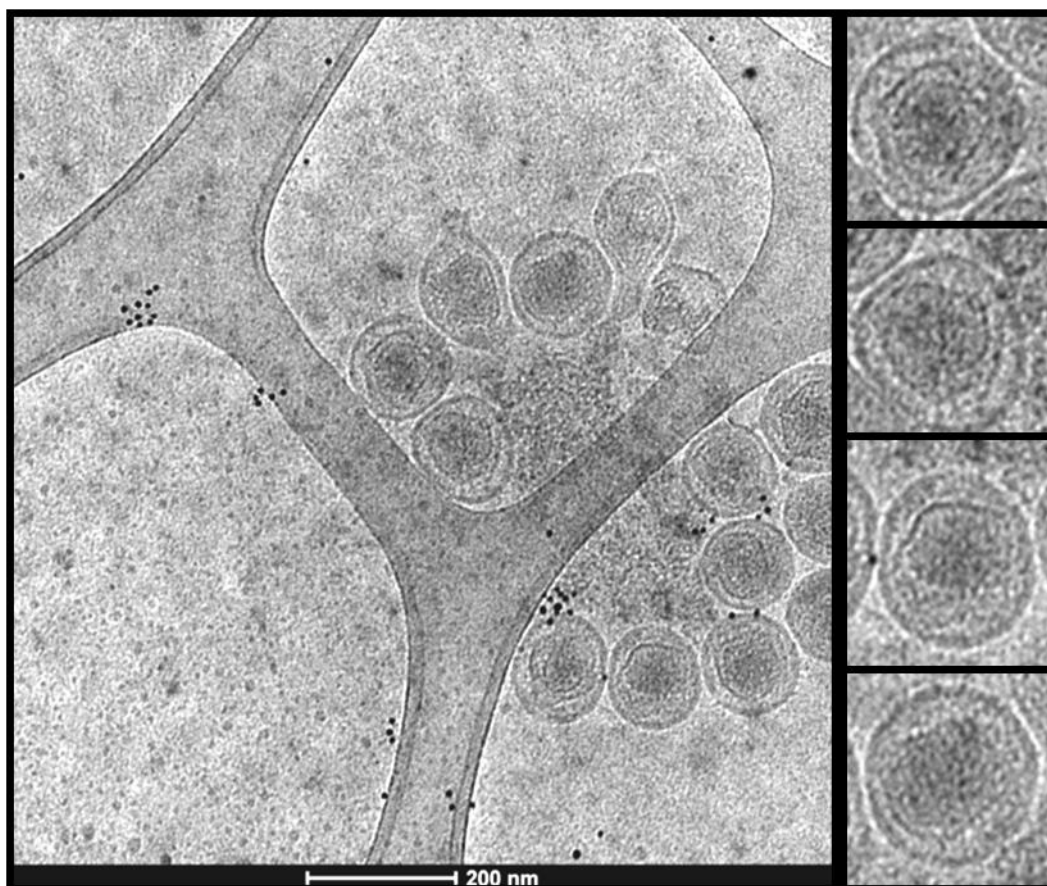


Figure 4.7 | Cryo-electron microscopy of purified MLV HER2.

Image shown on the left is representative of entire grid. Digitally zoomed images on right hand side show individual virus particles that are uniform in shape and size. Gold particles (6 nm) can be seen in the image due to immuno-gold labelling of HER2. Cryo-EM carried out by Dr Robert Gilbert (Wellcome Trust/Division of Structural Biology, University of Oxford).

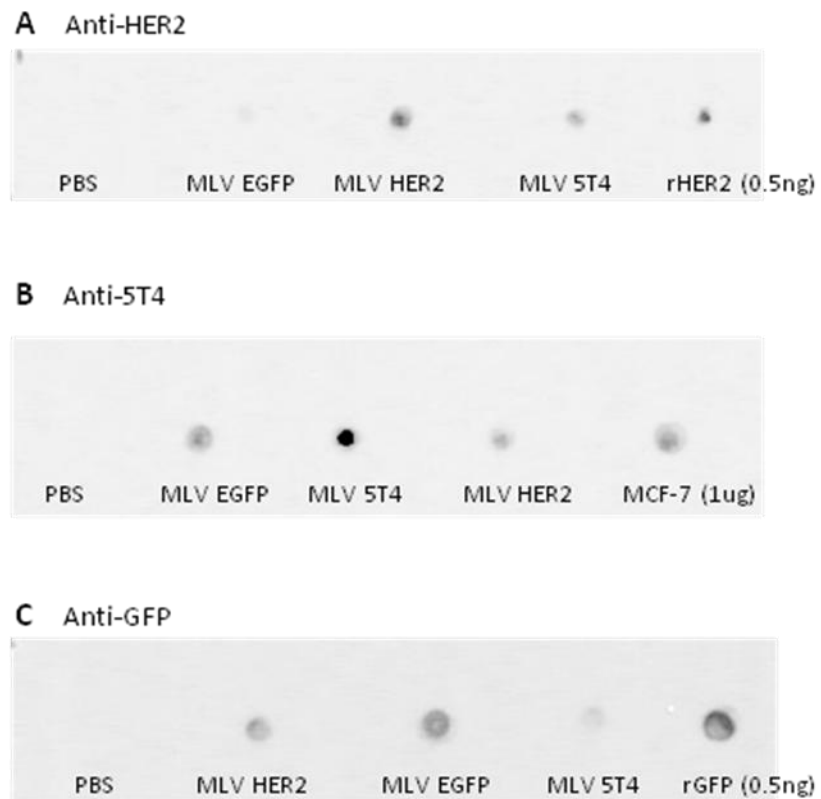


Figure 4.8 | Specific antigen is present in purified MLV particles.

(A) Blot for HER2, using MLV EGFP and MLV 5T4 as negative controls, and recombinant HER2 as a positive control. (B) Blot for 5T4, with MLV EGFP and MLV HER2 as negative controls, and MCF-7 cell lysate as a positive control (C) Blot for GFP, with MLV HER2 and MLV 5T4 as negative controls, and recombinant GFP as a positive control..

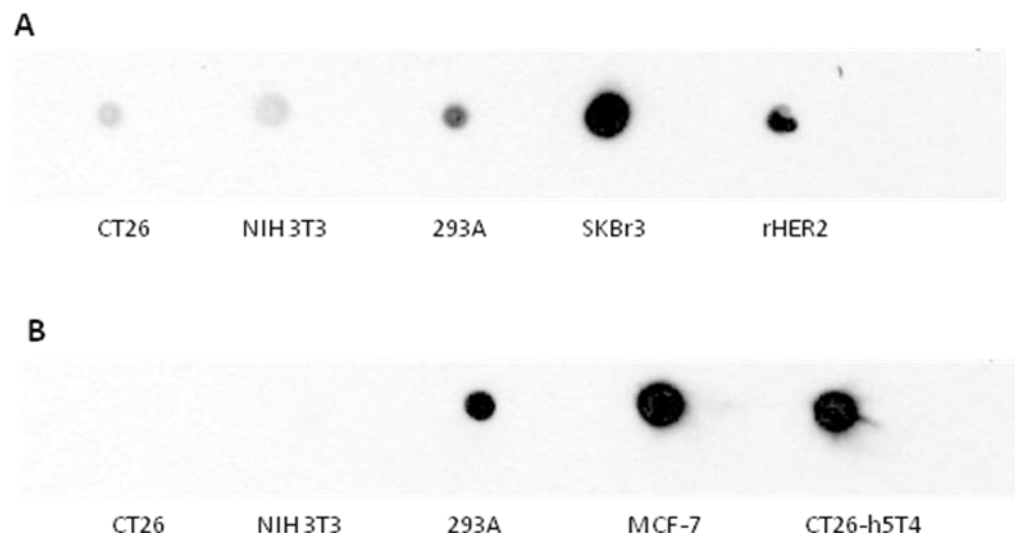


Figure 4.9 | Expression of endogenous HER2 or 5T4 on cells amenable to WT MLV infection. Mouse cell lines (CT26, NIH 3T3) and human cells (293A) were tested for (A) human HER2 expression or (B) human 5T4 expression using relevant positive controls (SKBr3 and recombinant HER2 for HER2, and MCF-7 and CT26-h5T4 for 5T4).

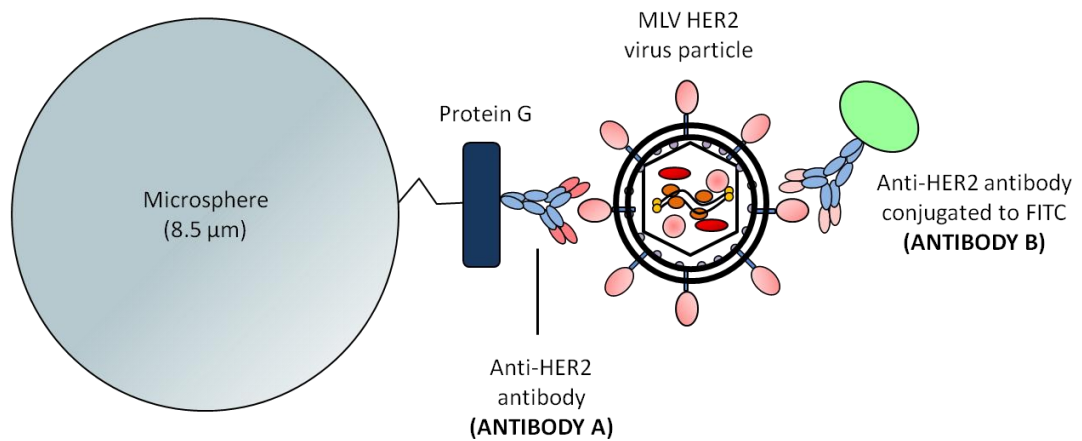


Figure 4.10 | Schematic diagram of bead-virus complex for flow cytometry.

Microspheres bound to Protein G are incubated with the an unconjugated anti-HER2 antibody. After incubation with MLV HER2 virus particles, the complex is labelled with a FITC conjugated anti-HER2 antibody for flow cytometry. Diagram not to scale.

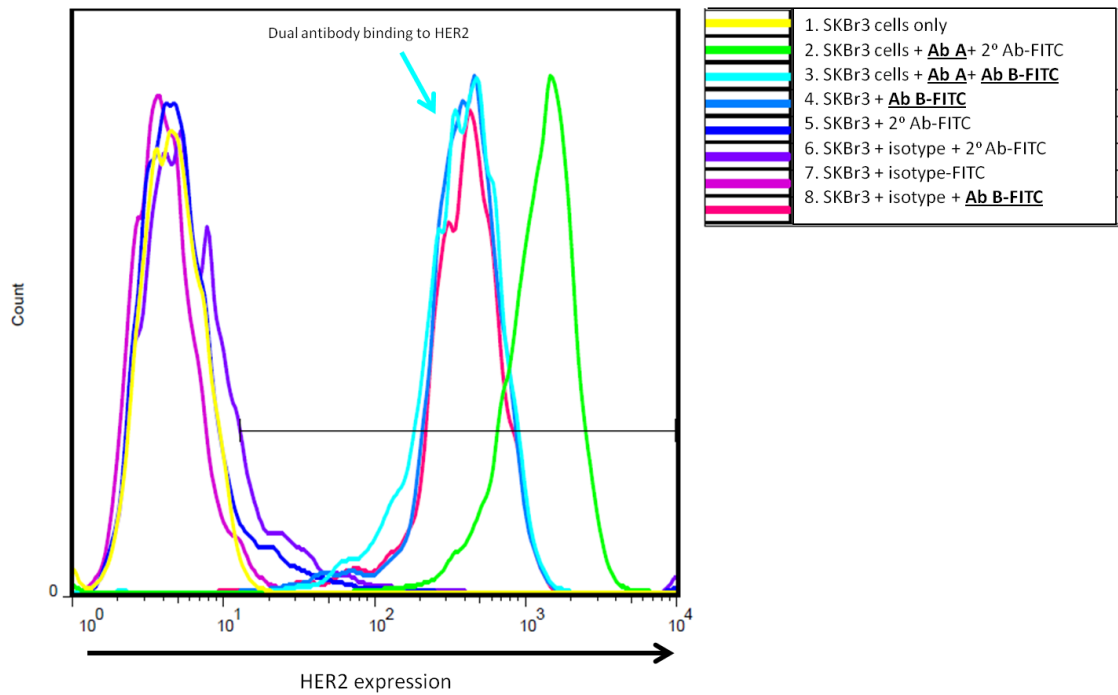


Figure 4.11 | Verification of anti-HER2 antibodies for flow cytometry with beads-virus complexes.

To confirm that the two antibodies used for beads-virus flow cytometry did not bind to the same epitope of HER2, SKBr3 cells were labelled with different combinations of both antibodies: (1) unlabelled SKBr3 cells (negative control). (2) SKBr3 cells labelled with Antibody A (Ab A, to be used as antibody link between Protein G coated bead and MLV HER2 virus) and secondary antibody conjugated to FITC, to verify specific Ab A binding to HER2. (3) SKBr3 cells labelled with Ab A and Ab B-FITC (to be used to label MLV HER2 virus particles), to confirm that both antibodies bind to HER2. (4) SKBr3 cells labelled with Ab B – FITC, to check that this antibody binds specifically to HER2. (5) SKBr3 cells labelled with secondary antibody conjugated to FITC, (6) SKBr3 cells labelled with isotype antibody and secondary antibody conjugated to FITC and (7) SKBr3 cells labelled with isotype antibody conjugated to FITC, to confirm that there is no non-specific binding to HER2. (8) SKBr3 cells labelled with isotype antibody and Ab B-FITC, to ensure that Ab B-FITC does not bind non-specifically to other antibodies.

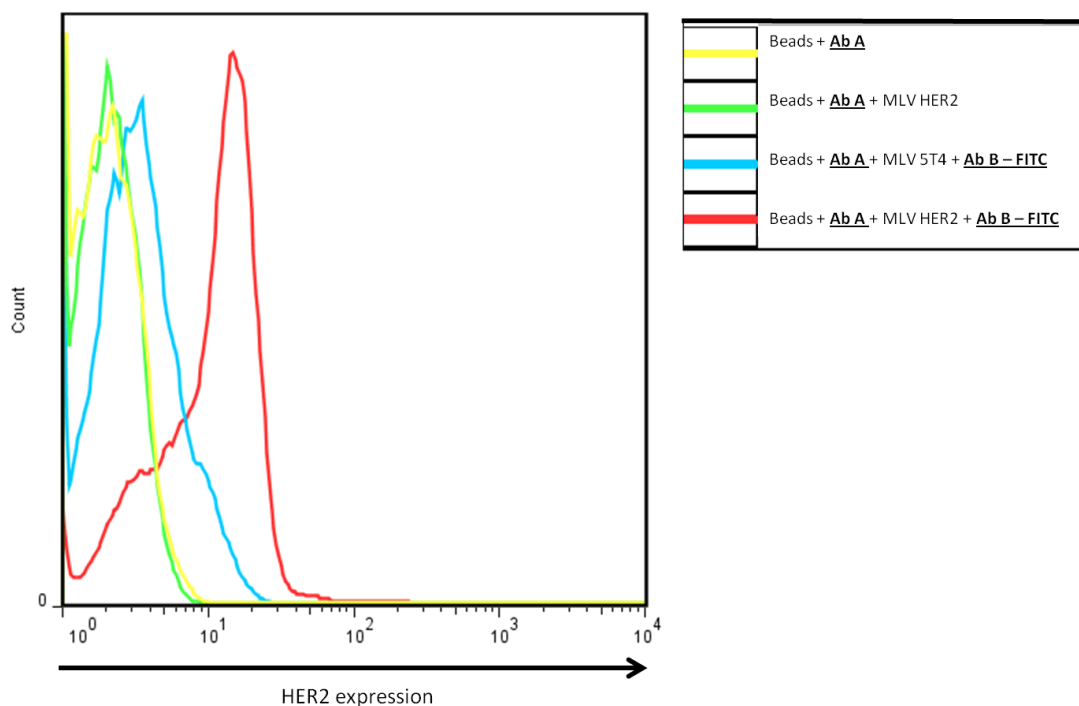


Figure 4.12 | HER2 can be found on the surface of MLV HER2 particles by flow cytometry of MLV HER2-bead complexes.

MLV HER2-bead complexes were analysed alongside negative controls for FITC fluorescence, indicating the presence of HER2 on the surface of the virus particle. Histogram is representative of two independent experiments.

4.4 Recombinant Adenoviruses

4.4.1 Transgene expression

As part of the production of recombinant adenoviruses (Chapter 3), ten viral clones were selected and assessed for transgene expression. QPCR was first carried out to determine viral titre, so that 293A cells could be infected with the same amount of virus (50 virus particles/cell). After 24 hours, cells were lysed and analysed by native PAGE and Western blotting for transgene expression. As all viral clones displayed similar levels of transgene expression (Figure 4.13), clone number 1 was picked from AdHER2 and Ad5T4 to grow for purification by caesium chloride banding. Banded viruses were used to infect 293A cells in various dilutions, and infected cells were again analysed for transgene expression. The purified recombinant viruses were confirmed to display the correct transgene product at high expression levels despite the viruses being diluted for infection (Figure 4.14), and hence were suitable for use in future *in vivo* experiments.

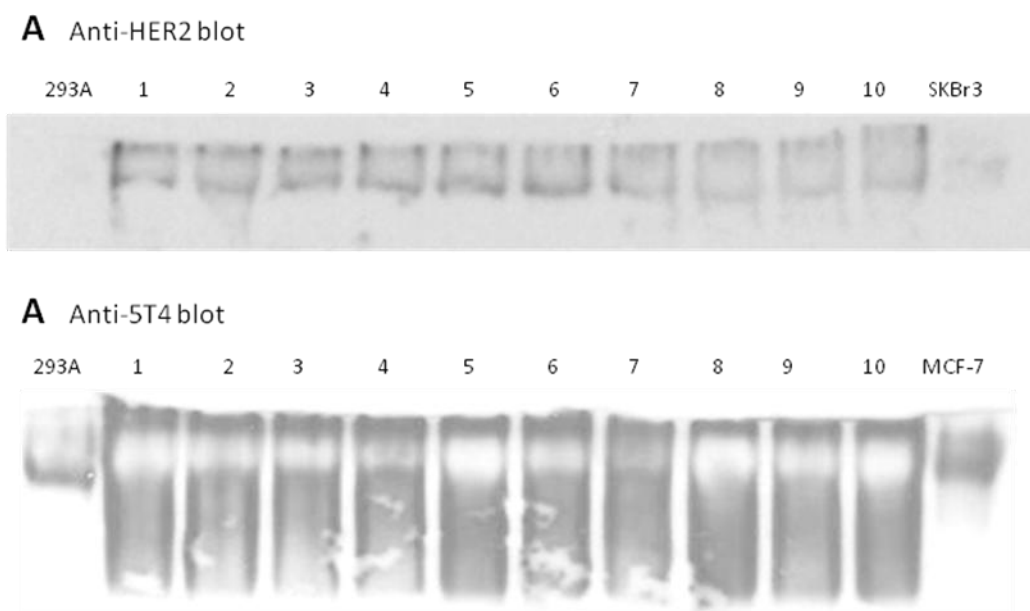


Figure 4.13 | Recombinant adenoviral clones express HER2 or 5T4.

Uninfected 293A cell lysate and either SKBr3 or MCF-7 cell lysates were run as negative and positive controls respectively. **(A)** HER2 transgene expression from AdHER2 viral clones, numbered 1 – 10. **(B)** 5T4 transgene expression from Ad5T4 viral clones, numbered 1 – 10. Band appears white due to overexposure of film.

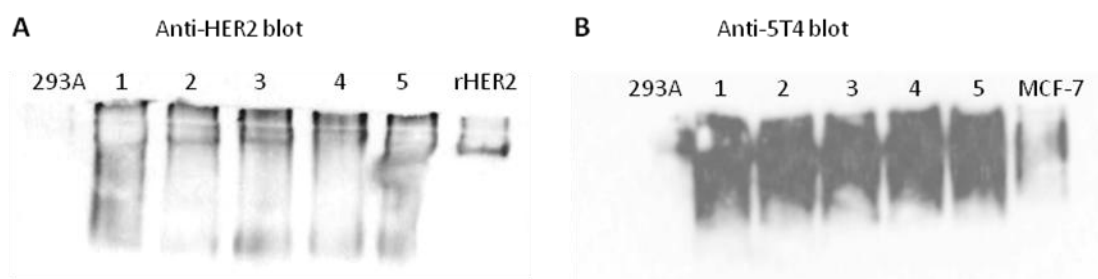


Figure 4.14 | Banded recombinant adenoviruses express HER2 or 5T4.

Viruses were diluted by 1 – 1:1000, 2 – 1:2000, 3 – 1:5000, 4 – 1:10000 and 5 – 1:20000 and used to infect 293A cells. Uninfected 293A cell lysate (293A) and the relevant positive controls were also run alongside infected cell lysates. **(A)** AdHER2 infected cell lysates. **(B)** Ad5T4 infected cell lysates.

4.5 Discussion

This chapter describes the characterisation of recombinant MLV virus particles by assessing the presence of the appropriate antigen either in virus-containing cells or on the vaccine viral particle itself. This step was necessary for ensuring that *in vivo* vaccinations would be carried out with the viral vectors presenting their cognate antigen correctly.

After completion of DNA cloning, viral plasmids were tested first by transient transfection into BHK cells. These packaging cells were confirmed to show expression of the appropriate antigen, indicating that cloning of the transgene had been successful. Stable cell lines were then created to produce the appropriate virus particles; after sorting by FACS, the sorted cell lines were also analysed for antigen expression before harvesting supernatant from the cells for virus purification. Although the 293A cells selected for generating stable lines also showed endogenous expression of both HER2 and 5T4, flow cytometry data displayed clear shifts in expression when comparing 293A cells with the stable cells. These results were backed up by the corresponding Western blots. As the antibodies used for flow cytometry and Western blotting were unable to recognise their cognate protein when fully denatured, it could be seen that the transiently expressed antigens, detected both intracellularly and on the cell surface, were correctly folded and presented as antibody binding was successful.

This work has demonstrated that it is possible to genetically modify MLV vectors to display an antigen-viral envelope fusion protein in place of the viral Env glycoprotein without affecting viral assembly and budding. It can be seen that the transgene products in this study can be successfully recognised by the appropriate antibodies, which only recognise cognate antigens in their native form. This indicates that the antigens have adopted the correct conformation for antibody recognition and binding. Therefore fusing the EC domain of a specific antigen to the viral TM and CP regions still results in the proper synthesis of the EC region which is unaffected by the fusion, and is still accessible to antibody binding.

Detection of the recombinant antigen in cells alone was not sufficient for creating a vaccine particle; post-purification of virus, it was also essential to verify the presence of the antigen on the surface of the virus particle itself, in order to produce an effective B cell targeting vaccine. Electron microscopy was carried out to visualise the virions, and to assess their shape and size after purification by ultracentrifugation. It was found that the modification of the MLV vectors does not affect the physical shape or size of the vaccine particle, indicating that by retaining

the virus TM and CP domain, the virus is able to form and bud off in the same manner as the wild type virus. Additionally, the method used for purification does not appear to affect or damage the surface proteins of the virus particle, as the purified virions maintain regular spherical shapes. This may be due in part to the double envelope of MLV which provides some structural integrity to the particle.

Dot blots were carried out and showed that the appropriate antigen was associated with purified virus particles. In order to prove that the antigen was present on the surface of the particle, which would be crucial for eliciting an antibody response *in vivo* through external antigens available for B cell binding, further analysis of the virus particle was required using a more sensitive method. Flow cytometry with virus particles using microspheres was optimised and confirmed that the virus particles did display tumour antigen on the surface, in a manner amenable to antibody binding.

Similar to the construction of the MLV vaccine vectors, the recombinant adenoviruses were also confirmed to show strong expression of the encoded transgene before continuing with each step of the production and purification process. As the same antibodies were used for detection of HER2 and 5T4 in the analysis of modified MLV, it can be noted that the transgenes expressed from the recombinant adenoviruses also displayed native folding and conformation. These properties are important in acting as a fair comparison to the MLV vaccines and eliciting the appropriate antibody response.

Having fully characterised the modified MLV vaccine particles and ensured that the recombinant adenoviruses were expressing the appropriate transgene correctly, both recombinant vaccine vectors can be tested *in vivo*.

5 Generation of Gag and 5T4 Recombinant Proteins

5.1 Introduction

This chapter describes the production of Gag recombinant protein, and the cloning and generation of 5T4 recombinant protein. It was necessary to produce these recombinant proteins for use in future *in vivo* and *in vitro* studies.

Although MLV is used frequently for cancer therapy as a retrovirus⁸⁰, there are few commercially available reagents relating to MLV as researchers often focus on the transgene of interest rather than the MLV vector itself. To investigate the hypothesis of this thesis fully, it was necessary to obtain a suitable vaccine that would lead to the activation of MLV-specific T cells, therefore “priming” these T cells for subsequent vaccinations. As detailed in the following chapter, MLV EGFP was initially generated as a prime vaccine but was found to also present human HER2 on the surface of the virus particle. This would affect its purpose as a prime vaccination, as the only antigens the prime and boost vaccines should have in common are the viral antigens, rather than HER2. This is in order to investigate the hypothesis that virus-specific T cells can activate B cells of a different specificity. Subsequently, heat-inactivated MLV was used as an alternative prime vaccination (Chapter 6), but the presence of the viral envelope glycoprotein appeared to be sufficient to divert the immune response from the antigen of interest. Hence it was decided to use a single internal viral protein as a prime; for this reason, MLV Gag was selected because the polyprotein is present in large quantities inside virus particles.

HER2 and 5T4 were selected as model antigens for this project; however unlike HER2, 5T4 is studied relatively infrequently and as a result has very few commercially available reagents. After *in vivo* vaccination with MLV 5T4 and Ad5T4, it was necessary to assess the antibody response to 5T4 by enzyme-linked immunosorbent assay (ELISA). As commercial recombinant 5T4 is produced in bacteria, it lacks the correct glycosylation patterns and hence may not be recognised by antibodies raised against native mammalian glycosylated 5T4, as these antibodies may be specific to carbohydrate and/or protein-carbohydrate epitopes. Therefore it was desirable to create stocks of purified recombinant 5T4 protein produced in mammalian cells.

Recombinant proteins can be produced by transfection of the relevant plasmid in mammalian cells, and either obtained from the cell lysate (for membrane bound or associated proteins) or the supernatant (for soluble proteins). In order to obtain purer preparations of the desired recombinant protein and to reduce any contaminants from cellular proteins, recombinant proteins can be fused to a tag to enable further purification. Both recombinant Gag and 5T4 were fused to a hexa-histidine (His) tag, a commonly used tag for protein purification either by nickel or cobalt columns, or by antibody binding.

5.2 Results

5.2.1 Production of MLV Gag-His protein

A DNA plasmid encoding full-length MLV Gag with a C-terminal hexa-His tag (Oxford Genetics) was used to transfect BHK cells in order to produce Gag-His fusion protein. BHK cells were used to express Gag-His as these cells do not contain any endogenous human HER2 or 5T4 in the cell membrane, and hence would minimise contamination by either of these antigens for the Gag prime vaccination. As Gag is a membrane associated polyprotein, cells were lysed and incubated with anti-His magnetic microbeads, before extraction on a column placed in a magnet (Miltenyi Biotec). Purified protein from cell lysate was analysed by Western blotting using antibodies probing for the His-tag. A clear band corresponding to Gag (~ 70kDa) can be seen in both the crude cell lysate and the purified elution (Figure 5.1), with less background in eluted lane, indicating that MACS purification was selective. However, as there may also be cellular proteins containing cross-reactive sequences, this method of purification is not specific to just the recombinant Gag-His protein, as seen in the non-transfected BHK cell lysate. The high levels of background seen in the crude Gag-His cell lysate could be as a result of the transfection process; however most of this background is eliminated during MACS extraction.

This synthesis method allowed for the small-scale production of recombinant Gag-His in microgram quantities. However for *in vivo* vaccination, large quantities of Gag protein were required which would not have been possible to achieve in this lab. Purified truncated Gag protein (~ 40 kDa) was kindly gifted by Dr. Siddharta Datta and Dr. Alan Rein (National Institutes of Health/National Cancer Institute) and used in a dose priming study in a small group of mice (Chapter 7). This was carried out to assess the optimum number of doses of Gag prime to be given to mice, in order to detect an anti-Gag T cell response by enzyme-linked immunosorbent spot (ELISPOT). Figure 5.2 shows a protein gel of the purified protein (provided by Dr Datta). Although this recombinant Gag protein was truncated (338 amino acids long as opposed to 560 amino acids), the immunogenicity of the protein was not expected to differ from the full-length version as the protein would be processed into linear peptides for presentation to T cells. Hence, this recombinant Gag protein was also used for all subsequent *ex vivo* analyses.

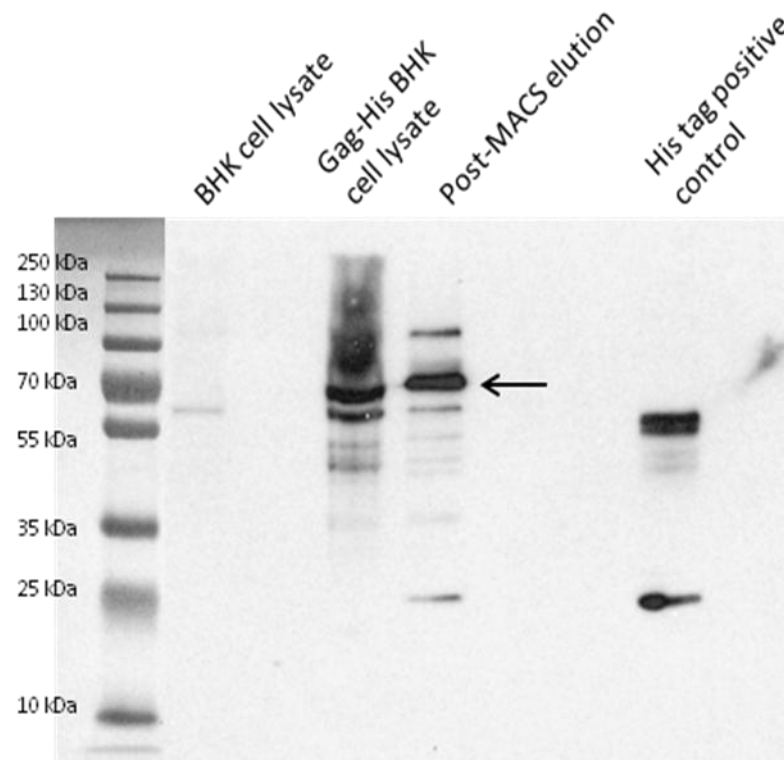


Figure 5.1 | MACS purification of recombinant Gag with His tag.

DNA plasmid containing Gag-His was transfected into BHK cells. Lysate was incubated with magnetic beads bound to anti-His antibody and purified through a MACS column. Samples were analysed by Western blotting, probing for His-tag before and after MACS purification. Positive control used was purified bacterial His-tag positive protein. Arrow indicates protein band for Gag (~ 70 kDa).

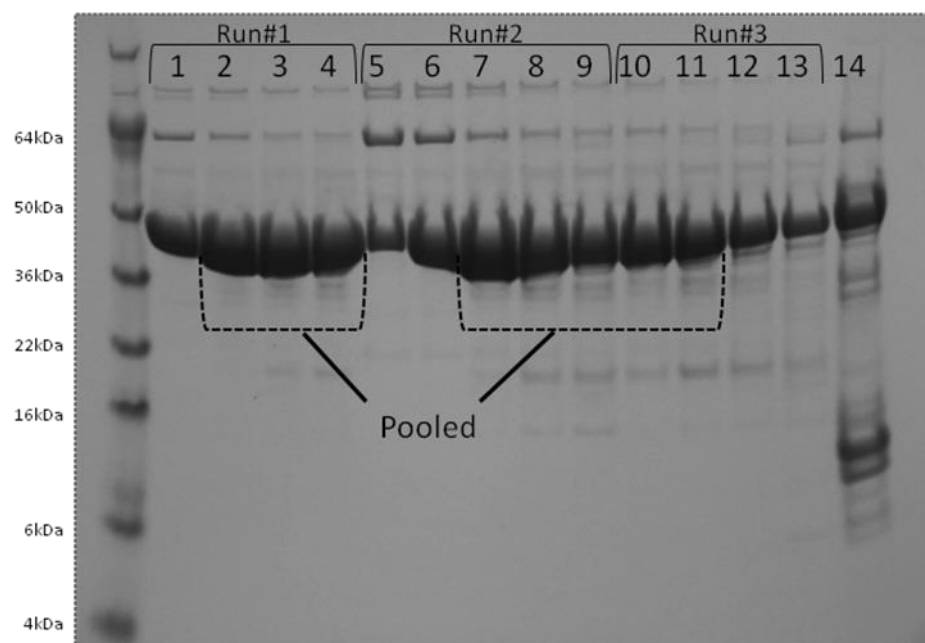


Figure 5.2 | Recombinant MLV Gag protein.

Protein purified by size exclusion chromatography and analysed by SDS-PAGE and Coomassie staining (molecular weight ~ 40 kDa). Image from Dr. Siddharta Datta (NIH/NCI).

5.2.2 Cloning and production of 5T4-His protein

In order to produce recombinant 5T4-His protein, a DNA plasmid was constructed encoding the EC domain of 5T4 followed by a C-terminal hexa-His tag, as summarised in Figure 5.3. PCR was carried out using MLV 5T4 DNA plasmid as a template in order to amplify the extracellular region of 5T4 flanked by restriction sites *NcoI* and *XhoI* (Figure 5.4). After this, both the purified 5T4 EC DNA and a plasmid encoding a C-terminal His tag (Oxford Genetics) were digested with *NcoI* and *XhoI*. This digest was verified by gel electrophoresis by comparing the undigested vector to the digested vector (Figure 5.5); The uncut vector appears at a lower size to the linearised vector as it may be coiled, and hence runs at a lower size than its true size of 4.359kb. Once digested, the vector is separated into two fragments – the backbone at 4.236kb and a small fragment at 0.023kb, which is too small to be visible by gel electrophoresis. The vector backbone and the PCR fragment were ligated together. Colonies containing the ligated DNA product were selected and amplified to produce a maxiprep of the 5T4-His fusion DNA plasmid.

Having confirmed that the sub-cloning of 5T4 was successful by restriction enzyme digest (Figure 5.5), the DNA plasmid encoding for 5T4-His was transfected into BHK cells with different amounts of DNA used. After 72 hours, both cell lysate and cell supernatant were analysed by Western blotting for the presence of 5T4-His fusion protein. Samples were run in duplicate gels, one native and one containing SDS for transfer onto separate nitrocellulose membranes; samples run on the native gel were probed with anti-5T4 mAb, while samples run on the denaturing gel were probed with anti-His antibody (which can recognise its target in a denatured form).

Development of both blots revealed that there was 5T4-His present in both transfected cells and supernatant (Figure 5.6). The predicted molecular weight of the 5T4 EC domain is 36 kDa; however, as the EC domain is heavily glycosylated¹⁰⁰, the apparent weight as seen on the blots is much larger at around 70 kDa. Although 5T4 is a membranous protein, as only the EC domain of 5T4 was encoded in the plasmid, there was no TM region produced to tether the protein to the cell membrane. Therefore after translation, the protein would have been trafficked to the cell surface and secreted as a soluble protein. This may account for the different molecular weights seen in the anti-5T4 blot, as 5T4 may be tethered to some cellular compartment in cell lysate and therefore have a larger apparent molecular weight than soluble 5T4. It is also possible that the cell lysate may contain 5T4 protein that has been misfolded,

and so is retained and formed into aggregates, rather than being secreted from the cell. However the anti-5T4 blot is a native gel, and so it is difficult to accurately calculate molecular weights from this type of gel as proteins are not separated by size. From the anti-His blot it is clear that there are cellular components containing cross-reactive sequences, based on the negative control (untransfected cell lysate), but no such background can be seen in the lanes containing supernatant only. It can be seen from the anti-His blot that there are two bands present in the supernatant when staining for anti-His; this may have arisen from different glycosylation forms of secreted 5T4, or perhaps various oligomerisation states of the protein. From these results, it was decided to purify 5T4-His fusion protein from the supernatant of transfected cells instead of the cell lysate, in order to minimise purification of any BHK cellular proteins containing cross-reactive sequences.

5T4-His fusion protein purification was carried out by a modified form of magnetic activated cell sorting (MACS) to isolate His-tagged proteins. Supernatant was first concentrated using a 10 kDa size filter in order to reduce the total volume, and incubated with anti-His beads. The supernatant and bead mixture was purified over columns bound to a magnetic field, and fractions of the flow-through and wash steps were retained. Finally, the recombinant protein was eluted from the column by pH shift and assessed by Western blotting for 5T4-His content. It can be seen that purification by MACS increases the amount of 5T4-His fusion protein compared to the crude supernatant, indicating that the anti-His beads are highly specific for the recombinant protein (Figure 5.7). Although there is a band present for the untransfected BHK elution (control supernatant) in the anti-5T4 blot, this could be due to some non-specificity of the 5T4 antibody, as there is no corresponding band in the anti-His blot. This indicates that this band is not derived from the recombinant fusion protein and must be background staining. The purified recombinant 5T4-His was used in subsequent *in vitro* assays.

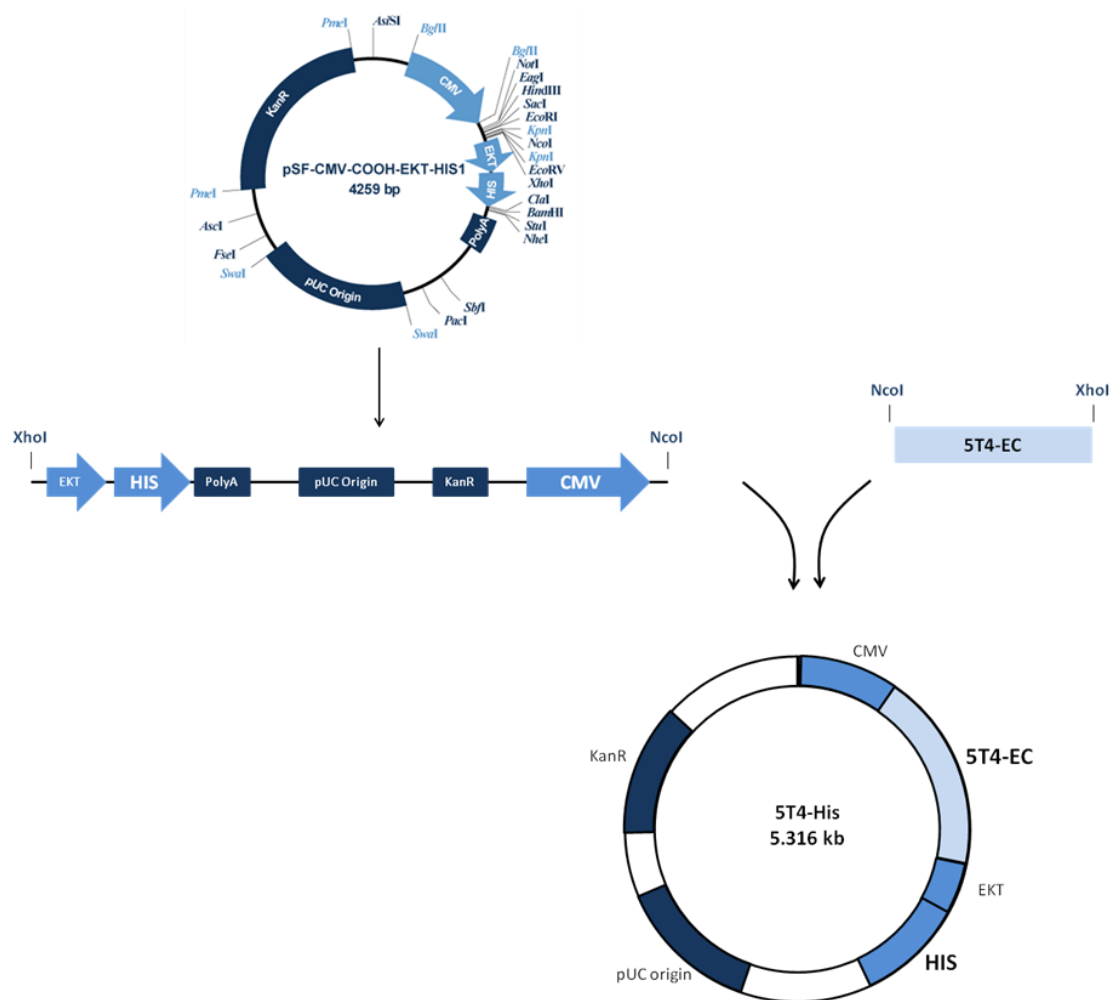


Figure 5.3 | Sub-cloning of the extracellular domain of 5T4 into a C-terminal His-tag containing DNA vector.

A DNA plasmid (Oxford Genetics) containing a CMV promoter and a hexa-His tag sequence was cut with restriction enzymes *NcoI* and *XhoI* to linearise the plasmid. This was ligated to a PCR product of the 5T4 extracellular domain flanked by the same restriction enzyme sites, in order to form a full plasmid containing the 5T4 gene with a C-terminal His-tag. CMV – cytomegalovirus (promoter), EC – extracellular domain, EKT – enterokinase cleavage site (not used), His – histidine site, pUC origin – origin of replication, KanR – kanamycin resistance, polyA – poly-adenosine tail.

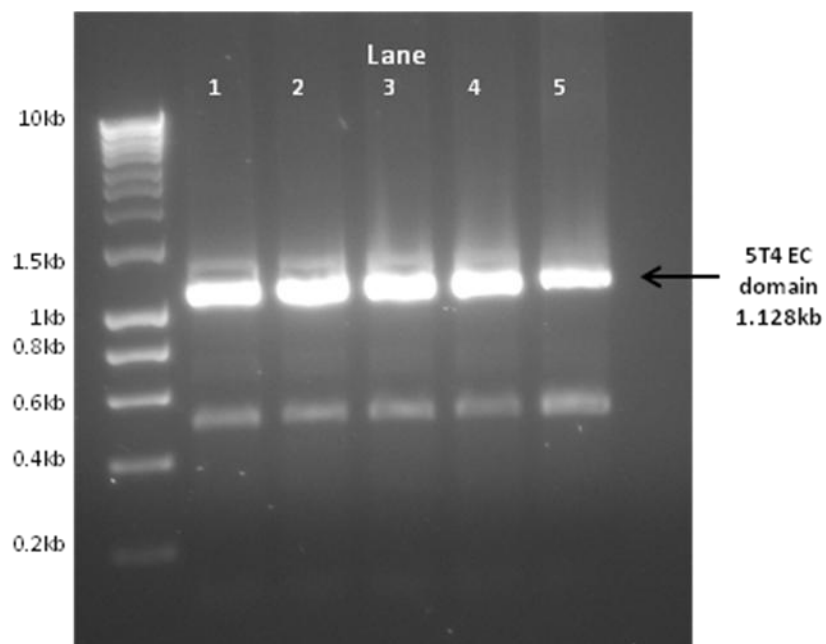


Figure 5.4 | Amplification of 5T4 extracellular region by PCR for cloning.

PCR was carried out to amplify the region encoding the EC domain of 5T4 flanked by restriction sites. PCR products were purified by gel extraction (not shown).

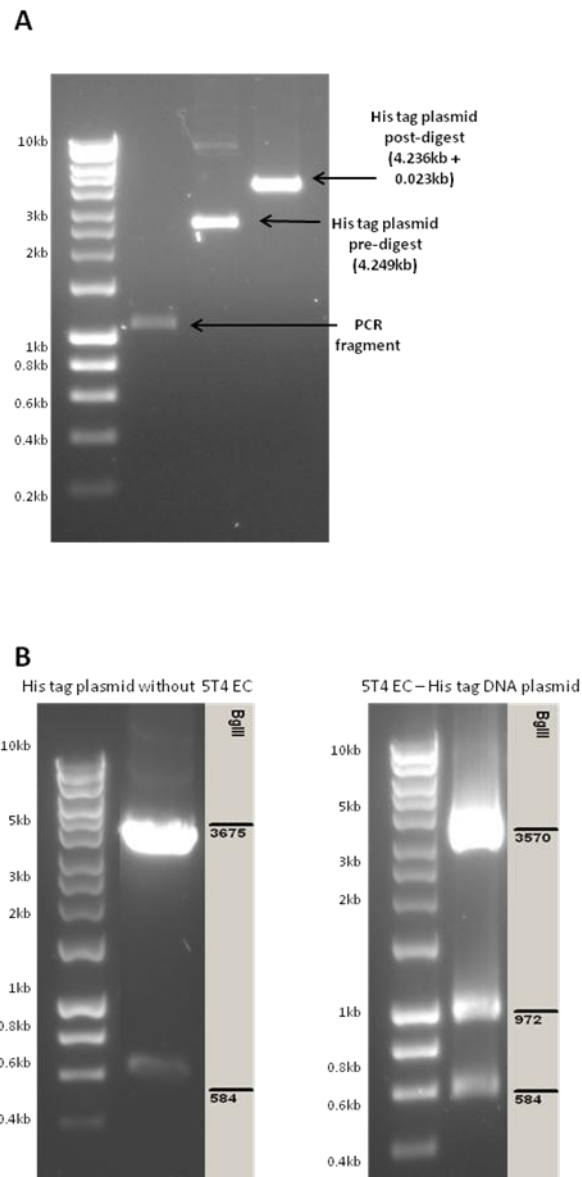


Figure 5.5 | The extracellular region of 5T4 was cloned into a plasmid to produce a 5T4 EC-His tag fusion gene.

(A) Both PCR fragment encoding the extracellular domain of 5T4 and a DNA plasmid with a His tag were digested by restriction enzymes and ligated together. (B) DNA from colonies with correct ligated product were amplified by maxiprep and checked by restriction enzyme digest (right), compared to the original plasmid without 5T4 (left).

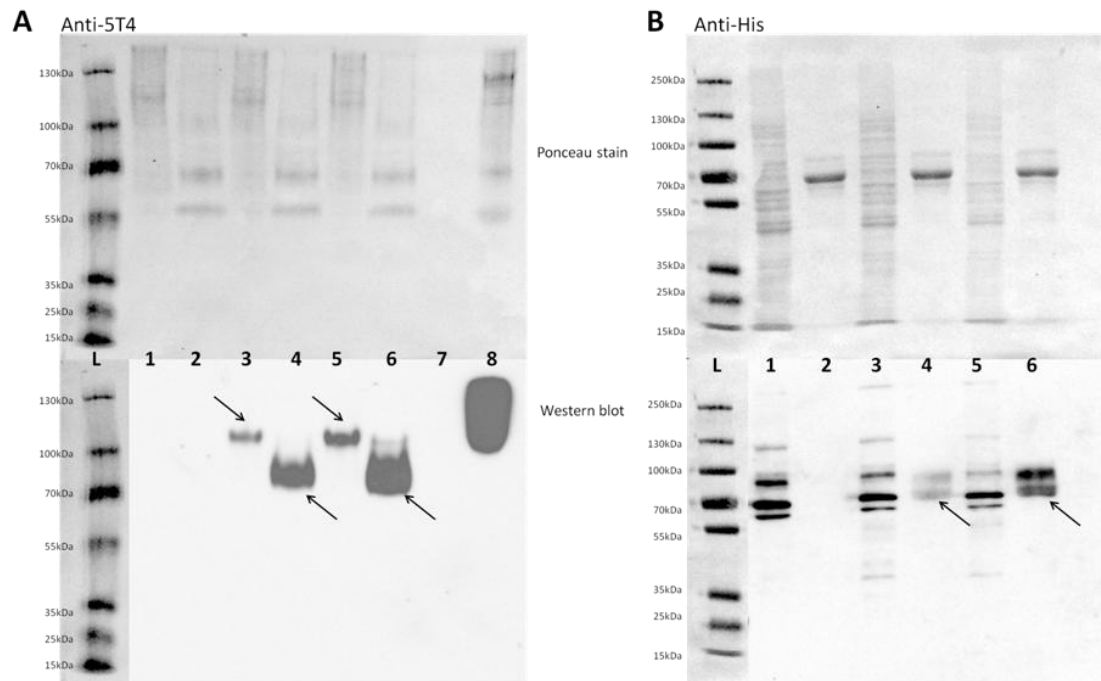


Figure 5.6 | BHK cells transfected with 5T4-His DNA plasmid show expression of 5T4-His fusion protein.

Lanes – L: Protein ladder; 1: untransfected BHK cell lysate; 2: untransfected BHK supernatant; 3: 1 μ g DNA transfected BHK cell lysate; 4: 1 μ g DNA transfected BHK supernatant; 5: 2 μ g DNA transfected BHK cell lysate; 6: 2 μ g DNA transfected BHK supernatant; 7: empty lane; 8: MCF-7 cell lysate (5T4 positive control).

(A) Cell lysates and concentrated supernatant were loaded onto a native gel and transferred onto a nitrocellulose membrane for probing with anti-5T4 antibody. (B) Cell lysates and concentrated supernatant were also loaded onto a denaturing gel and transferred onto a nitrocellulose membrane for probing with anti-His antibody. Arrows indicate 5T4-His fusion protein.

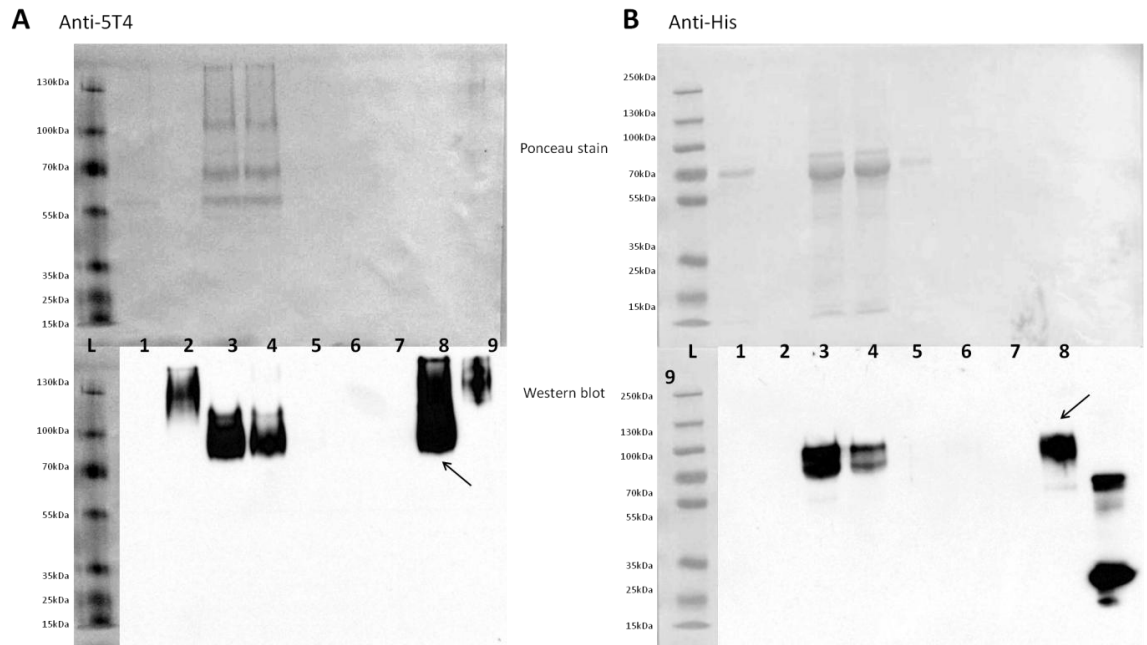


Figure 5.7 | 5T4-His fusion protein purified by magnetic sorting.

Lanes – L: Ladder; 1: BHK supernatant; 2: BHK elution; 3: 5T4-His transfected supernatant; 4: 5T4-His supernatant flow-through; 5: 5T4-His supernatant wash 1; 6: 5T4-His supernatant wash 2; 7: 5T4-His supernatant wash 3; 8: purified 5T4-His; 9: MCF-7 cell lysate (5T4 positive control) or His-tag positive control.

(A) Samples were run on a native gel before transferring to a nitrocellulose membrane and probing with anti-5T4 antibody. (B) Samples were run on a denaturing gel before transferring to a nitrocellulose membrane and probing with anti-His antibody. Arrows indicate purified recombinant 5T4-His.

5.2.3 Verification of recombinant 5T4-His for ELISA

The aim of producing recombinant 5T4 was to test for the presence of anti-5T4 antibodies in serum of vaccinated mice. Previous ELISAs (not shown) using commercially available recombinant 5T4 produced in bacteria had proved negative for both vaccinated mice sera and anti-5T4 mAb (as previously used in flow cytometry and Western blotting). Having produced pure recombinant 5T4 by His purification and shown that it is recognised by the same mAb in Western blotting, it was necessary to test if it could be used to coat ELISA plates and still be detected.

Both crude supernatant and purified 5T4-His were diluted in PBS and used to coat an ELISA plate overnight, along with BHK supernatant and elution as controls. Successful coating was detected using the same monoclonal anti-5T4 antibody used for Western blotting and flow cytometry (Figure 5.8). This ELISA shows that recombinant 5T4 can be purified by MACS and after coating to the appropriate plate surface, is still able to bind antibody. Therefore 5T4-His purified by this method is suitable to use in an assay to detect anti-5T4 antibodies in mice sera.

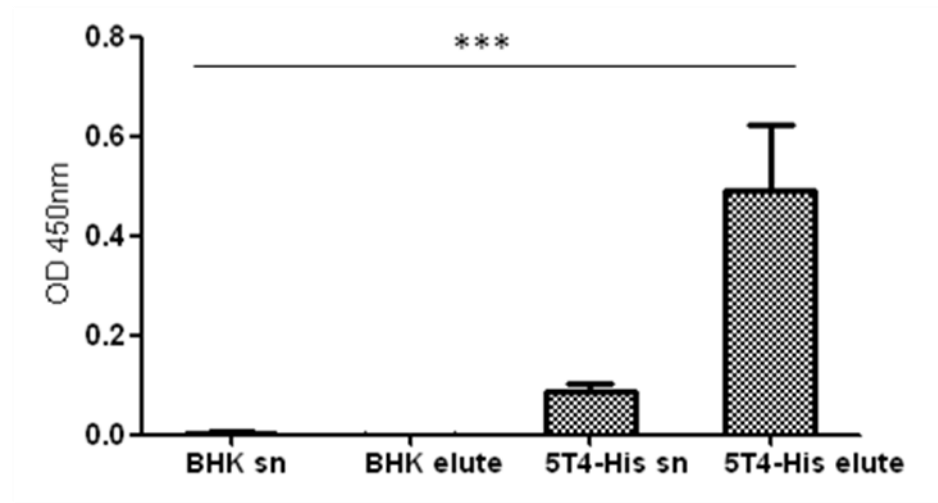


Figure 5.8 | Recombinant 5T4-His protein can be detected by ELISA.

Recombinant 5T4-His fusion protein was used to coat an ELISA plate. Successful binding to the plate was verified by using a mAb against 5T4. Crude supernatant (sn) was compared to purified protein (elute) as a control. Data analysed by one way ANOVA where *** = $p < 0.001$; data significant compared to 5T4-His elute. Errors represent standard deviation (technical replicates $n = 3$).

5.3 Discussion

This chapter describes the production and purification of His-tagged proteins. For future *in vivo* experiments it was necessary to obtain purified Gag protein for vaccinations. His tag purification was chosen as it is relatively simple to fuse a hexa-His tag to proteins by cloning, and there was a wide variety of purification methods available as it is a popular form of protein tagging. A small amount of Gag-His protein was purified in this way; however due to contaminants in BHK cell lysate, and the fact that large amounts were required for vaccination, it was desirable to use purer Gag stocks from an external source.

As described in previous chapters, MLV 5T4 and Ad5T4 were generated for vaccination in mice. Hence it was essential to develop a method of detecting anti-5T4 antibodies in mice sera. During optimisation of an anti-5T4 ELISA, it was necessary to generate recombinant 5T4 protein produced from mammalian cells, in order to detect the appropriate antibodies (as both MLV 5T4 and Ad5T4 were generated from mammalian cells, and hence would show mammalian glycosylation patterns). Recombinant 5T4-His fusion protein was first generated through sub-cloning to obtain the correct fusion genes, then protein was collected from the supernatant of transfected BHK cells. This protein was further purified by MACS and assessed by Western blotting and ELISA. Although only a small amount of 5T4-His was produced by this method, this was sufficient for use in ELISAs to analyse vaccinated mice serum.

6 Optimisation of Vaccination Studies

6.1 Introduction

This chapter describes the optimisation of the *in vivo* studies necessary to fully test the hypothesis of B cells acquiring help from T cells of a different specificity. Modified MLV vaccine particles, which had been previously developed and characterised, were to be tested in mice to establish a working vaccination protocol.

Adjuvants can enhance the immune response against specific antigens and increase the efficacy of vaccines. Different adjuvants were trialled in conjunction with the MLV vaccine particles. Freund's Complete Adjuvant (CFA) and Freund's Incomplete Adjuvant (IFA) (Sigma-Aldrich) are the gold standard used for *in vivo* vaccinations, as they can induce high antibody responses. Both CFA and IFA contain paraffin oil and mannide monooleate (a surfactant), while CFA also contains heat-killed *Mycobacterium tuberculosis* for TLR activation (leading to a TH1 response). The oil components in these adjuvants is thought to work assisting the formation and subsequent stabilisation of the antigen-adjuvant emulsion. This emulsion acts as a depot for slow and controlled antigen release, assisting antigen transport through the lymph to immune cells by acting as a vehicle and interacting directly with antigen presenting cells¹²⁸. Due to the presence of *M. tuberculosis*, CFA can only be used once in the same animal as subsequent vaccinations with CFA would skew the immune response to the bacteria and ablate desired response towards the vaccine of choice. CFA is commonly used for initial vaccinations, with IFA used for subsequent vaccinations¹²⁹. Although CFA and IFA can induce good immune responses, the use of these adjuvants can also lead to the formation of granulomas and lesions as well as inflammation at the site of injection. Due to the toxic nature of these side effects, Freund's adjuvant has never been licensed for use in humans¹²⁸.

An alternative to Freund's adjuvant was also tested, Titermax Gold (Sigma-Aldrich) which contains a proprietary block copolymer (for TLR activation) and squalene (for formation of the emulsion). Like Freund's adjuvant, it is also a water-in-oil formulation and so requires homogenisation or vortexing with the desired antigen to form an emulsion, but is not reported to have the same severe side effects displayed by mice vaccinated with Freund's¹³⁰.

A third adjuvant was also used *in vivo*, working by a slightly different mechanism. Alhydrogel (Brenntag Biosector) is composed of 3% aluminium hydroxide and due to its positive charge, is able to adsorb neutral or negatively charged antigens. It has several mechanisms of action, including the creation of an antigen depot at the site of vaccination to enable the slow release of antigen, increasing antigen uptake by APCs and activation of complement, eosinophils and macrophages (inflammatory response)¹³¹. Aluminium-based adjuvants are widely used and are safe to use in humans.

In order to properly investigate the hypothesis of this thesis, it was necessary to obtain an effective vaccine that would lead to the expansion of virus-specific CD4+ T cells *in vivo* before administering subsequent vaccinations containing the antigen of choice. In this case, the CD4+ T cells would be “primed” against viral antigens, and would be available to provide help to B cells following the “boost” vaccinations (ie the modified vaccine particles containing the weak antigens). The boost vaccinations are different from conventional heterologous prime-boost techniques where both prime and boost steps use different vectors encoding the same antigen; in this case, the prime and boost vaccinations are composed of the same viral vector, but only the boost vaccine contains the antigen of choice. The purpose of the prime vaccine is to stimulate virus-specific T cells only; these activated cells will then provide help to antigen-specific B cells. To assess the hypothesis, it will be necessary to compare the difference between a prime-boost vaccination and a boost only vaccination, as this will reveal the role of primed virus-specific CD4+ T cells in activating the immune response against weak tumour antigens.

This method of “homologous” prime-boosting will be compared to conventional viral vectors such as adenovirus. Anti-vector antibodies can often hinder an immune response against a immunogenically weak transgene product, as shown with adenovirus vaccines⁹³. In contrast, the modified MLV vaccine will also raise a strong anti-vector response, but this will benefit the immune response against the weak antigen instead of hindering it. The generation of virus-specific CD4+ T cells would be expected to augment the B cell response against antigens. Any anti-MLV antibodies generated would not be expected to affect the immune response against the weak antigens as the modified vaccine particles do not contain any exposed viral epitopes for antibody binding, and hence would not be expected to be cleared from the circulation by neutralising antibodies.

6.2 Preliminary Experiments to Determine Optimum Parameters

6.2.1 Viral dosage and schedule

Mice were vaccinated three times in week 1 and three times in week 4 with 1×10^8 MLV EGFP or MLV HER2 virus particles per mouse, and tail bled once a week until week 6 (Section 2.11.3 in Materials and Methods). This dose was calculated as Maximum Feasible Dose (MFD) that could be given at the time, as purification methods were still in the process of being optimised and viral titres were low. Diluted sera were tested for the presence of anti-GFP or anti-HER2 antibodies following the administration of the boost vaccination (Figure 6.1).

No antibody responses against HER2 or GFP above background level (set by serum from control mice) were detected in the sera of vaccinated mice. At the current dose, the vaccine particle did not appear to be sufficiently immunogenic to generate a detectable antibody response. This may have been due to a lack of corresponding CD4⁺ T cell help, which was not tested for in this preliminary study. Therefore it was desirable to optimise the vaccination protocol further by increasing the viral dose and vaccinating alongside different adjuvants.

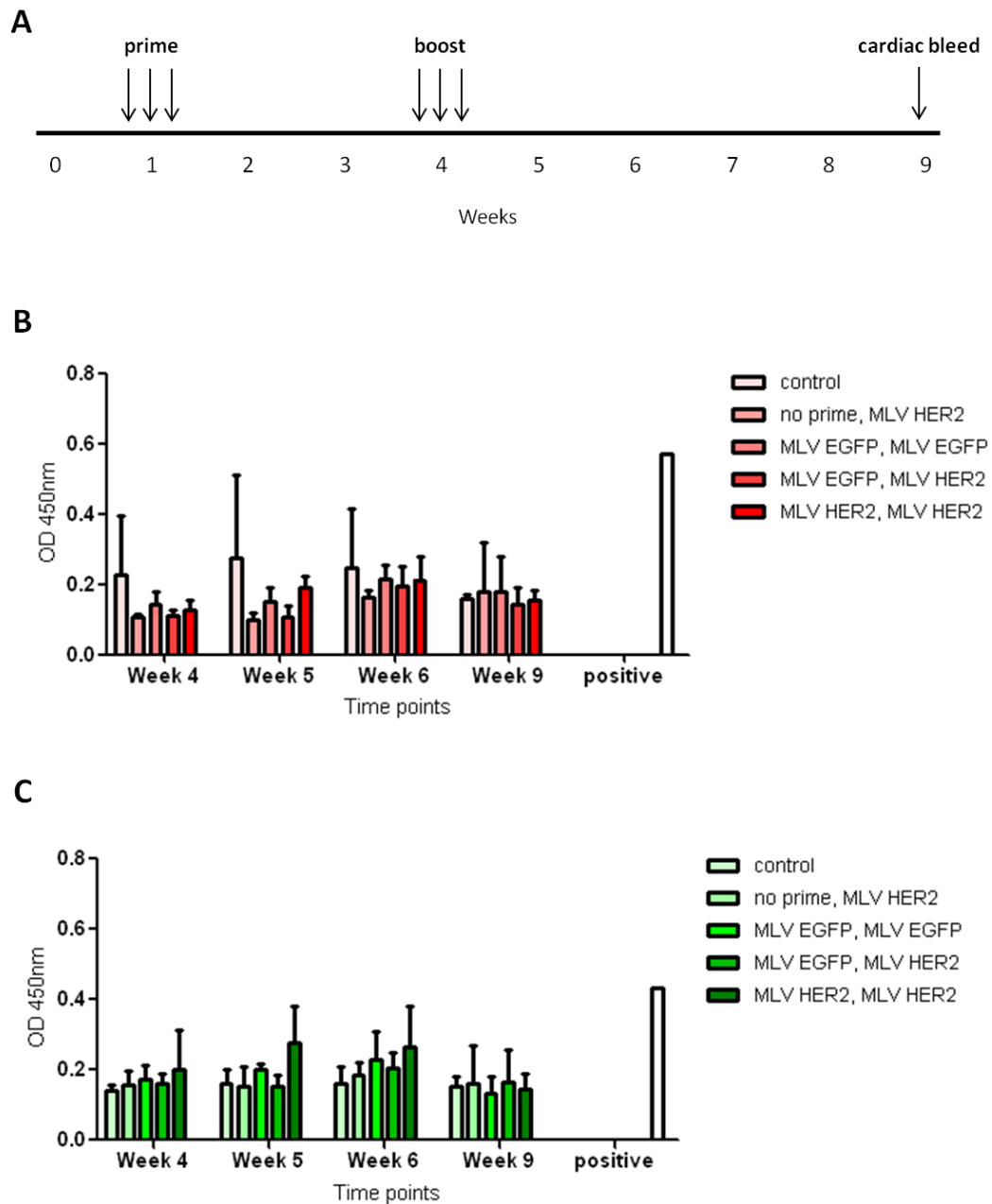


Figure 6.1 | Prime-boosting with low doses of MLV EGFP and MLV HER2 does not show any antibody responses.

(A) Mice were primed three times in week 1 with MLV EGFP or MLV HER2, and boosted three times in week 4 with MLV EGFP or MLV HER2, all doses at 1×10^8 vp. Serum from mice was tested by ELISA using a 1:7 dilution (except for week 9, serum undiluted) for the presence of (B) anti-HER2 or (C) anti-GFP antibodies in the weeks following the boost vaccination. Groups are as follows: (1) control, PBS injections only (2) no prime and MLV HER2 boost (3) MLV EGFP prime and MLV EGFP boost (4) MLV EGFP prime and MLV HER2 boost (5) MLV HER2 prime and MLV HER2 boost. Each bar represents the average of each group, where error bars show standard deviation representing biological variation rather than experimental error. Control group, $n = 3$; all other groups $n = 5$. Positive and negative controls for ELISAs were commercial monoclonal antibodies to HER2 or GFP, or PBS only.

6.2.2 Selection of adjuvant to augment immune responses

In order to enhance an immune response against HER2, different adjuvants were used in conjunction with the viral vaccine particle. Water-in-oil adjuvants Titermax Gold and Freund's adjuvant were mixed with vaccine particles to form emulsions. These emulsions were tested to be properly prepared and stable by pipetting a small drop on the surface of water, and if the droplet retained its shape and did not diffuse then it was ready for use¹²⁴. Alhydrogel was also tested alongside Freund's and Titermax Gold adjuvants.

These three adjuvants were used in conjunction with MLV EGFP and MLV HER2 in a prime-boost regimen, as well as being tested alongside mice vaccinated with virus particles only (Table 2.11 in Materials and Methods). All vaccinations were given at 2×10^9 vp per dose, 20 times higher than the previous *in vivo* experiment; this was possible because of improved purification techniques. Mice were vaccinated with MLV EGFP prime two weeks apart (weeks 1 and 3), and subsequently with three booster vaccinations of MLV HER2 at two week intervals (weeks 5, 7 and 9).

Diluted sera taken from mice by tail bleeding was assessed for anti-GFP and anti-HER2 antibodies (Figure 6.2). Data from ELISAs performed on mice sera indicated the presence of anti-HER2 antibodies one week after the second prime vaccination. This was unexpected, as all mice were primed with MLV EGFP only. One possibility was that the recombinant HER2 protein (R&D Systems) used to coat the ELISA plates contained an Fc region, to which some antibodies in the polyclonal mice sera could have bound. To clarify this, a different recombinant HER2 protein without an Fc region was purchased from Prospecc and a small scale ELISA was carried out using serum from mice vaccinated with virus only (Figure 6.3). Serum against both types of recombinant HER2 proteins showed similar OD values after the prime vaccination (week 4 time point), indicating that the antibody response seen at this time point is actually against HER2 rather than a different component of the recombinant protein. The presence of anti-HER2 antibodies in mice sera after the MLV EGFP prime vaccinations may have arisen due to low levels of HER2 being present on all virus particles, as the virus-producing 293A cells contain endogenous HER2 on their cell surface. Therefore it is expected that MLV EGFP would also contain some HER2, derived from the cellular membrane that the virus originally budded.

At week 11, mice were put down by cardiac puncture and serum from this bleed was again tested for antibodies by ELISA (Figure 6.4). No statistically significant difference can be seen

between mice vaccinated with either adjuvant or virus alone at this time point. This could be due to the higher dilution of serum used compared with previous ELISAs (Figure 6.2).

From this *in vivo* experiment, it can be seen that Titermax Gold was superior compared to the Freund's adjuvant and Alhydrogel. However by increasing the viral dose 20 fold from the previous vaccination study (Figure 6.1), antibody responses against HER2 and GFP can be raised using virus only with no adjuvant; for example, anti-GFP responses after the higher dose vaccination with Titermax results in an OD of approximately 1.3, whereas the lower dose of the vaccine particle MLV EGFP without adjuvant gives no increase over the background level. It was decided to continue using Titermax Gold and a higher dose of vaccine particles for further *in vivo* experiments, with the added benefit that Titermax Gold was not associated with any adverse side effects as seen with other water-in-oil emulsions.

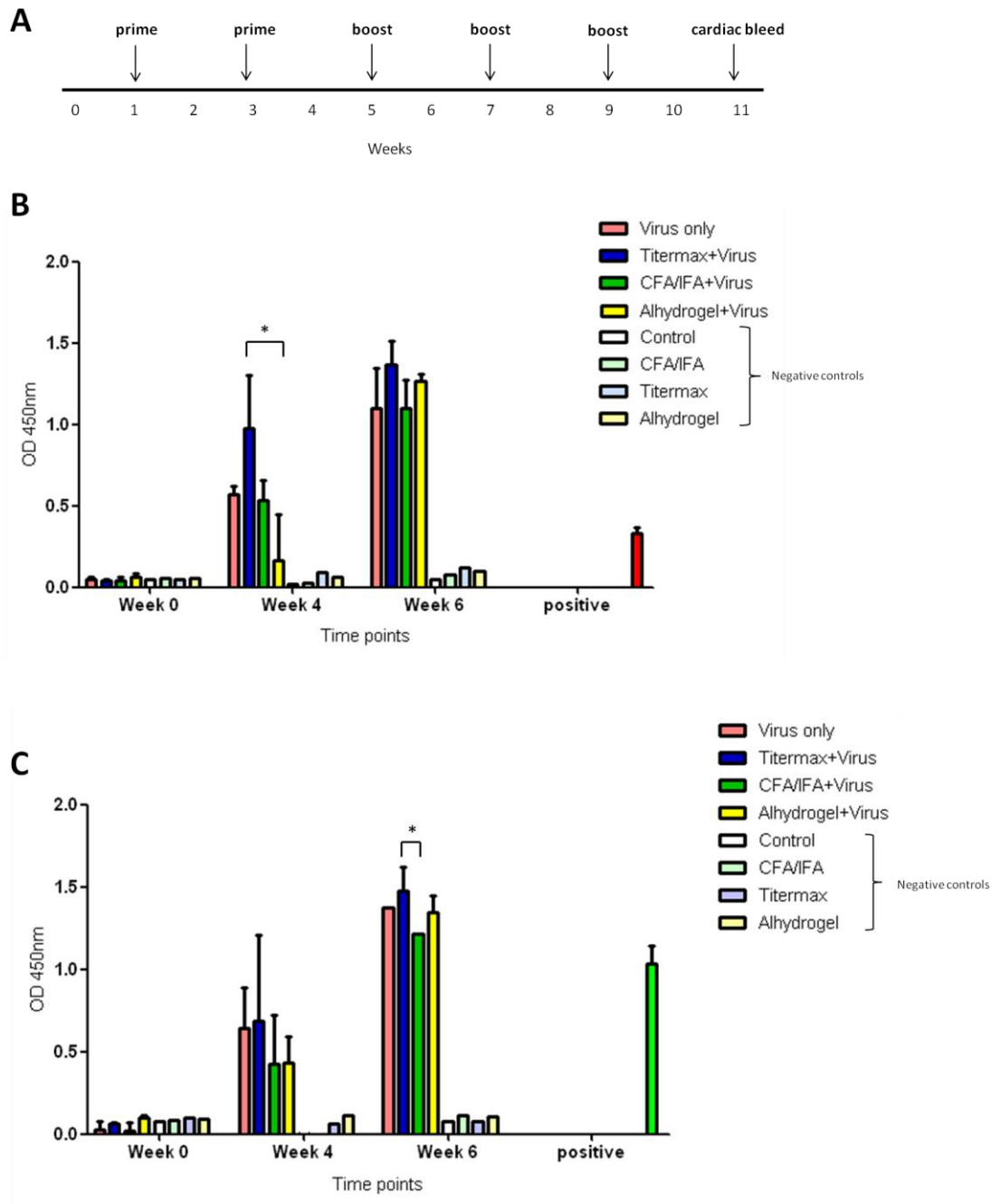


Figure 6.2 | Vaccination with different adjuvants results in anti-HER2 and anti-GFP antibodies after two prime and one boost vaccination.

(A) Mice were primed twice in weeks 1 and 3 with MLV EGFP and boosted three times with MLV HER2 in weeks 5, 7 and 9. All doses administered at 2×10^9 vp. Serum from mice taken at weeks 0, 4 and 6 was tested against (B) recombinant HER2 protein for anti-HER2 antibodies and (C) recombinant GFP for anti-GFP antibodies. Serum was diluted 1:8 for ELISA. Each bar represents the average of each group, where error bars show standard deviation representing biological variation rather than experimental error. Data was analysed by one-way analysis of variance, where * = $p < 0.05$. Negative control groups, $n = 1$; all other groups $n = 3$. Positive controls for ELISAs were commercial monoclonal antibodies to HER2 or GFP.

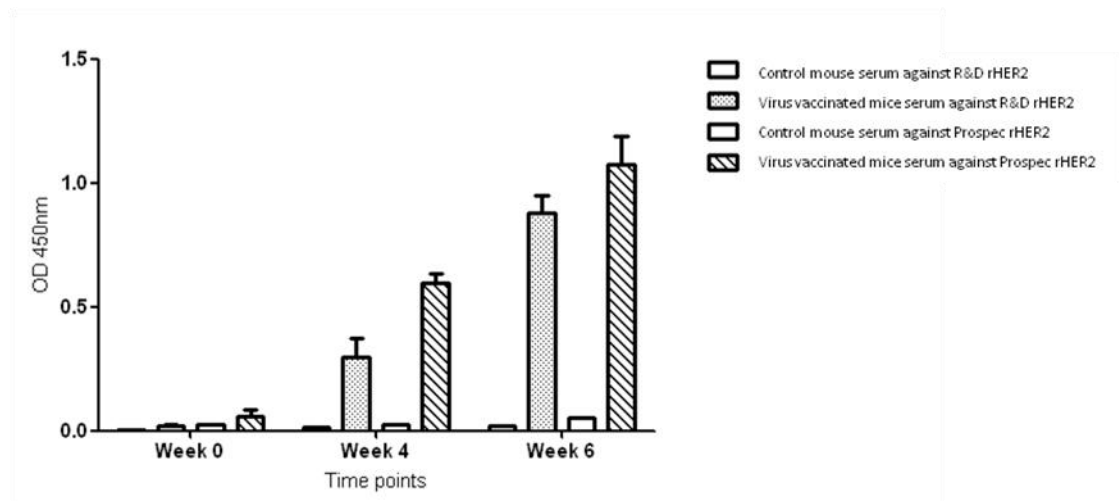


Figure 6.3 | Assessing the presence of Fc region linked to recombinant HER2 protein.

Serum from control mice and virus only vaccinated mice (same from Figure 6.2) were used to test for genuine specific anti-HER2 antibodies. Antibodies can be detected against both forms of recombinant HER2 protein, showing that the antibody response is specific to HER2 only and not any other component of the recombinant protein. Error bars represent standard deviation from the mean, $n = 3$ for vaccinated mice serum, $n = 1$ for control mice.

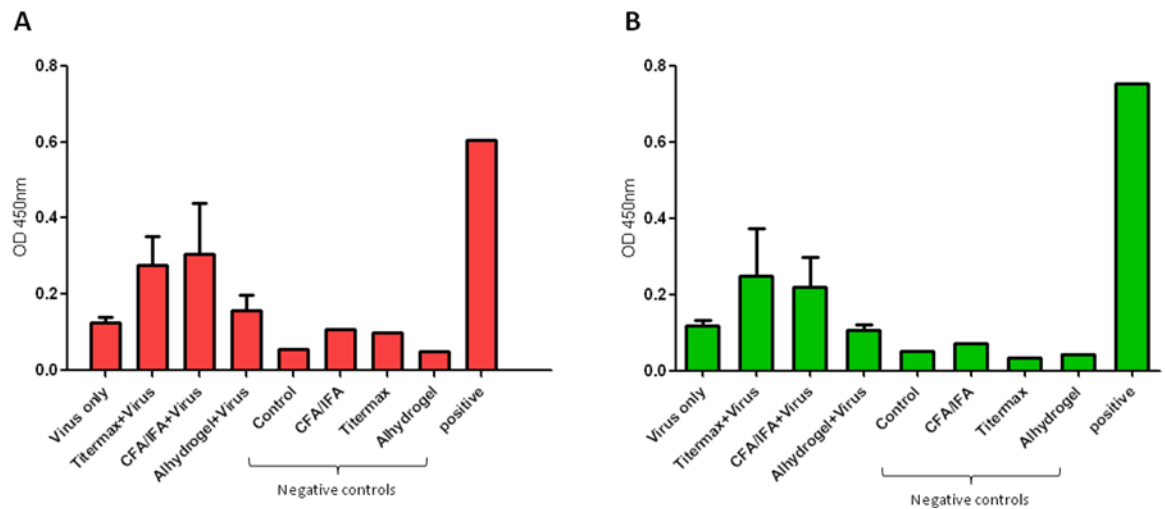


Figure 6.4 | Antibody responses after two MLV EGFP prime and three MLV HER2 boost vaccinations.

Serum from cardiac bleeds (week 11, Figure 6.2A) was tested for **(A)** anti-HER2 antibodies and **(B)** anti-GFP antibodies. Serum was diluted 10 times more than the ELISA shown in Figure 1.2. Each bar represents the average of each group, where error bars show standard deviation representing biological variation rather than experimental error. Positive controls for ELISAs were commercial monoclonal antibodies to HER2 or GFP.

6.2.3 Development of a prime step without HER2 contamination

The purpose of a priming vaccination for this strategy was to enhance the numbers of virus-specific T cells (or “prime” them) before the administration of the “boost” vaccination containing the desired antigen. The idea of using the MLV EGFP virus to prime for MLV HER2 was that the only common component between the two viruses would be the internal viral proteins; however as it appeared that MLV EGFP also contained endogenous HER2 (incorporated into the vaccine particle during budding from packaging 293A cells), it would seem that an alternative prime had to be found composed only of viral components.

To this end, heat inactivated WT MLV was used as an alternative priming agent, as it would contain the desired internal viral antigens necessary to prime virus-specific T cells, and would be easier to grow to higher titres. WT MLV readily infects 293A cells, but as these cells contain both endogenous HER2 and 5T4 on the cell surface, an alternative producer cell had to be found. To produce WT MLV, the DNA plasmid encoding the virus was transfected into cells which would subsequently release progeny viruses. These virions could then infect further cells, leading to the creation of a stable cell line continuously producing higher titres of WT MLV than simply transient transfections. Non-human cell lines BHK and CT26 were assessed for human HER2 and 5T4 expression by dot blots and found to be negative (Chapter 4). This was expected, as the cells are of hamster and murine origin respectively. However it was found that although BHK cells were easily transfected with DNA (not shown), they were not permissive to infection by WT MLV, whereas CT26 cells were difficult to transfect but were susceptible to WT MLV infection (Figure 6.5). In order to achieve a stable WT MLV producing cell line, 293A cells were transfected with DNA, as these cells have high transfection efficiencies due in part to their larger surface area. The supernatant from these cells, containing WT MLV, was then taken and used to infect CT26 cells by spin infection, resulting in WT MLV producer CT26 cells.

Although CT26 cells were permissive to WT MLV infection, the cells did not display as high fluorescence as WT MLV-infected 293A cells, and subsequent purification led to low virus titres. To increase viral titres obtained from CT26 producer cells, three rounds of ultracentrifugation were carried out using the virus-containing supernatant, instead of the usual one round. Purified virus was rendered non-infectious through heat inactivation at 56°C for 1 hour (Figure 6.6).

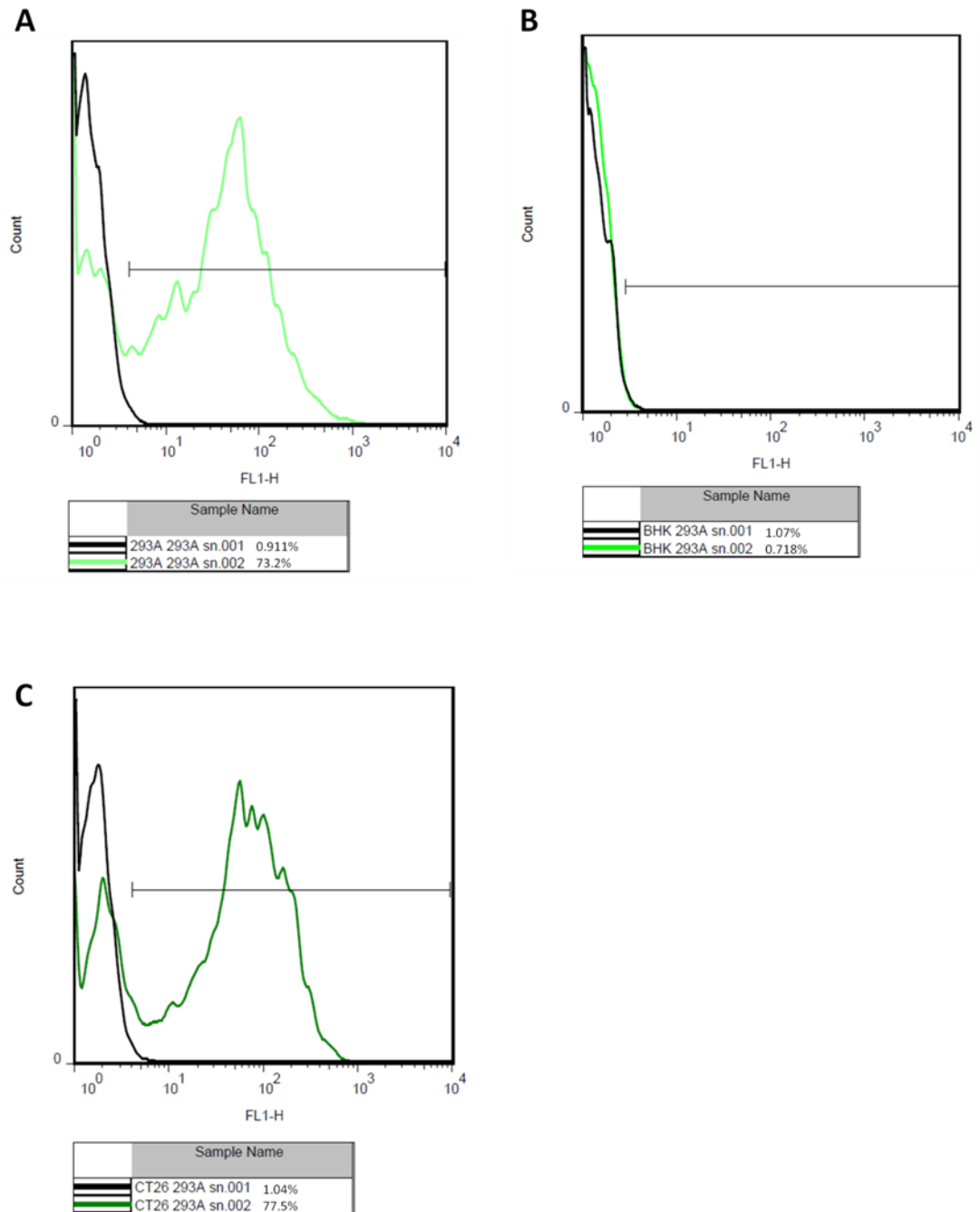


Figure 6.5 | Infectivity of WT MLV on different cell lines.

293A cells were transfected with WT MLV DNA plasmid (MLV EMD), and supernatant was collected 72 hours later. This supernatant, containing infectious WT MLV, was added to **(A)** 293A cells (as a positive control), **(B)** BHK cells and **(C)** CT26 cells. Cells were analysed for GFP fluorescence by flow cytometry 72 hours post infection (green lines), compared to cells “infected” with supernatant from non-transfected 293A cells (negative control, black lines). Supernatant from 293A cells transfected with GFP plasmid was also spun down on the three cell types, no fluorescence was seen (data not shown).

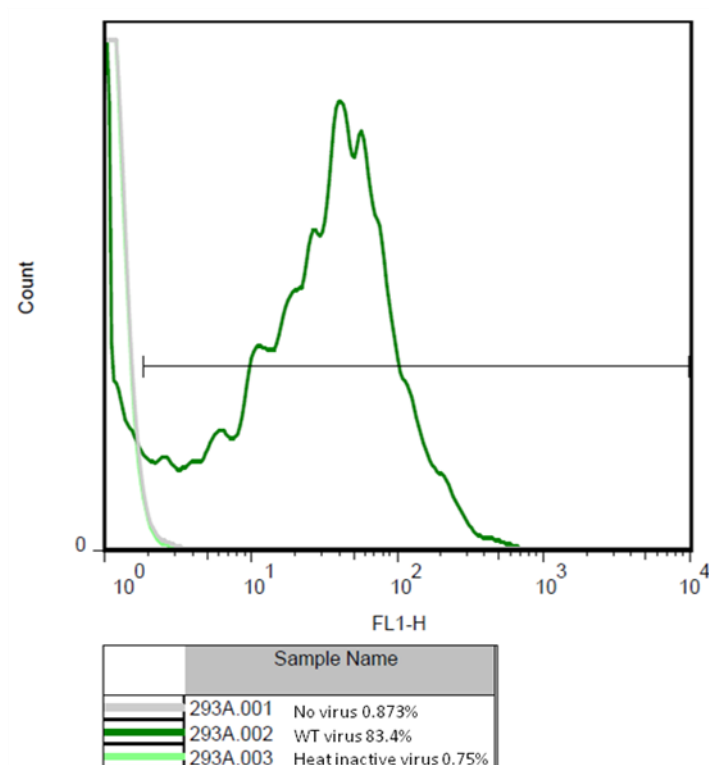


Figure 6.6 | Heat inactivation of WT MLV for use as a prime vaccination.

293A cells were infected with infectious WT MLV or heat inactivated WT MLV and assayed for GFP fluorescence by flow cytometry. Non-infected 293A cells were also analysed as a negative control.

6.3 Assessment of Prime-Boost Regimes and Comparison with Conventional Adenoviral-Based Vaccines

WT MLV was used as a new prime vaccine to replace MLV EGFP (section 6.2.3). Based on the previous *in vivo* work conducted with different adjuvants (see section 6.2.2), Titermax Gold was selected as a suitable adjuvant as mice vaccinated with Titermax Gold showed the highest antibody responses, without the adverse side effects shown with Freund's vaccination.

A large scale *in vivo* study was carried out with two arms:

1. To test the over-arching hypothesis by measuring whether there was an increased antibody response when the vaccination regime contained a virus priming step. For this, mice were primed with inactivated WT MLV and subsequently boosted with MLV HER2 or MLV 5T4.
2. To compare the two-stage (prime-boost) MLV-based system as a vaccine platform against conventional adenoviral based vaccines.

Adenoviral-based vaccines provide an opportunity to compare the MLV-vaccination strategy with a potent vaccine, using a similar two-step vaccination approach. One crucial difference is that Ad vaccines do not contain the target antigens as proteins within the virus particle itself; they are simply encoded as transgenes, and therefore depend on successful infection of cells to allow transgene expression. Another crucial difference between the MLV- and Ad-based strategies is that priming with Ad would be likely to produce Ad-specific antibodies, which are predicted to be detrimental to the subsequent boost vaccinations by neutralising their infection. This should not occur with the MLV approach, as any MLV-specific antibodies generated by the WT MLV prime will not neutralise the boost vaccines, as MLV HER2 and MLV5T4 do not contain any virus specific proteins on the surface of the virus particle.

Non-replicating adenovirus (AdZ5) was previously modified to express either the EC domain of HER2 (AdHER2) or 5T4 (Ad5T4) as a transgene. Unmodified AdZ5 was used as a "prime" to mimic the homologous prime-boost regime of MLV vaccination, followed by either AdHER2 or Ad5T4 as a "boost" vaccine. Mice were primed once (week 1) and subsequently boosted three times (weeks 3, 4, 7) (see Table 2.12 for vp doses, Materials and Methods). Serum samples were taken in the weeks between (weeks 0, 2, 5, 6, 7, 9) and analysed by ELISA for anti-tumour antigen responses (HER2 or 5T4) and anti-vector responses (MLV or adenovirus).

6.3.1 Comparison of MLV HER2/AdHER2 prime-boost vaccinations

Figure 6.7A shows the analysis of anti-HER2 responses from the HER2 arm of the *in vivo* study. It can be seen that for several weeks after the first and second boost vaccinations, only the adenovirus vaccinated mice show any measurable antibody response, with the primed group higher than the non-primed mice. Anti-HER2 responses can be detected in both groups of MLV vaccinated mice by week 9; although there appears to be a higher anti-HER2 antibody response in the primed mice over the non-primed mice, this difference was not significantly significant (t-test, $p = 0.0869$). Serum from week 9 was diluted further to fully compare the difference between adenovirus and MLV vaccinated mice (Figure 6.7B). At higher dilutions (1:150 and 1:300) it can be seen that the adenovirus vaccinated mice have significantly higher anti-HER2 responses than the MLV vaccinated mice ($p < 0.001$), showing that although the MLV mice have a good anti-HER2 response by week 9, it is still not as strong as the adenovirus treated mice.

Anti-vector responses were also tested by ELISA. It can be seen that mice not primed with MLV showed much weaker anti-MLV responses than primed mice, whereas both groups of Ad vaccinated mice showed similar anti-Ad antibody titres throughout (Figure 6.8). This shows that the initial prime of WT MLV administered in week 2 for the primed mice group was sufficient to generate anti-MLV antibodies, as these mice show statistically significant higher anti-MLV responses at week 5 ($p < 0.01$) and week 6 ($p < 0.001$), even though both groups received MLV HER2 vaccinations in weeks 3 and 4. Subsequent MLV HER2 vaccinations in the non-primed group did not generate any detectable anti-MLV antibodies until week 9. This exemplifies the difference in structure between the modified MLV and Ad viruses; the boost vaccinations of MLV do not display any viral antigen on their surface, whereas there is no external difference between the viral capsids of WT Ad or the recombinant AdHER2.

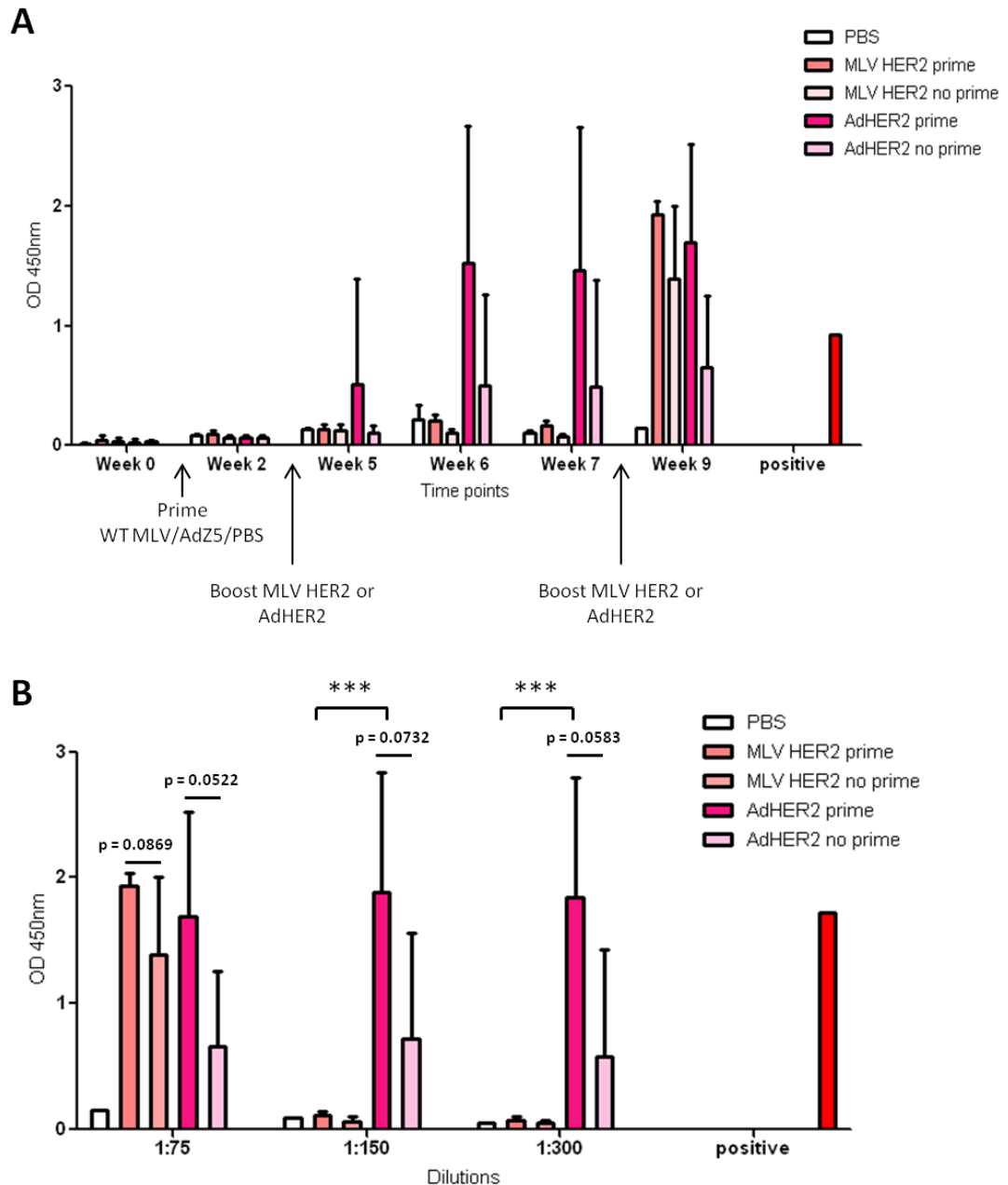


Figure 6.7 | Mice primed with WT MLV or Ad, and boosted with MLV HER2 or AdHER2 show anti-HER2 responses.

Serum samples from mice were tested in recombinant HER2-coated ELISA plates. Mice were primed once (week 1) and boosted three times (weeks 3, 4 and 7). As a control, a group of mice were vaccinated with PBS and adjuvant only (PBS) (A) Serum was diluted 1:75 in PBS. A mAb was used as indicator that the assay had worked. (B) Serum obtained by cardiac puncture (week 9) was tested again at higher dilutions to fully investigate the anti-HER2 response (and to calculate endpoint titre). Each bar represents average of the whole group, and error bars show standard deviation which represents biological variation between members of each group. Control mice, $n = 3$, all other groups $n = 5$. Data analysed by t-test between MLV prime vs non-prime, and Ad prime vs non-prime; differences were not significant, p values listed on graph. Data analysed by one-way ANOVA between MLV prime vs Ad prime, *** = $p < 0.001$.

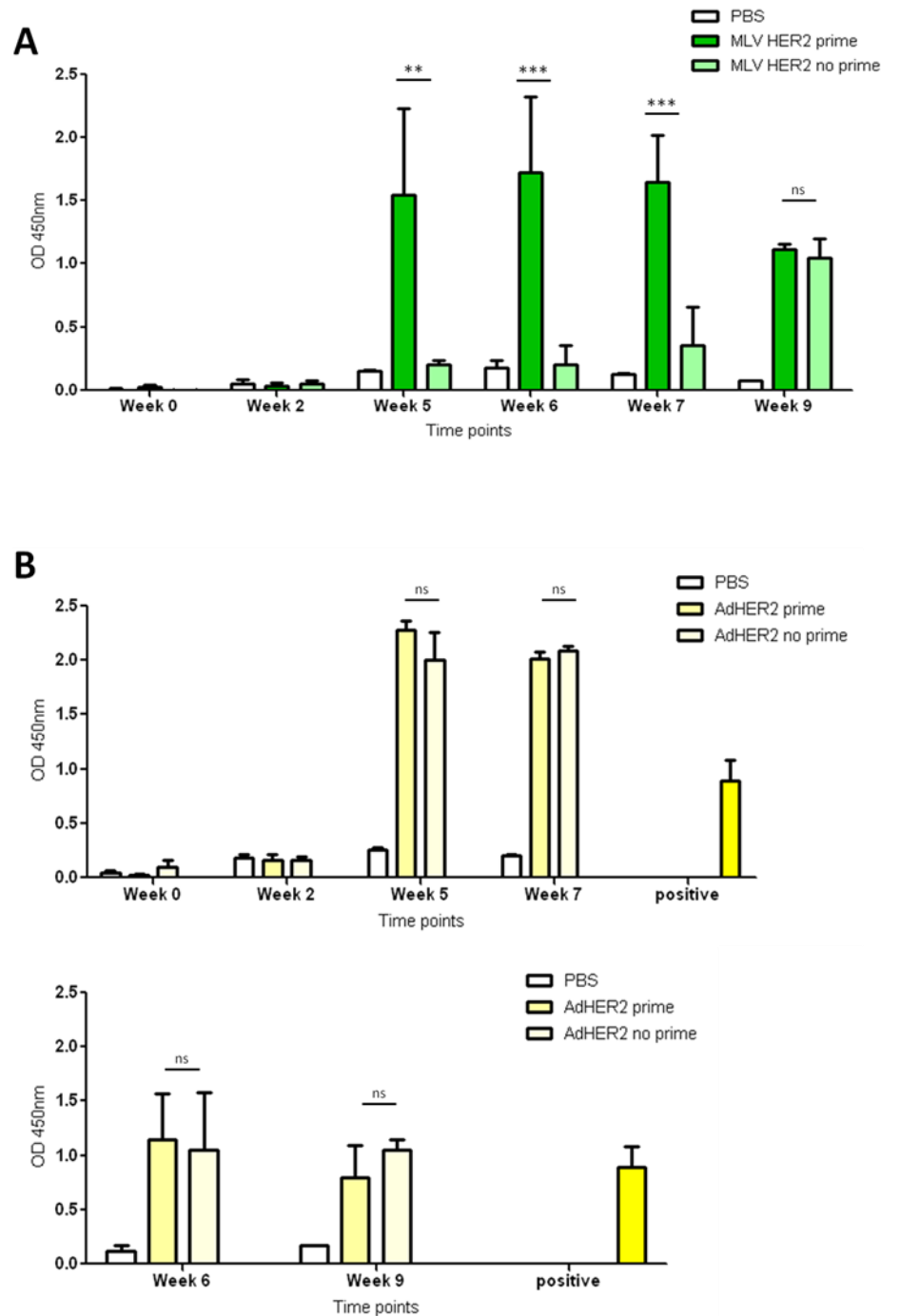


Figure 6.8 | Anti-viral antibodies were detected in MLV/Ad primed-MLV HER2/AdHER2 boosted mice.

ELISAs were carried out to detect anti-vector responses. Serum (same as Figure 6.7) was diluted 1:75. **(A)** Anti-MLV antibodies were detected using plates coated with whole WT MLV particles (heat inactivated). **(B)** Anti-Ad antibodies were detected using plates coated with Ad5T4 (top graph) or AdZ5 (bottom graph). Each bar represents average of the whole group, and error bars show standard deviation which represents biological variation between members of each group. Data analysed by one-way ANOVA, where ns = not significant, ** = $p < 0.01$ and *** = $p < 0.001$.

6.3.2 Comparison of MLV 5T4/Ad5T4 prime-boost vaccinations

The 5T4 arm of the MLV/Ad vaccination study was analysed by ELISA for antibodies against 5T4, MLV or Ad. In contrast to the data obtained from the MLV HER2/AdHER2 vaccination study, it appears that priming with WT MLV and subsequent boosting with MLV 5T4 induces statistically significantly higher anti-5T4 responses than boosting with MLV 5T4 alone (Figure 6.9). This supports the hypothesis that mice receiving an additional vaccination to prime virus-specific T cells will show higher antibody responses against the antigen found in the boost vaccinations, as the prime vaccine will expand the population of virus-specific T cells which can activate 5T4-specific B cells based on linked recognition and T cell-dependent B cell activation. However the difference between antibody responses in primed mice versus the non-primed group is only significant ($p < 0.05$) at week 9, after a single prime at week 1 and three boost vaccinations at weeks 3, 4 and 7. In comparison to MLV, 5T4 is a weaker antigen as anti-MLV antibodies can be detected by week 5 in MLV primed mice (Figure 6.10).

Interestingly, Ad5T4 does not seem to be as effective as MLV 5T4 at eliciting anti-5T4 antibodies; MLV 5T4 vaccinated mice show significantly higher anti-5T4 responses than Ad5T4 vaccinated mice ($p < 0.001$). This could be due to the differences between the two vaccines – 5T4 cell surface expression from Ad5T4-infected cells will not be as dense or highly organised as 5T4 molecules on the surface of MLV 5T4 virus particles, which is highly immunogenic⁶⁰. It is also possible that due to the anti-Ad antibodies detected in vaccinated Ad mice sera (Figure 6.10), that the Ad5T4 may have been cleared before infection and transgene expression could occur. Similar patterns of anti-vector responses can be seen with the 5T4 vaccinated mice and the HER2 vaccinated mice, once again indicating that the modified MLV vaccine particle on its own is unable to produce as high anti-vector antibodies as WT MLV, while both recombinant adenovirus and WT Ad induce comparable responses.

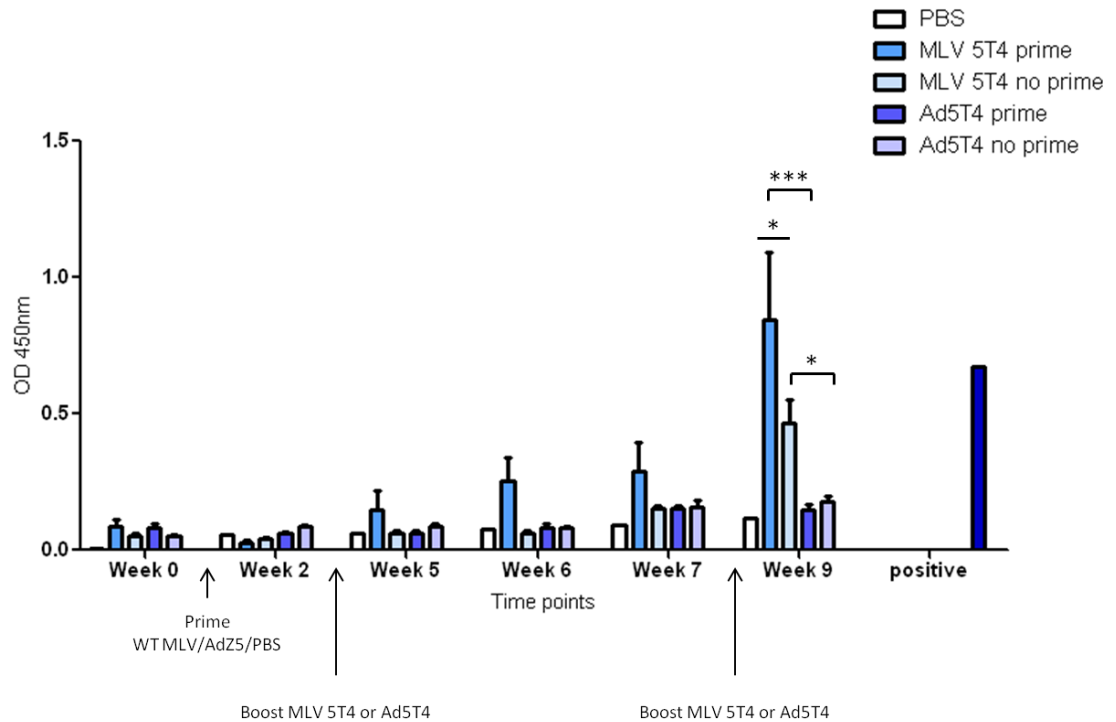


Figure 6.9 | Mice primed with WT MLV or Ad, and boosted with MLV 5T4 or Ad5T4 show anti-5T4 responses.

Diluted serum samples (1:75) from mice were tested in recombinant 5T4 coated ELISA plates. Mice were primed once (week 1) and boosted three times (weeks 3, 4 and 7). As a control, a group of mice was vaccinated with PBS and adjuvant only (PBS). A mAb was used to verify that the assay had worked. Each bar represents average of the whole group, and error bars show standard deviation which represents biological variation between members of each group. Control mice $n = 3$, all other groups $n = 5$. Data analysed by t-test between MLV prime vs no prime, $* = p < 0.05$. Data analysed by one-way ANOVA between MLV prime vs Ad prime and MLV no prime vs Ad no prime, where $*** = p < 0.001$.

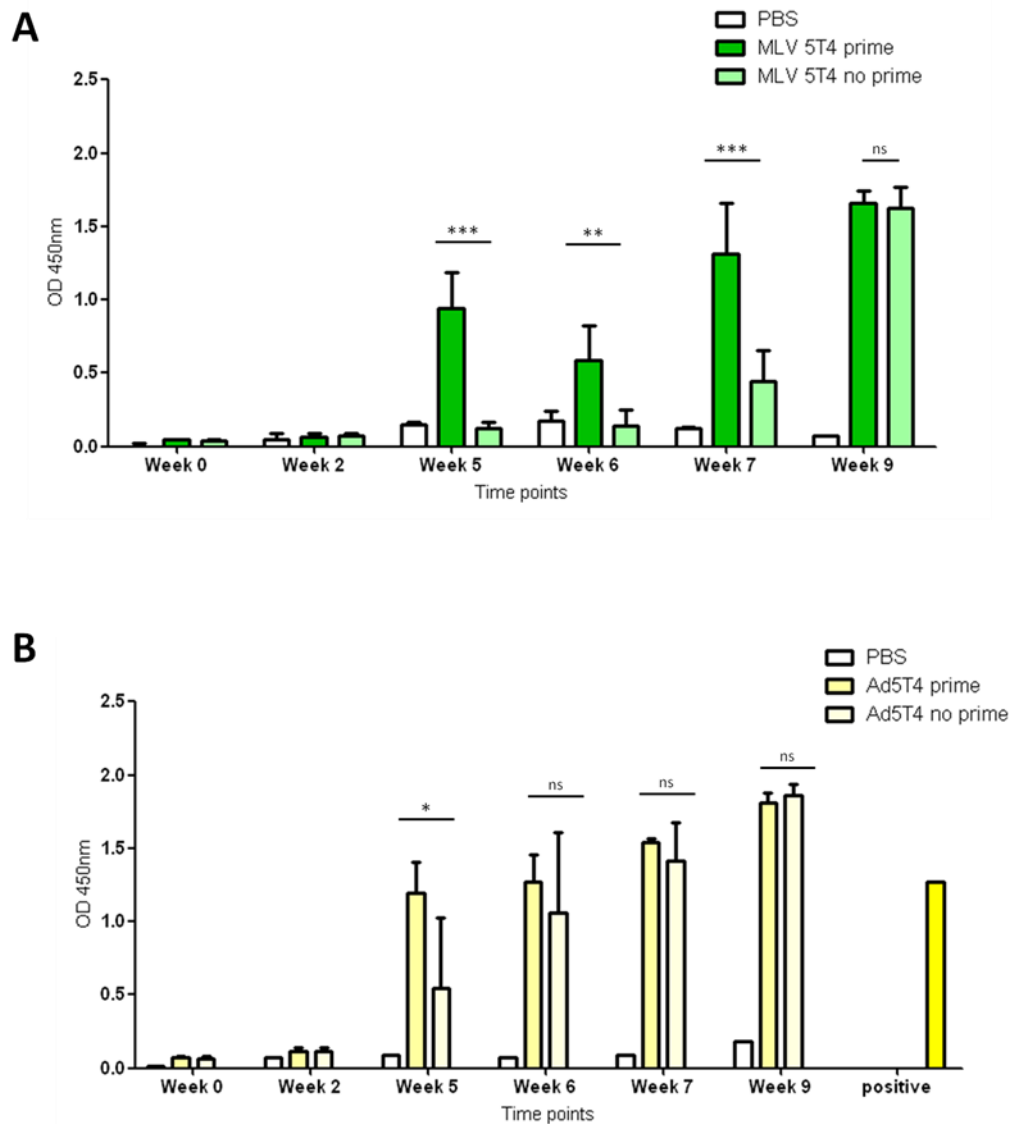


Figure 6.10 | Anti-viral antibodies were detected in MLV/Ad primed-MLV 5T4/Ad5T4 boosted mice.

ELISAs were carried out to detect anti-vector responses. Serum (same as Figure 6.9) was diluted 1:75. **(A)** Anti-MLV antibodies were detected using plates coated with whole WT MLV particles (heat inactivated). **(B)** Anti-Ad antibodies were detected using plates coated with AdHER2. Each bar represents average of the whole group, and error bars show standard deviation which represents biological variation between members of each group. Data was analysed by one-way ANOVA where ns = not significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

6.4 Discussion

This chapter has explored different strategies in order to establish an effective vaccination regimen, by carrying out preliminary experiments to determine a set protocol and schedule. The work detailed in this chapter supports the hypothesis of virus-specific T cells providing intermolecular help to antigen-specific B cells, as it can be seen that primed mice show higher antibody responses against endogenous antigens than non-primed mice. However, further work is required to elucidate the exact mechanisms.

An initial *in vivo* study testing different combinations of prime-boost immunisations using MLV EGFP and MLV HER2 found that no detectable antibody response could be raised (Figure 6.1). In an attempt to rectify this, an adjuvant screen was carried out using MLV EGFP and MLV HER2 again as examples of prime-boosting. It was decided that Titermax Gold would be an appropriate adjuvant to use for future *in vivo* studies, and the viral dose was increased from 1×10^8 vp to 2×10^9 vp, resulting in high antibody responses detectable by ELISA (Figure 6.2).

Unfortunately MLV EGFP produced from stable 293A cells was found to be an inappropriate prime vaccination, as the vaccine particles contained endogenous HER2, incorporated during virus budding. The use of MLV EGFP produced in this way as a prime would not test the hypothesis fully, as a virus-specific prime and an antigen-exposed boost were required; by using MLV EGFP with its endogenous HER2 on the surface, the prime would not longer contain solely viral antigens and in effect would be no different from the boost vaccination.

Consequently, a different strategy for generating a prime vaccination was required. Firstly, murine CT26 cells were used as new producer cells to generate MLV instead of human 293A. This was to eliminate the possibility of incorporating any human HER2 or 5T4 into the viral membrane. However, as CT26 cells were difficult to transfect compared to 293A, an altered transfection and purification protocol was optimised. WT MLV was produced in CT26 cells instead of MLV EGFP, as it was quicker to generate infectious virus instead of the modified retrovirus particles. WT MLV DNA could be transfected once and the resulting infectious particles would infect and integrate into producer cells, without the need for VSV-G pseudotyping and FACS, both of which were relatively time-consuming processes.

Having generated a viable prime vaccine, mice were primed with WT MLV and boosted with either MLV HER2 or MLV 5T4. The MLV 5T4 prime-boosted vaccinated mice showed a

statistically higher anti-5T4 antibody response compared to those that received the boost only ($p < 0.05$). In the MLV HER2 arm of the study, there was also a higher anti-HER2 response in the primed mice compared with non-primed mice, however this difference was not significant ($p = 0.0869$). These results support the hypothesis that primed mice would display higher antibody responses due to the enhancement of virus-specific T cells that would be available to activate antigen-specific B cells following the boost vaccination.

One reason for this difference in significance between the 5T4 and HER2 studies may be because human 5T4 has a restricted expression profile compared to human HER2; under normal conditions human 5T4 is expressed only in the placenta, whereas human HER2 is expressed ubiquitously on most cell types, albeit in lower levels than cancer cells. Based on the specific functions of both 5T4 and HER2, mice and humans would also show similar expression profiles in normal cells¹³². As human HER2 and mouse HER2 protein sequences show 85% sequence similarity (BLAST search), it can be inferred that mice may have some pre-existing tolerance to HER2. On the other hand, mice are less likely to show the same tolerance against 5T4 due to its non-ubiquitous nature, and hence MLV 5T4 prime-boosted mice are able to raise a statistically significant antibody response against 5T4 as both mouse and human 5T4 also share sequence similarities (80%). Overcoming such a tolerance may require a more specific prime vaccination or further optimisation of the vaccination protocol and schedule.

It is also possible that the lack of a significant difference in antibody responses between MLV HER2 primed and non-primed mice could be due to the presence of an exposed immunodominant epitope on the WT MLV prime, namely the viral Env glycoprotein. This may have skewed the CD4⁺ T cell repertoire to select for Env-specific T cells during the priming stages, which, following the boost immunisations, would have failed to provide help to antigen-specific B cells as the MLV HER2 and MLV 5T4 do not contain the corresponding Env protein in their particle. In order to elucidate the differences in antibody responses between primed and non-primed mice, a new prime vaccination was required with the same viral antigens as the boost vaccinations, to prevent off-target activation of the immune system.

MLV vaccine particles were also compared to Ad-based viral vectors encoding the equivalent endogenous antigen. Interestingly, AdHER2 prime-boosted mice showed higher antibody responses against HER2 than mice receiving AdHER2 only, despite both groups displaying high anti-Ad antibodies. In the 5T4 arm of the *in vivo* study, MLV was superior to Ad as a vaccine vector, as the Ad5T4 immunised mice showed no 5T4 antibody responses. This could be due to

the differences between the two vaccines – Ad5T4 expresses 5T4 which will be trafficked to the cell membrane (as it is still fused to MLV signal peptide and transmembrane domains), and displayed in a similar manner to host cell surface proteins. On the other hand, MLV 5T4 displays multiple membranous 5T4 molecules on the virion surface in a dense and organised fashion, which is will be more immunogenic⁶⁰. While it is not clear why AdHER2 seemed superior to Ad5T4 in eliciting antibodies against the transgene product, both primed and non-primed mice exhibited high anti-Ad antibodies, highlighting the difference between MLV and Ad viral capsids.

This chapter has described the optimisation of an appropriate vaccination protocol to carry out prime-boost immunisations. Prime vaccinations produced from different producer cell lines have also highlighted the risk in contaminating endogenous proteins incorporated into the virus particle, and the impact that these have on antibody responses. The data obtained from the MLV HER2/MLV 5T4 vaccinations with Titermax adjuvant have shown that it is possible to generate good antibody responses against HER2 and 5T4. In the case of 5T4, priming mice first with an agent that activates T cells against antigens that are hidden within the structure of the “boost” vaccine results in a significantly higher antibody response compared to non-primed mice. This supports the hypothesis that pre-existing viral-specific T cells are able to provide intermolecular help to antigen-specific B cells. However further work was required to investigate the nature of CD4+ T cells.

7 Humoral and Cellular Responses to Vaccine Particle

7.1 Introduction

This chapter describes the *in vivo* work carried out for this thesis in order to test the overarching hypothesis rigorously, by further investigation into the mechanisms of virus-specific T cells and the role they play in providing help to antigen-specific B cells. An alternative third priming strategy was also utilised.

To detect whether antigen-specific B cells receive the required help from virus-specific T cells, it was important to look for CD4⁺ T cell responses. The ELISAs previously carried out in Chapter 6 were specific for IgG isotype antibodies, thus indicating that the B cells that produced the antibodies in the vaccinated mice must have been activated by CD4⁺ T cells in order to induce isotype switching. However a more direct method of detecting CD4⁺ T cells, and in particular their specificity, was required. For this, it was necessary to optimise an assay that would allow for the detection of activated CD4⁺ T cells only, and not CD8⁺ T cells, which would not be expected to play a role in B cell stimulation and antibody production. Accordingly a protocol was required that could deplete CD8⁺ T cells from a pool of immune cells, as well as the development of an ELISPOT assay to determine the specificity of the remaining activated T cells.

The previous chapter explored the use of two different priming strategies; however neither was found to be ideal. The use of MLV EGFP as a prime was flawed due to the incorporation of endogenous HER2 within the viral envelope, derived from the packaging cell lines used. Subsequently the use of inactivated WT MLV, while not containing any contaminating endogenous HER2 or 5T4, did contain viral Env glycoprotein, which is not present in the boost vaccine particle. It is possible that Env may have been sufficiently immunodominant to detract from the usefulness of the priming step, which is required for generating CD4⁺ T cell responses against the internal viral proteins of the MLV boost particle. An alternative third priming strategy was explored, based on purified Gag protein.

7.2 Assessment of CD4+ T Cell Help

7.2.1 Optimisation of ELISPOT assays

T cell responses generated in vaccinated mice were assessed using ELISPOT assays to detect interferon- γ (IFN- γ) secretion from activated T cells. The overarching hypothesis of this thesis states that T cells activated by prime vaccination will provide help for B cells to produce antibodies against the weaker antigen in the following boost vaccination; hence any T cell assay should focus on CD4+ T cells rather than CD8+ T cells. Antibodies present in the mice sera were of the IgG class (as detected by IgG-specific ELISAs), which represents a CD4+ T cell response. This is normally characterised by ELISPOT and the detection of IFN- γ production from activated CD4+ T cells. However, CD8+ T cells also express the same cytokine when activated; therefore it was desirable to remove CD8+ T cells in order to focus on the detection of IFN- γ secretion by activated CD4+ T cells only.

Magnetic activated cell sorting (MACS) was used to deplete cell populations of CD8+ T cells; this method was preferable over extracting CD4+ T cells from a pool of cells, as it was necessary to retain APCs in the population to enable CD4+ T cell activation. Mouse splenocytes were incubated with anti-CD8 magnetic beads and run through a magnetic column. After labelling with a fluorophore conjugated anti-CD8 antibody, splenocytes were analysed by flow cytometry. Total cells before sorting contained approximately 20% CD8 positive cells. After depletion of CD8+ cells, the remaining pool contained less than 9% positive cells, although this could be attributed to background staining as there is no clear peak seen for CD8 staining (Figure 7.1). Meanwhile the CD8+ population was relatively pure at almost 94%; hence this method could be used to remove CD8 positive cells from mouse splenocytes, so that the remaining cells could be used for ELISPOT assays.

The ELISPOT protocol was optimised using non-specific T cell activation by the plant lectin concavalin A (ConA)¹³³. Three mouse spleens were processed and CD8+ cells were removed by MACS. The remaining cells were seeded in ELISPOT plates (previously coated with mouse IFN γ capture antibody) at varying cell densities. Each cell density was incubated with different concentrations of ConA. Plates were analysed for IFN γ secretion 20 hours after cell activation (Figure 7.2). It can be seen that cells respond best to the highest concentration of ConA, but there is little difference between seeding 2.5×10^5 or 5×10^5 cells per well. It should also be

noted that mouse spleens showed a range of responses to ConA activation, indicating wide biological variation. Although ConA activates immune cells in a non-specific manner, it was still important to optimise the assay for CD4⁺ T cells, as future ELISPOTs would be carried out looking for peptide-specific CD4⁺ T cells as evidence of T cell help for activated B cells.

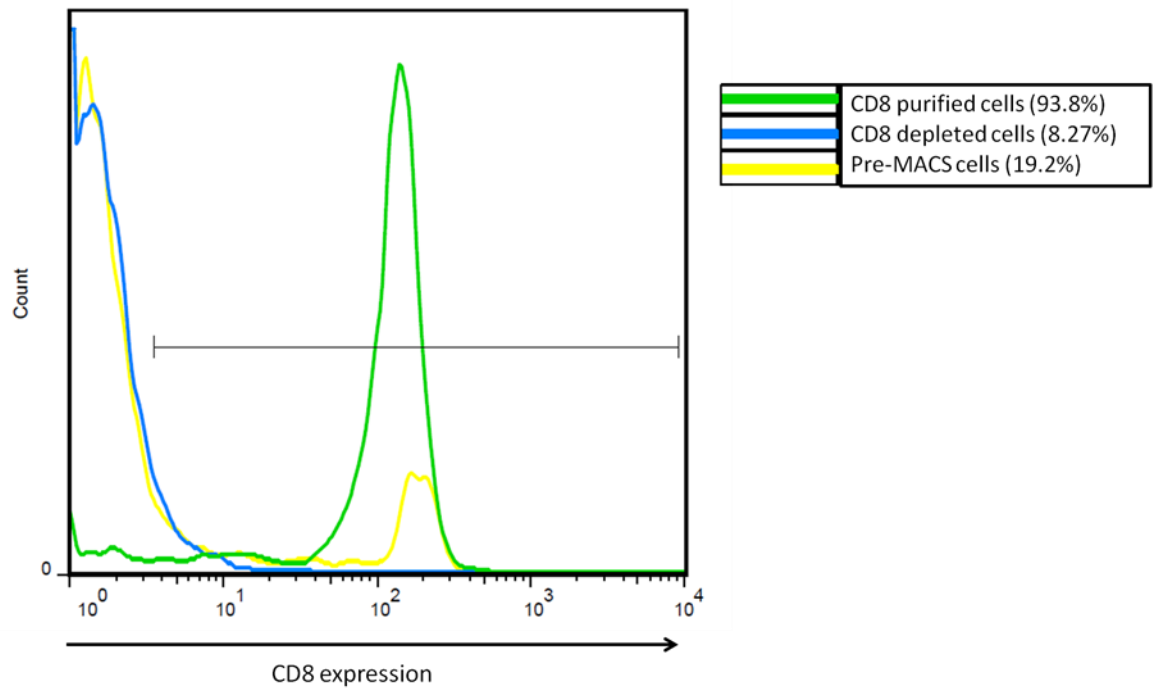


Figure 7.1 | Depletion of CD8 positive cells from mouse splenocytes by MACS.

Cells sorted by MACS were labelled with anti-CD8 antibody conjugated to Alexa488, and analysed by flow cytometry.

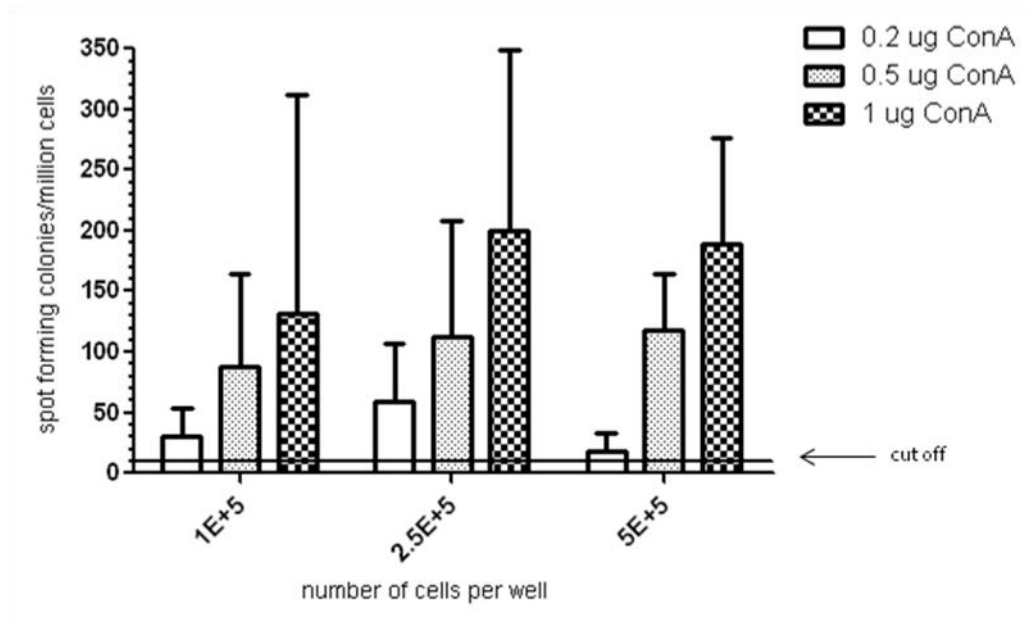


Figure 7.2 | ConA activated non-CD8+ T cells from mouse splenocytes.

Three mouse spleens were seeded at different densities and activated with varying concentrations of Con. After a period of incubation, IFN γ secretion was detected by spot forming colonies. Each bar represents the average of three mouse spleens. Error bars show standard deviation and represent biological variation between spleens.

7.2.2 Analysis of Gag prime vaccination

It was thought that one reason why the WT MLV prime vaccinations may have failed to result in a significantly higher anti-HER2 response (Chapter 6, Figure 6.7) could be because the WT MLV prime vaccination used contained all viral proteins, whereas both MLV HER2 and MLV 5T4 only possess the internal viral components (Gag and Pol). It is possible that the virus Env glycoprotein may have skewed the immune response away from the Gag and Pol proteins, leading to the generation of Env-specific CD4⁺ T cells which would have failed to enhance the MLV HER2/5T4 response, as these modified vaccine particles do not contain any virus Env. Hence, an alternative system for priming was required, and priming of mice with solely Gag protein (a kind gift from Dr Siddharta Datta and Dr Alan Rein, NIH/NCI) was carried out.

To determine the optimum number of doses of Gag protein to elicit a Gag-specific T cell response, mice were vaccinated with either 1, 2 or 3 doses of 25 µg recombinant Gag and Titermax, at one week intervals (Table 2.13, Materials and Methods). At week 4, spleens were removed from mice and analysed for Gag-specific T cells by ELISPOT. CD8⁺ cells were removed from total splenocytes before plating into ELISPOT plates and stimulating with Gag, in order to assess the contribution of IFN γ secretion from activated CD4⁺ T cells only (Figure 7.3). ConA was used as a positive control for the assay as it is a non-specific stimulator of T cells; hence incubation with ConA should activate all T cells present in the ELISPOT plate. To this end, there was no significant difference in IFN γ secretion between splenocytes from all groups of mice. Unusually, mice that received two doses of Gag seem to have fewer Gag-specific T cells than those that received only a single dose. However the overall ConA response in mice vaccinated twice is lower than all other groups too, so there may have been fewer immune cells within these splenocytes. There is a statistically significant difference ($p < 0.05$) in the number of IFN γ secreting cells from mice vaccinated with three doses of Gag compared to the control group; hence it was decided to prime all mice with three doses of Gag for future *in vivo* studies.

Antibody responses were also analysed, using serum collected from mice at weekly intervals. Despite all mice receiving their first vaccination at week 1, anti-Gag antibody responses only begin to show at week 3 for all groups (Figure 7.4). This is increased by week 4 for all groups, even though one group only received a single dose of Gag three weeks previously. As all ELISAs carried out only detected IgG antibodies, this demonstrates that there are still anti-Gag CD4⁺ T cells present in the mice in order to stimulate isotype switching and sustain a long-lasting immune response against Gag.

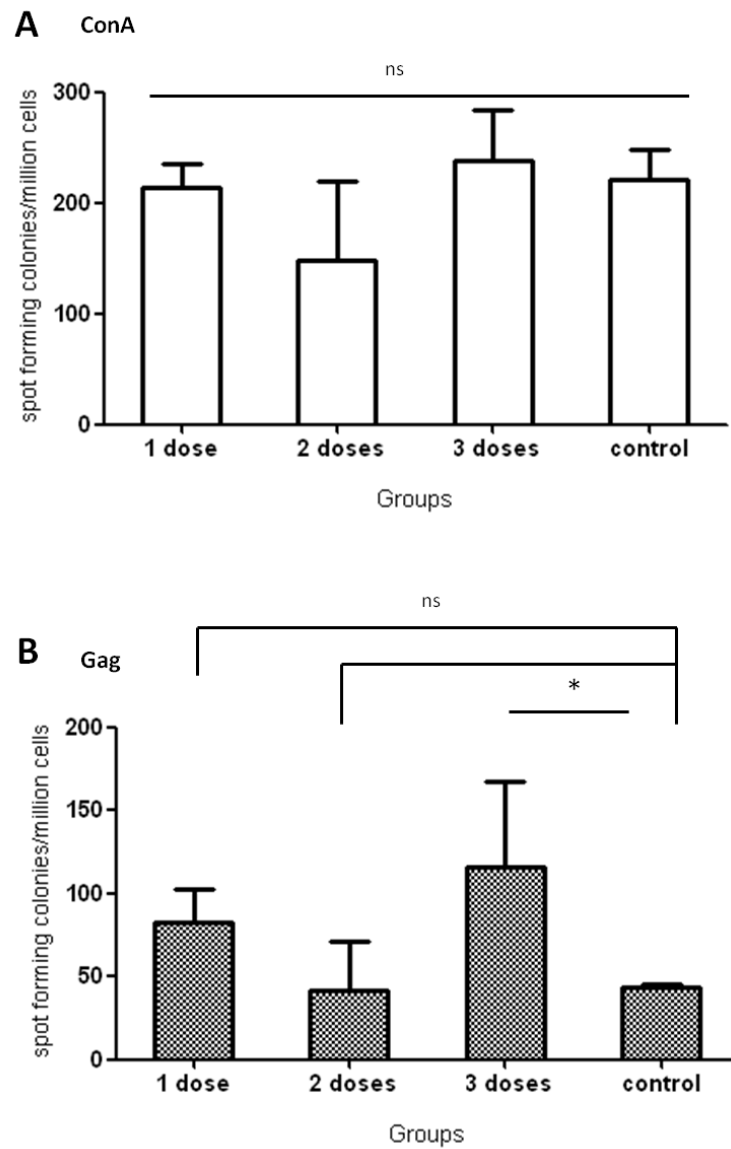


Figure 7.3 | ELISPOT analysis of splenocytes from Gag primed mice.

Mice were administered 1, 2, 3 doses of Gag or none (control) over a period of weeks. Splenocytes were analysed for Gag-specific CD4⁺ T cells. **(A)** ConA activation of non-CD8⁺ cells. **(B)** Gag-specific cells from non-CD8⁺ cells. Each bar represents average of the whole group, and error bars show standard deviation which represents biological variation between members of each group (n = 4). Data analysed by one-way ANOVA where ns = not significant, * = p < 0.05.

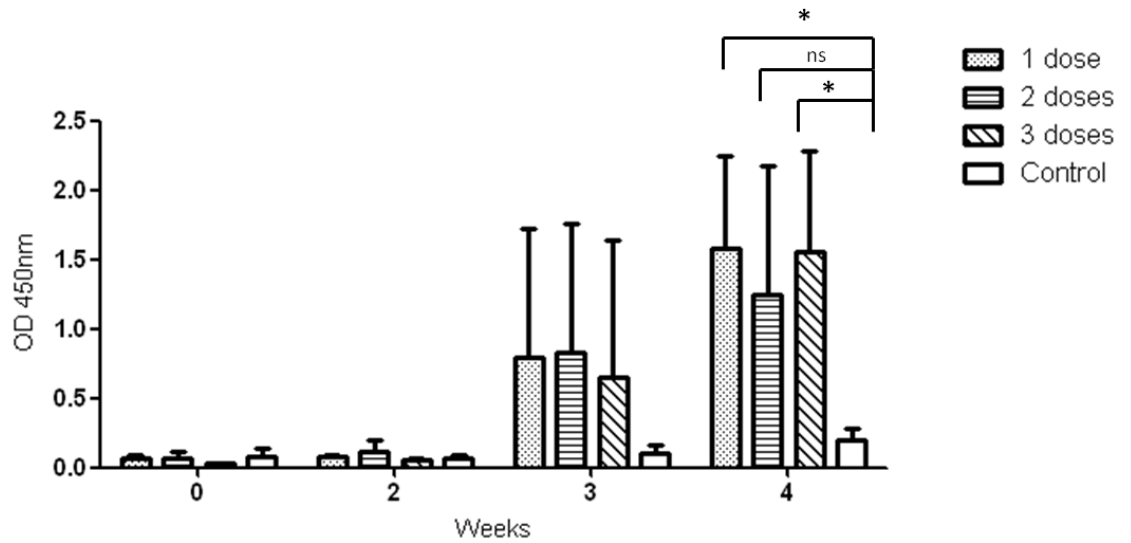


Figure 7.4 | Anti-Gag responses after Gag vaccinations.

Serum from vaccinated mice was analysed by ELISA for anti-Gag antibodies at weekly intervals. Each bar represents average of the whole group ($n = 4$), and error bars show standard deviation which represents biological variation between members of each group. Data analysed by ANOVA where ns = not significant and $* = p < 0.05$.

7.3 MLV HER2 Vaccination with Gag Prime

Having established a protocol for the activation of virus-specific CD4⁺ T cells, the hypothesis that virus-specific T cells can activate antigen-specific B cells could now be assessed *in vivo*. Mice were vaccinated three times with recombinant Gag combined with Titermax adjuvant, and subsequently administered three vaccinations of MLV HER2. These boost vaccinations were not used in conjunction with Titermax adjuvant as it was seen in previous *in vivo* work that the virus particles without adjuvant still elicited a detectable antibody response (Chapter 6, Figure 6.2). It was also thought that the combination of Titermax and vortexing to produce an emulsion may result in the lysing of virus particles which would expose the viral antigens in the boost vaccinations, which might be one reason why non-primed mice eventually acquire an anti-MLV response (Chapter 6, Figure 6.8 and Figure 6.10). Hence for this *in vivo* study, only the Gag prime vaccination was administered with Titermax.

Mice were primed three times with recombinant Gag and boosted three times with MLV HER2 (Table 2.14, Materials and Methods). Serum samples taken between vaccinations were tested for anti-Gag and anti-HER2 antibody responses by ELISA. As expected, mice in the prime-boost group show good sustained antibody responses towards Gag. Interestingly the mice in the boost only group, which do not receive recombinant Gag protein but are still exposed to Gag within the boost viral vaccine particle, do not show any anti-Gag antibodies (Figure 7.5). However when tested for anti-HER2 antibodies, vaccinated mice do not seem to show any significant responses compared with non-vaccinated control mice. This lack of antibody response could be because Titermax adjuvant was not used for the boost immunisations, and as mice may have some tolerance towards HER2, additional immune stimulation may be required. It is also possible that fewer doses of Gag prime would be sufficient in raising a moderate T cell response against Gag, while at the same time not skewing the antibody response away from HER2.

T cells from mouse splenocytes were also analysed by ELISPOT for specificity. It can be seen that spleens from mice in the prime-boost group show significantly higher Gag-specific T cells than spleens from mice administered the boost vaccine only (Figure 7.6). Mice receiving the boost vaccination only showed significantly higher Gag-specific T cells compared to the control group. As the boost-only group did not show any antibody responses against Gag, it can be inferred that T cells are exposed to the internal viral antigens contained in the boost vaccine particle, but that these antigens are not available for BCR binding on B cells, as there would

have been an anti-Gag antibody response seen after the boost vaccinations. Both groups of mice also show low HER2-specific T cell responses, however there was no significant difference between both groups as mice were exposed to the same number of virus particles, and hence the same amount of HER2. This also supports the data seen in the serum ELISAs, as there were no HER2 antibodies detected; hence any HER2-specific B cells present did not receive the required help from HER2-specific T cells.

This *in vivo* study was useful in showing that the virus vaccine particles remain intact upon vaccination and that the internal viral antigens are able to elicit good T cell responses but not B cells. However an alternative boosting strategy was required in order to obtain detectable anti-HER2 responses.

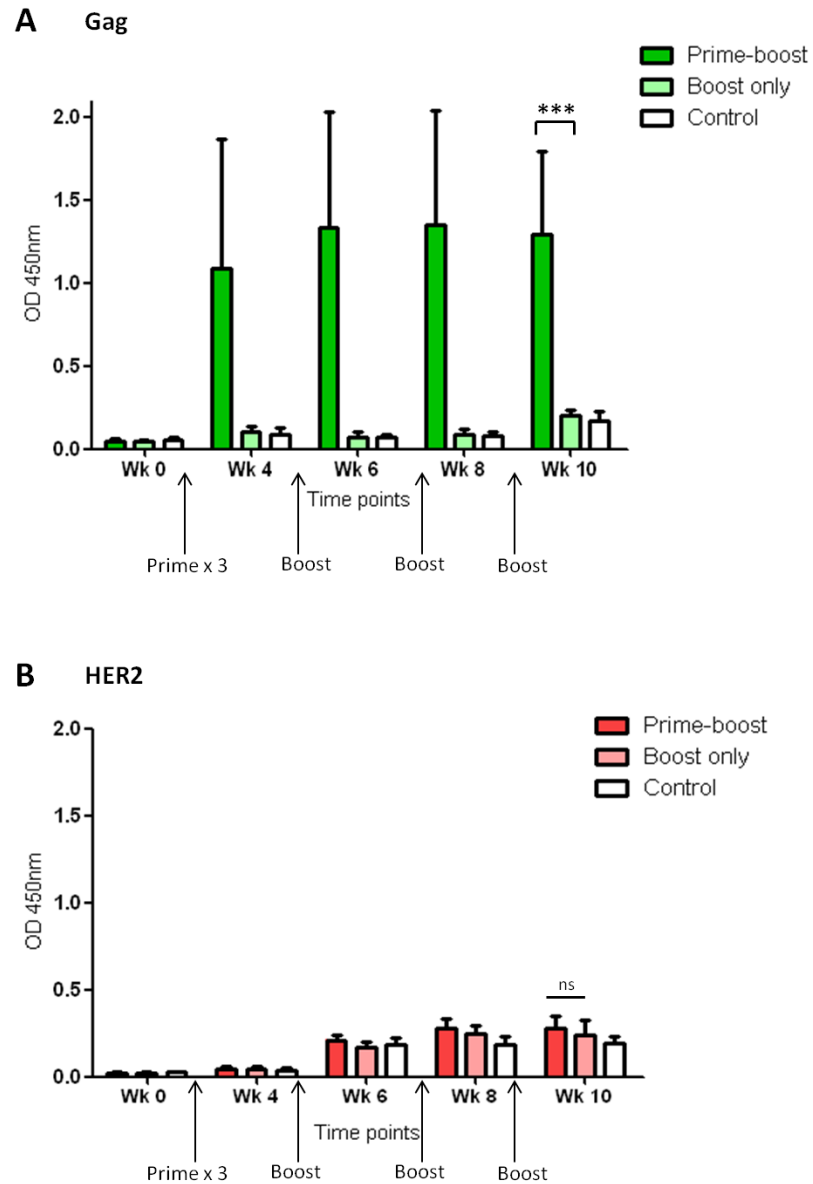


Figure 7.5 | Antibody responses against Gag and HER2 after prime-boost vaccination

(A) Anti-Gag antibody responses. Serum was diluted 1:75 before analysis by ELISA. (B) Anti-HER2 antibody responses. Serum was diluted 1:25 before analysis by ELISA. Each bar represents average of the whole group, and error bars show standard deviation which represents biological variation between members of each group ($n = 5$). Data was analysed by one-way ANOVA where *** = $p < 0.001$, ns = not significant.

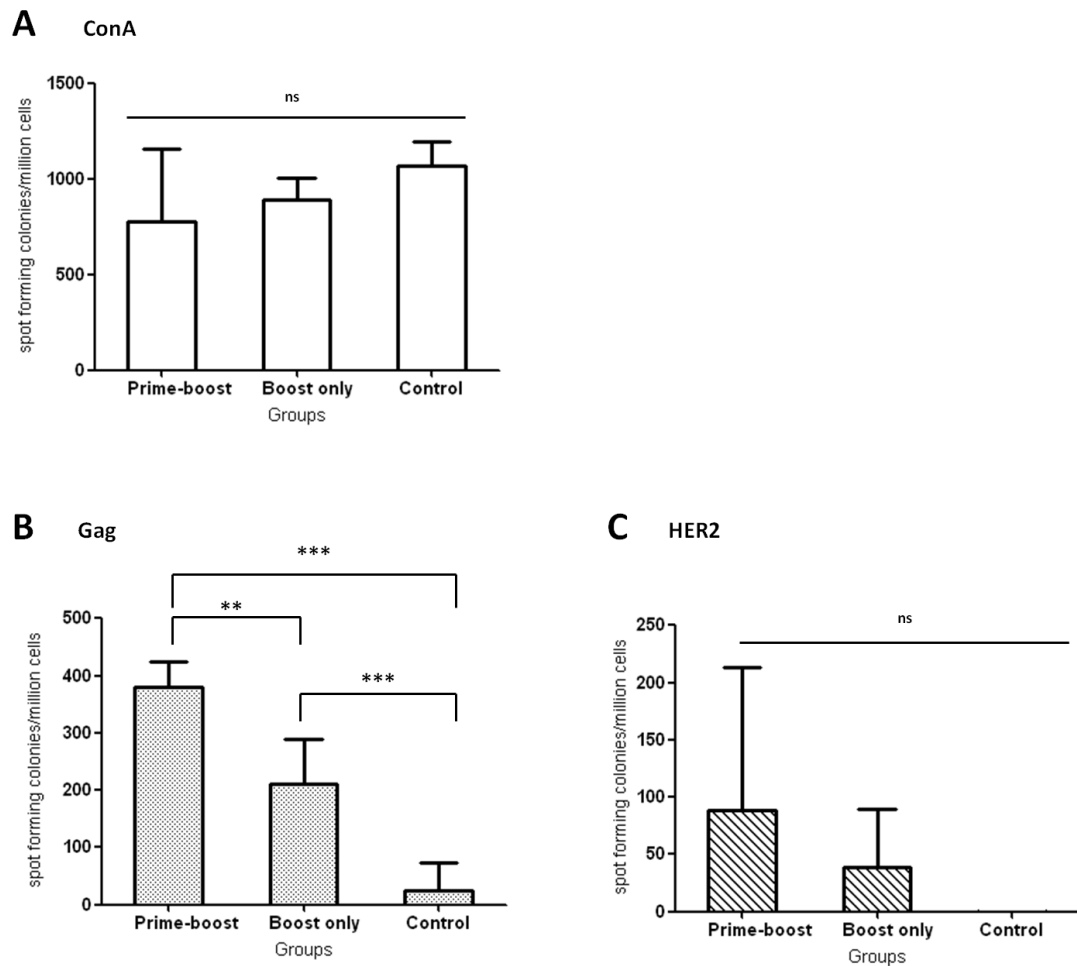


Figure 7.6 | Immune cell specific responses after prime-boost vaccination

ELISPOT analysis of (A) ConA; (B) Gag; (C) HER2 stimulated splenocytes (after removal of CD8+ cells) at week 10. Data was analysed by one-way analysis of variance (ANOVA) where ns = not significant, ** = $p < 0.01$ and *** = $p < 0.001$. Error bars represent standard deviation, and are representative of the biological variation within each group.

7.4 Administration of Prime and Boost Vaccinations with Adjuvant

It was decided to return to the use of Titermax adjuvant, as even with a prime vaccination, the antigens in the boost vaccination are not sufficiently immunogenic without an adjuvant to raise a measurable antibody response. As 5T4 antibody responses had previously been detected in *in vivo* studies (Chapter 6, Figure 6.9), it was decided to investigate MLV 5T4 as well as MLV HER2 in boost vaccinations. The number of doses of Gag as a prime vaccination was reduced to a single dose, as seen previously that Gag as a viral protein can elicit high antibody responses, and repeated doses of Gag could skew the antibody response from HER2 or 5T4.

7.4.1 MLV HER2 boost immunisation

Mice were administered a single priming vaccination of 25 µg Gag, followed by three doses of MLV HER2 at 5×10^9 vp per dose (Table 2.15, Materials and Methods). Serum samples were collected in the weeks between vaccinations, and analysed by ELISA for antibody responses.

It can be seen in Figure 7.7B that after three administrations of the boost vaccine, primed mice show higher anti-HER2 responses than non-primed mice ($p = 0.344$), although this difference is not statistically significant. Even though the primed mice only received a single dose of Gag at week 1, the anti-Gag response is sustained through to week 8 (Figure 7.7A). It can also be seen by this time point that non-primed mice also show an increasing anti-Gag response, perhaps due to the use of Titermax adjuvant along with the boost immunisations.

Mice spleens were also analysed for the presence of virus-specific T cells by ELISPOT. Both primed and non-primed groups show similar levels of Gag-specific T cells, yet there are no HER2-specific T cells present (Figure 7.8). These data suggest that only Gag-specific T cells are present in mice, and these are responsible for the antibody response against HER2, supporting the intermolecular help model of B cell activation by T cells of different specificities.

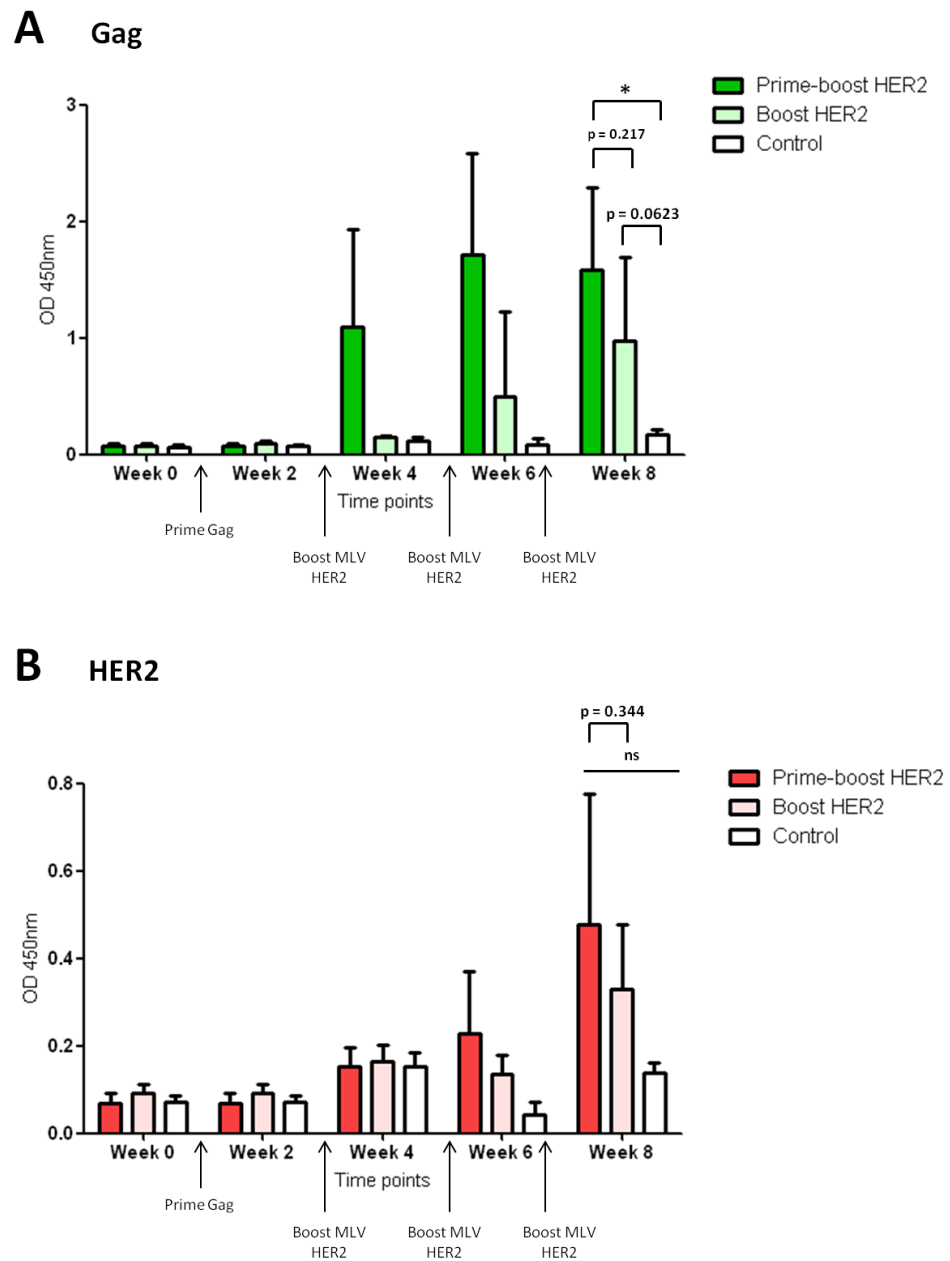


Figure 7.7 | Antibody responses in Gag primed and non-primed mice, boosted with MLV HER2 vaccine.

Mice were primed with Gag in week 1 and boosted with MLV HER2 in weeks 3, 5 and 6. Serum samples from mice were analysed by ELISA against (A) Gag and (B) HER2. Serum was diluted 1:75. Each bar represents average of the whole group, and error bars show standard deviation which represents biological variation between members of each group ($n = 5$ except control group week 8 $n = 4$). Data were analysed by one-way ANOVA where $*$ = $p < 0.05$, ns = not significant.

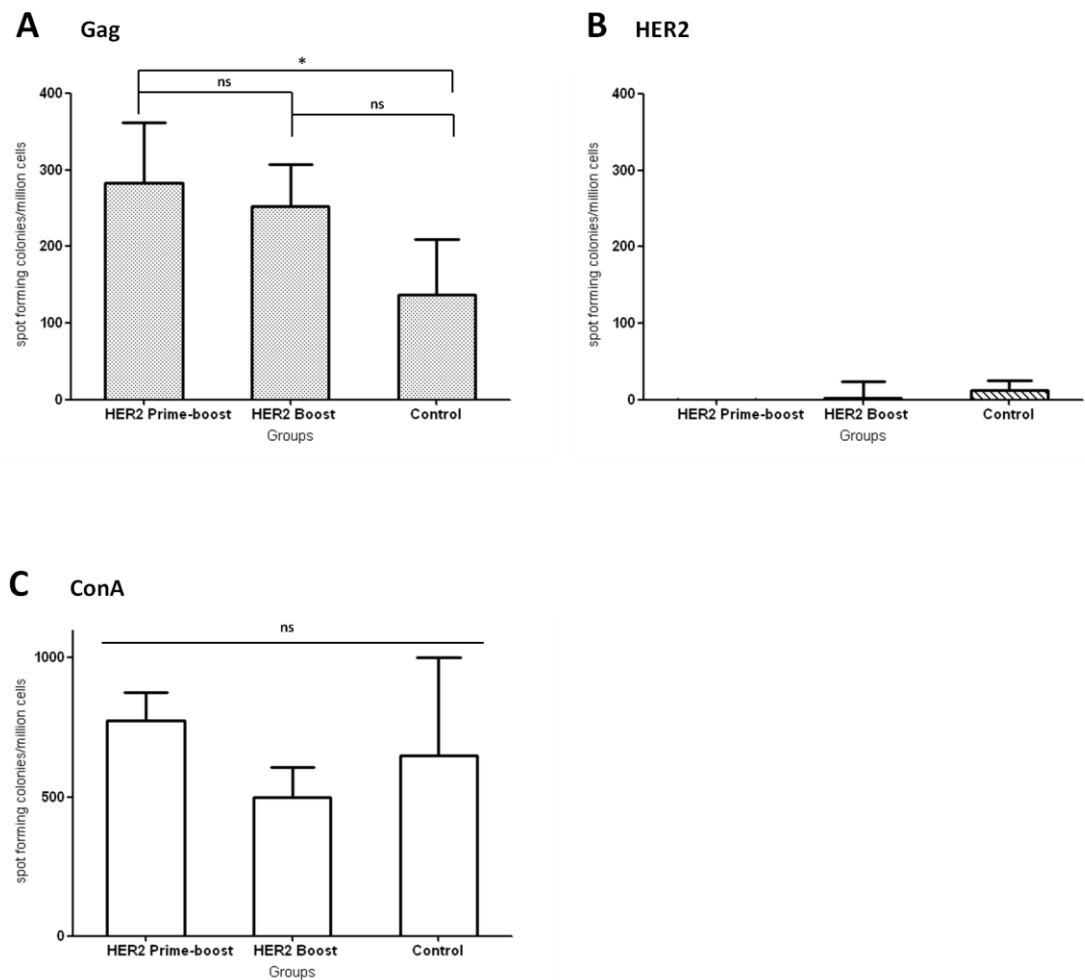


Figure 7.8 | Virus specific T cells are generated in Gag primed or non-primed, MLV HER2 boosted mice.

ELISPOT analysis of (A) ConA; (B) Gag; (C) HER2 stimulated splenocytes (from mice in Figure 7.7) at week 8. Data were analysed by ANOVA where ns = not significant and * = $p < 0.05$. Error bars represent standard deviation, and are representative of the biological variation within each group (n = 5 except control group, n = 4).

7.4.2 MLV 5T4 boost immunisation

Mice were administered a single priming dose of Gag recombinant protein followed by three boost vaccinations of MLV 5T4 (Table 2.15, Materials and Methods). Serum samples were collected at time points between vaccinations and two weeks after the final boost immunisation.

Antibody responses against Gag and 5T4 were detected by ELISA. Although the data shows that there is a slight increase in anti-5T4 antibodies from primed mice compared to non-primed mice, this difference is not significant (Figure 7.9B). It can also be seen that the primed mice exhibit a prolonged anti-Gag antibody response despite only receiving a single dose at week 1 (Figure 7.9A); however this could have been enhanced through the administration of the boost vaccine with Titermax, as it can be seen that by the final time point, non-primed mice also display anti-Gag antibodies.

T cells obtained from mouse splenocytes were analysed for specificity by ELISPOT. Interestingly, both primed and non-primed mice show the same level of Gag-specific T cells, as Gag is present in both prime and boost vaccines (Figure 7.10). Both groups also lack any 5T4-specific T cells; therefore it may be inferred that the 5T4 antibody response seen in MLV 5T4 primed and non-primed mice is derived from Gag-specific T cells providing help to 5T4-specific B cells. This supports the idea of intermolecular help.

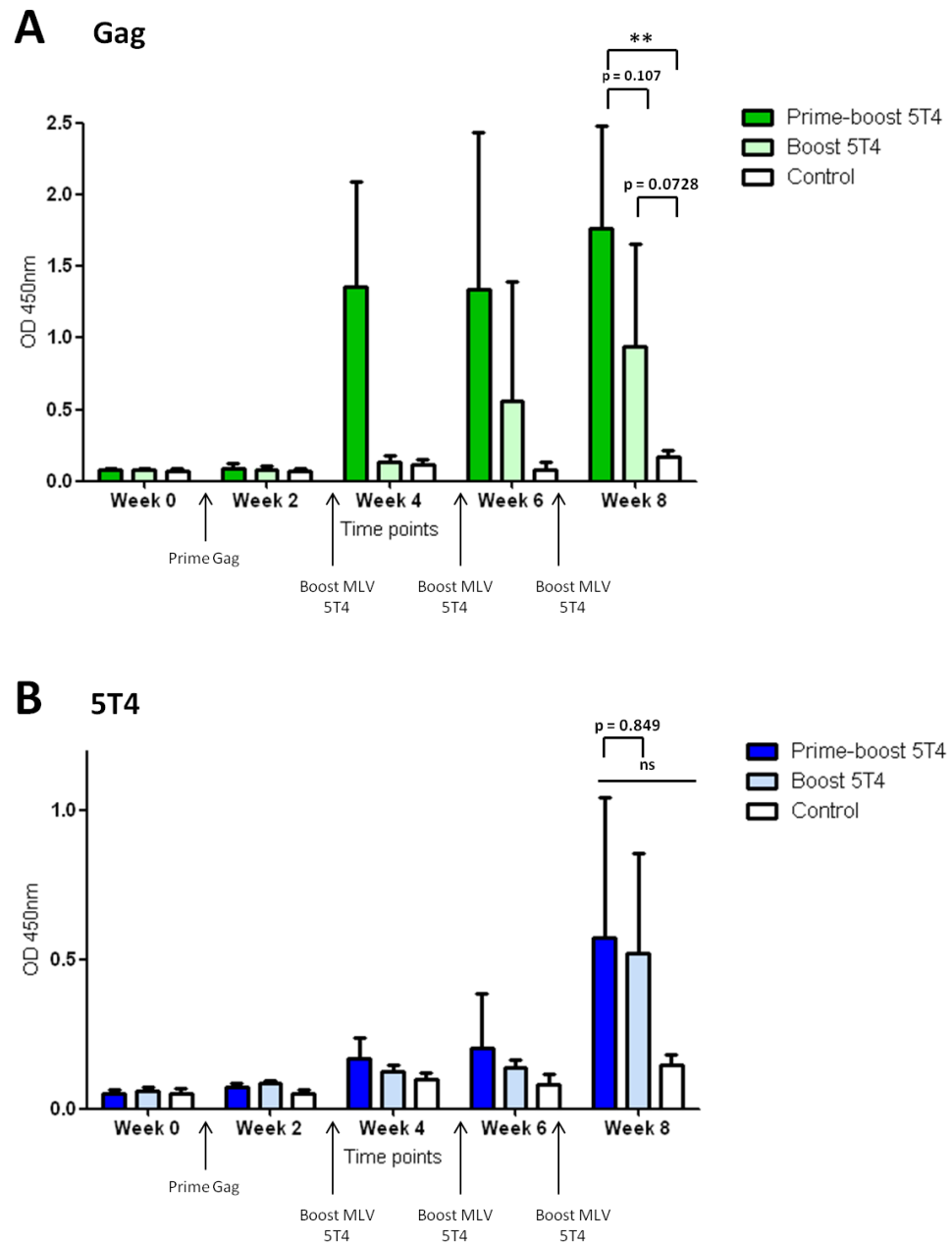


Figure 7.9 | Antibody responses in Gag primed and non-primed mice, boosted with MLV 5T4 vaccine.

Mice were primed in week 1 and boosted with MLV 5T4 in weeks 3, 5 and 6. Serum samples from mice were analysed by ELISA against (A) Gag and (B) 5T4. Serum was diluted 1:75. Each bar represents average of the whole group, and error bars show standard deviation which represents biological variation between members of each group ($n = 5$ except control group week 8 $n = 4$). Data was analysed by one-way ANOVA where $** = p < 0.01$, ns = not significant.

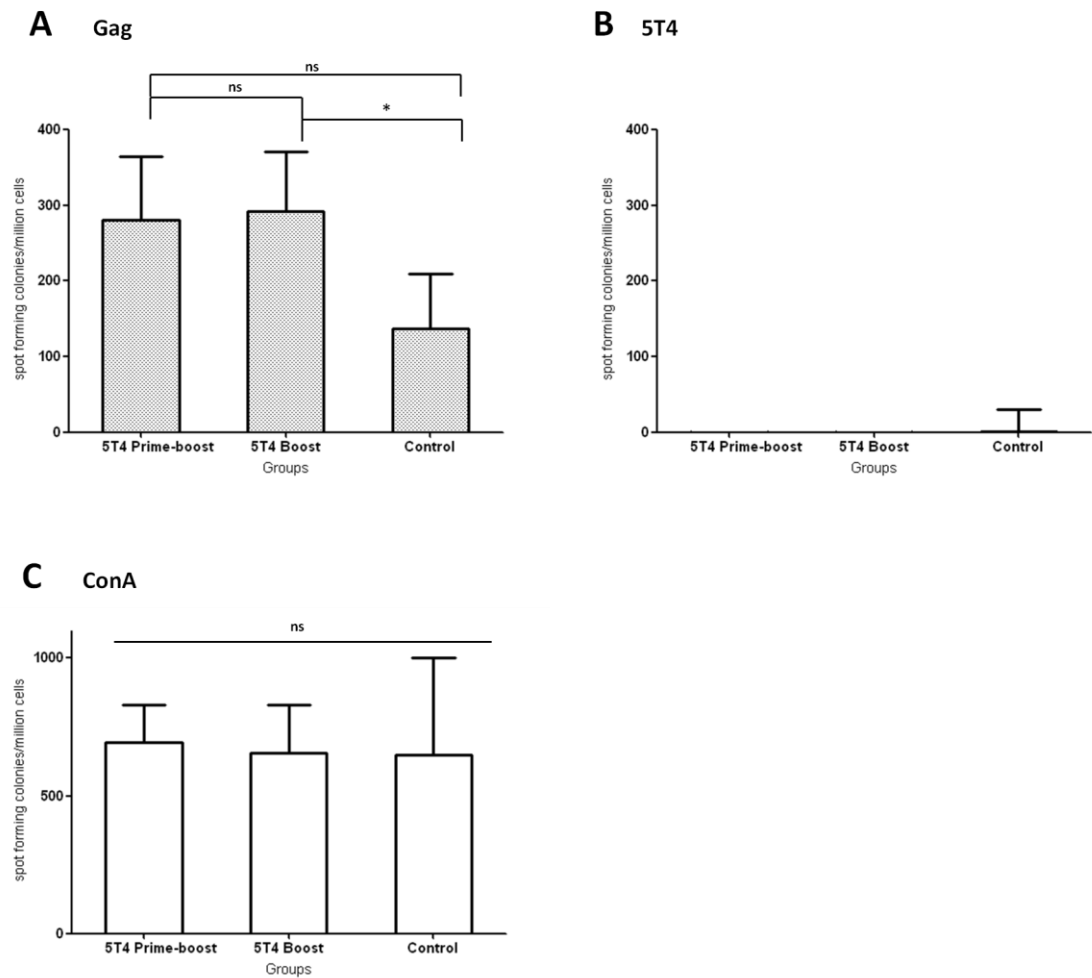


Figure 7.10 | T cell responses after Gag priming or no prime, MLV 5T4 boost vaccinated mice. ELISPOT analysis of (A) ConA; (B) Gag; (C) 5T4 stimulated splenocytes (from mice in Figure 7.9) at week 8. Data was analysed by ANOVA where ns = not significant and * = $p < 0.05$. Error bars represent standard deviation, and are representative of the biological variation within each group ($n = 5$ except control group, $n = 4$).

7.5 Discussion

This final results chapter describes the culmination of the knowledge gathered in the previous chapters and applied to further *in vivo* vaccination studies.

A new prime vaccination, purified recombinant Gag, was used in the *in vivo* studies detailed in this chapter. A preliminary study was carried out where different doses of Gag were used to vaccinate mice in order to optimise a vaccination schedule and an immune readout for activated CD4⁺ T cells. From Figure 7.3 and Figure 7.4, it can be seen that mice vaccinated with a single dose of Gag were capable of strong antibody and CD4⁺ T cell responses up to several weeks after immunisation. In order to generate the maximum number of Gag-specific CD4⁺ T cells, however, it was decided to prime mice in the subsequent *in vivo* study using three doses of Gag.

A further optimisation *in vivo* study was carried out using three doses of Gag prime followed by three boost vaccinations with MLV HER2 without adjuvant. While the mice failed to show any anti-HER2 antibody response, it was encouraging to note that non-primed mice did not exhibit any anti-Gag antibodies either, although from ELISPOT data (Figure 7.6), the mice show significantly higher Gag-activated CD4⁺ T cells than the control mice. This indicates that the MLV HER2 vaccine particle remains intact after immunisation, so that the internal viral proteins are still hidden from BCR binding but are available for MHC class II presentation to T cells, a major criterion in the hypothesis of this thesis.

The lack of any anti-HER2 antibody response in both the primed and non-primed mice could arise from the fact that Titermax adjuvant was not used in the boost immunisations, and as HER2 is a weak antigen, additional immune stimulation from an adjuvant may have been necessary. Although an anti-HER2 response was detected in previous *in vivo* studies (Chapter 6, Figure 6.2), this was effectively after three doses of HER2-containing MLV vaccine particles, as it was found that the prime vaccination used previously had endogenous HER2 on the surface. Another possibility could be that due to the high immunogenicity of recombinant Gag, and its repeated doses, this selected for B cells specific to Gag at the expense of HER2-specific B cells, of which there would be fewer in the first place due to tolerance.

As a result, it was decided for the final *in vivo* study to use a single prime of Gag, followed by three boost vaccinations with Titermax of MLV HER2 or MLV 5T4, in order to maximise any

antibody response to the endogenous antigens. The addition of Titermax adjuvant resulted in detectable anti-HER2 or 5T4 responses. The data obtained from this final *in vivo* study suggests that primed mice exhibit a higher anti-HER2 or 5T4 response than their non-primed counterparts, however in both cases these differences were not statistically significant ($p = 0.344$ and $p = 0.849$ respectively). In contrast to the previous *in vivo* study where no adjuvant was used with the boost vaccinations (Figure 7.5), the mice boosted with Titermax adjuvant along with MLV HER2 or MLV 5T4 show anti-Gag antibody responses by the final time point. Hence it may be inferred that Titermax may affect the particle in some way to expose the internal viral antigens, so that they are available for BCR binding. It is possible that the sustained anti-Gag responses seen in both primed and non-primed mice results from exposed Gag antigens during vaccination, which bind to Gag-specific B cells at the expense of the tumour antigen.

Despite the lack of significance, it can still be seen from Figure 7.7 and Figure 7.9 that three boost vaccinations of MLV HER2 or MLV 5T4 are sufficient to generate an anti-tumour antigen antibody response. From ELISPOT data, it can be seen that there are no HER2 or 5T4-specific T cells present in any mice (Figure 7.8 and Figure 7.10); therefore it can be presumed that Gag-specific T cells present in the mice are responsible for providing the necessary stimulation to HER2 or 5T4-specific B cells, resulting in antibody responses against the tumour antigen. This supports the intermolecular help model, where T cells are able to provide help to B cells of different specificities, as the B cells internalise the MLV vaccine particle by BCR-tumour antigen binding, and present the internal viral antigens to T cells via MHC class II complexes for T cell help.

8 Discussion

8.1 Cancer Vaccination

In principle, cancer vaccination is not dissimilar to vaccination against infectious diseases as both utilise the body's natural immune response. However, tumour antigens are poorly immunogenic, which has contributed to the slow progress in the field of cancer vaccines. T cell targeting vaccines have been thought to be more effective due to a cytotoxic lymphocyte effect; although many are currently in clinical trials, only one DC-based vaccine, Provenge (Sipuleucel-T), has been approved in the US²⁰. This is because T cells must be activated through TCR binding to MHC class I-peptide complexes, and as potent APCs, DCs have been pursued as attractive vehicles for T cell stimulation. However, there are limitations with the use of DCs such as the inability to expand DCs *ex vivo*, and poor antigen delivery to lymphoid tissue due to a failure of DCs to migrate to lymph nodes after injection, leading to poor efficacy in clinical trials¹³⁴.

Even if successful DC trafficking occurs, it is unlikely that there will be T cells present that are specific for self-antigen, as T cell tolerance is a rigorous process that results in the deletion of self-reactive T cells. On the other hand, mature self-reactive B cells can be found in the periphery, as B cell tolerance utilises different mechanisms¹³⁵. These B cells often do not result in autoimmunity as they still require T cell help for activation; as it is unlikely for self-reactive T cells to escape to the periphery, self-reactive B cells will remain in a state of anergy or ignorance.

The use of mAbs against tumour antigens has shown that antibodies are capable of eliciting effective anti-tumour responses, functioning through inhibition of cell signalling and ADCC¹³⁶. However due to increasing resistance to mAbs, it is desirable to target self-reactive B cells with active immunotherapy to generate a polyclonal antibody response. Tumour antigens can be administered by different means, for example encoded in viral vectors. However as stated previously, tumour antigens are often poorly immunogenic, and the viral vector itself will often dominate any immune response generated from the vaccine¹³⁷.

The hypothesis for this thesis is that by using a viral vector as a vaccine platform, weak antigens presented on the surface in a highly organised and clustered fashion can stimulate B cells. Internal viral antigens, hidden from BCR binding, can be presented to pre-existing viral specific T cells, which in turn can provide help to B cells in order to stimulate antibody production against the weak antigen. T cell confirmation of B cells is necessary to prevent aberrant B cell activation against self antigen, and as T cell selection is more stringent than the equivalent processes for B cells, it is unlikely that there will be T cells specific to weak or self antigens. Hence under normal circumstances, these self reactive B cells will not be stimulated. However fusing weak antigens to viral epitopes will result in the recruitment of virus-specific T cells upon MHC class II presentation by the B cell. Based on the intermolecular help model, these T cells will be able to activate anergic or ignorant B cells to produce antibodies against weak antigens.

By substituting the tumour antigen in place of the highly immunogenic viral envelope protein, the particle should not be cleared by any neutralising antibodies against the virus, and should retain its native form to mimic a wild type virus and fully activate an immune response. Immunisation would require a homologous prime-boost approach, where the initial vaccine would contain only the strong viral antigens in order to prime the population of CD4⁺ T cells. This would be followed by the boost vaccination, which differs from conventional prime-boost strategies as this boost would contain the same viral antigens as the prime but also the desired endogenous antigens. In contrast to existing heterologous prime-boost strategies, the priming of virus-specific T cells would benefit the subsequent boost vaccination and enhance the immune response to the endogenous antigens.

8.2 Construction of a Novel Vaccine Particle

The viral vector chosen to test this hypothesis was MLV, a relatively simple retrovirus for cloning and viral production. Chapter 3 discusses in detail the generation of the novel recombinant viruses, where the EC domain of the viral Env glycoprotein is replaced by the EC domain of a membranous tumour antigen through sub-cloning. Purification of the recombinant retroviruses was optimised to produce high titres required for *in vivo* vaccination studies. Adenoviruses have long been used as a vaccine vector, and two recombinant adenoviruses, expressing the same tumour antigens as the modified retroviruses, were generated through recombination to allow *in vivo* comparison with the MLV vaccine particles.

Having successfully produced and purified the viruses, it was vital to fully analyse the retroviral particles and adenoviral transgene expression to ensure that the transgene products were folded and presented properly. Chapter 4 details the various methods used to characterise retroviral and adenoviral transgene expression in infected or transfected cells, and the retrovirus particles themselves. Adenovirus particles were not examined closely as the modification made to the viruses only allowed for the insertion of a transgene, rather than changing the appearance of the adenovirus capsid itself.

Not only was it important to confirm that all viruses were expressing the appropriate antigen properly, it was crucial that the retroviral particles in particular presented the weak antigens on the surface of the vaccine particles in order to fully test the hypothesis. Without this spatial separation of external tumour antigens and internal viral components, it would be difficult to determine the exact immune mechanisms of any subsequent antibody response. Virus particles were found by flow cytometry to possess the desired weak antigens on the surface in a manner to elicit antibody binding, enabling the vaccine particles to be used *in vivo*.

8.3 Testing the Hypothesis In Vivo

In order to test the hypothesis that pre-existing viral immunity can be used to enhance an antibody response against a weak antigen, it was necessary to generate a prime vaccination that would ensure the activation of virus-specific T cells before administration of the tumour antigen-virus vaccine particle (the boost vaccine). To this end, a modified MLV particle expressing EGFP on its surface was created. However it was found that the 293A stable cell lines used for long term production of the retroviruses exhibited endogenous tumour antigen expression themselves; therefore by priming with MLV EGFP (Chapter 6), small amounts of HER2 contained within the virus envelope after virus budding from 293A cell membranes also acted to boost the immune response to the tumour antigens, rendering the purpose of the prime vaccination obsolete.

A different approach was subsequently trialled, using heat inactivated WT MLV particles (Chapter 6). It was necessary to use a non-human cell line in order to reduce the chance of creating a vaccine particle with endogenous tumour antigens; as BHK cells had previously been found negative for both HER2 and 5T4 (Chapter 4), they were naturally the first choice.

However it was discovered that BHK cells were not permissible to MLV infection; subsequently mouse cell lines CT26 and NIH3T3 were used, although both cell lines displayed much lower rates of infectivity compared to human 293A cells.

Chapter 6 describes the use of WT MLV as a prime vaccine in a homologous prime-boost approach, boosting with either MLV HER2 or MLV 5T4. After a single prime and three boost vaccinations, it was found that the primed mice groups showed a higher antibody response against the tumour antigen of interest compared with the non-primed mice. In mice vaccinated with MLV 5T4, this difference proved to be statistically significant, supporting the hypothesis of B cells receiving help from T cells of different specificities (the intermolecular help model).

Both MLV HER2 and MLV 5T4 vaccines displayed good antibody responses against the tumour antigen compared with their conventional adenoviral counterparts. Although mice administered with the AdHER2 vaccine exhibited good anti-HER2 responses, the Ad5T4 vaccine failed to show any equivalent anti-5T4 responses. The disparity between these two adenoviruses is unclear, as both viruses were selected based on transgene expression before and after purification, and administration of both vaccines results in anti-Ad responses. It is possible that the anti-Ad antibodies generated from Ad5T4 vaccination was sufficient to neutralise any Ad5T4 before infection could occur, but the AdHER2 vaccine may have been able to undergo infection and transgene expression before clearance by anti-Ad neutralising antibodies. It is also possible that any HER2 or 5T4-specific B cell response may have been dampened by the existence of antigen-specific regulatory T cells.

The use of whole WT MLV as a prime should have generated sufficient virus-specific T cells to boost any antibody response to the weak tumour antigens; however *in vivo*, it was thought that the presence of the WT viral Env protein on the virion surface was a strong enough immunogen to result in an early antibody response towards viral antigens, with the antibody response against the weaker tumour antigens arising slower. This is based on the data obtained in Chapter 6, and the lack of significance (Figure 6.7 and Figure 6.9). It is possible that the prime vaccination resulted in the activation of Env-specific CD4⁺ T cells, which would not stimulate antigen-specific B cells as there is no viral Env in the recombinant MLV particles. To avoid the presence of any detrimental immunodominant CD4⁺ T cell epitopes, it was decided to use the internal viral antigens solely as a prime vaccine, namely Gag. Chapter 5 describes the effort to generate recombinant Gag, and the subsequent gift of purified recombinant Gag

from Dr Datta (NIH/NCI). This was used as a prime vaccination in the *in vivo* studies detailed in Chapter 7.

Chapter 7 describes the further investigation into the role of virus-specific CD4⁺ T cells in the antibody response against the tumour antigens. After optimisation of immune readouts, the hypothesis was tested by priming mice with Gag recombinant protein and boosting with MLV HER2. Mice were boosted without Titermax adjuvant and did not show any anti-HER2 antibody responses indicating that the MLV HER2 vaccine particle is not sufficiently immunogenic on its own to generate an immune response. Interestingly non-primed mice did not exhibit any anti-Gag antibodies, yet still showed that there was a population of Gag-specific T cells; therefore this indicates that the internal Gag antigens are shielded from BCR binding in the MLV HER2 vaccine particle, but are available for MHC class II loading to present to T cells.

A final *in vivo* study was carried out where mice were primed with a single dose of Gag and boosted with MLV HER2 or MLV 5T4 with adjuvant. Although in both cohorts, the primed mice showed higher antibody responses against HER2 or 5T4 compared with the non-primed mice, these differences were disappointingly not statistically significant. As corresponding ELISA data showed that both primed and non-primed mice had anti-Gag antibodies, it can be inferred that the Titermax adjuvant, while essential for enhancing the immune response against the tumour antigens, was also affecting the structure of the MLV vaccine particle in that the internal Gag proteins were exposed for BCR binding. Although previous ELISA data in Chapter 6 had showed a significant difference between the antibody response seen in primed and non-primed mice, the viruses used in Chapter 6 were from a different stock to those administered in Chapter 7; this could account in some part to the variation in antibody responses. Nonetheless, it is clear that MLV HER2 and MLV 5T4 are capable of inducing antibody responses against the desired antigen, and from ELISPOT data, these antibody responses are achieved through help from Gag-specific T cells, not HER2 or 5T4-specific T cells. This also supports the model of intermolecular help.

8.4 Future Directions and Final Conclusion

The work carried out in Chapters 6 and 7 have yielded interesting results that verify the intermolecular help model; however additional studies could be carried out to investigate this model further.

The nature of the MLV vaccine particle is crucial in spatially separating the tumour antigens and the internal viral components. Further characterisation of the vaccine particles could be carried out, for example the bead-virus complexes analysed by flow cytometry in Chapter 4 could also be assessed by electron microscopy, as seen with HER2 exosomes complexed to Protein G beads¹³⁸. This would have provided quantitative data, which could be supported by RT-QPCR to determine number of virus particles bound to the flow cytometry beads.

The *in vivo* work carried out in this thesis highlighted the importance of the prime vaccination in enhancing the virus-specific T cells population, yet not eliciting an antibody response which would be detrimental to the humoral response against the tumour antigen. The first prime vaccine utilised, MLV EGFP, was found to also contain endogenous HER2 on the surface of the particle as it was produced from 293A cells, and was replaced by WT MLV produced from CT26 cells. However the presence of viral Env on the surface of this prime vaccine may have skewed the immune response away from the tumour antigens and was ineffective in priming as there was no Env present in the MLV vaccine particles. Finally, recombinant Gag was used as a prime, and was sufficient in stimulating virus-specific T cells and generating antibody responses against the tumour antigens. However, the mice primed with Gag could have shown significantly higher antibody responses if the Gag antigen was shielded from B cells in both the prime and the boost vaccinations. It would seem that the optimal prime vaccine would be an MLV particle produced from mouse cells with no protein on the surface, but containing the internal viral proteins necessary for virus assembly and budding. Cell transfected with Gag and Pol are capable of producing “bald” MLV particles¹³⁹, which could then be purified and characterised before use as a prime vaccine.

Having produced MLV HER2 and 5T4 vaccine particles that shielded Gag and Pol, it was disappointing to discover that the Titermax adjuvant used may have affected the structure of the particle to expose these internal antigens. Further studies could be carried out to determine the composition of the vaccine particle after emulsifying with Titermax adjuvant, or further adjuvant screening to find a suitable adjuvant that would not affect the vaccine particle but would still be capable of enhancing immune responses.

It would have also been interesting to investigate the nature of the antibodies generated through vaccination. ELISAs carried out in this thesis only detected IgG antibodies, as these are the predominant class found in the circulation⁴¹. Corresponding data could be gathered on the

different isotypes and classes of vaccine-induced antibodies, to elucidate the time course of the immune response against the tumour antigen. More importantly, the efficacy of the MLV vaccines could be tested in tumour models in immune-competent mice, to assess whether the antibodies generated through recombinant MLV vaccination would be sufficient to generate an anti-tumour response.

The overarching hypothesis of this thesis is based on the intermolecular help model, applied for vaccination against weak endogenous antigens. By priming T cells for specific internal viral antigens and subsequently administering a boost vaccination containing both the internal viral and external endogenous antigens, the immune response against the weak surface antigens can be enhanced. This is through help provided by the activated virus-specific T cells to antigen-specific B cells. This is contrary to conventional viral-based vaccines, where any immune response against the virus is detrimental to the antigen response and must be avoided. This method of intermolecular help has already been demonstrated through the RTS,S vaccine against malaria, which is a conjugate vaccine consisting of the circumsporozoite protein fused to hepatitis B surface antigen in a VLP¹⁴⁰. This vaccine has already demonstrated to halve the number of cases of malaria in young children in the 18 months after the first vaccination¹⁴¹.

The work carried out in this thesis has shown that this hypothesis is valid *in vivo* through the use of a recombinant MLV vaccine vector. Mice vaccinated with both the prime and boost vaccination have shown higher antibody responses against the target antigen compared to mice that were not administered the prime vaccine. The *in vivo* studies detailed in this thesis have highlighted the importance of establishing a prime vaccination that will only activate T cells specific for the viral antigens shared between the prime and boost vaccines, in order to avoid any off-target effects.

While the concept of a cancer vaccine is not new, this method of generating immunity to tumours is certainly novel and has many advantages when compared to current methods. By exploiting the dual mechanism of surface antigen recognition by B cells, linked with T cell confirmation by MHC class II presentation of internalised antigen to pre-existing CD4+ T cells, B cell activation can be focused on the desired antigen. The production of a vaccine particle with surface presentation consisting solely of the intended antigen, coupled with internal strong epitopes that are not accessible to the BCR but are available to CD4+ T cells after MHC class II presentation, it should be possible to trigger the expansion of antigen-specific B cell clones.

Recombinant MLV an ideal candidate to achieve this as it is a simple enveloped virus, possessing only one viral surface glycoprotein that can be replaced by cloning or pseudotyping. This novel approach has unique potential for the development of both prophylactic and therapeutic vaccines for a range of cancer types.

9 References

1. CRUK. Cancer in the UK. (Cancer Research UK, 2012).
2. Dougan, M. & Dranoff, G. Immune Therapy for Cancer. in *Annual Review of Immunology*, Vol. 27 83-117 (2009).
3. Ellebaek, E., Andersen, M.H., Svane, I.M. & Straten, P.T. Immunotherapy for metastatic colorectal cancer: Present status and new options. *Scandinavian Journal of Gastroenterology* **47**, 315-324 (2012).
4. Keller, M.A. & Stiehm, E.R. Passive immunity in prevention and treatment of infectious diseases. *Clinical Microbiology Reviews* **13**, 602-614 (2000).
5. Nadler, L.M. et al. Serotherapy of a Patient with a Monoclonal-Antibody Directed against a Human Lymphoma-Associated Antigen. *Cancer Research* **40**, 3147-3154 (1980).
6. Dillman, R.O. Magic bullets at last! Finally - Approval of a monoclonal antibody for the treatment of cancer!!! *Cancer Biotherapy and Radiopharmaceuticals* **12**, 223-225 (1997).
7. Oldham, R.K. & Dillman, R.O. Monoclonal antibodies in cancer therapy: 25 years of progress. *Journal of Clinical Oncology* **26**, 1774-1777 (2008).
8. Abès, R. & Teillaud, J.-L. Modulation of tumor immunity by therapeutic monoclonal antibodies. *Cancer Metastasis Rev* **30**, 111–124 (2011).
9. Griggs, J. & Zinkewich-Peotti, K. The state of the art: immune-mediated mechanisms of monoclonal antibodies in cancer therapy. *British Journal of Cancer* **101**, 1807-1812 (2009).
10. Nimmerjahn, F. & Ravetch, J.V. Antibodies, Fc receptors and cancer. *Current Opinion in Immunology* **19**, 239-245 (2007).
11. Weiner, L.M., Surana, R. & Wang, S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nature Reviews Immunology* **10**, 317-327 (2010).
12. Hudis, C.A. Drug therapy: Trastuzumab - Mechanism of action and use in clinical practice. *New England Journal of Medicine* **357**, 39-51 (2007).
13. Ferrara, N., Hillan, K.J., Gerber, H.P. & Novotny, W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature Reviews Drug Discovery* **3**, 391-400 (2004).
14. Leveque, D., Wisniewski, S. & Jehl, F. Pharmacokinetics of therapeutic monoclonal antibodies used in oncology. *Anticancer Research* **25**, 2327-2343 (2005).
15. Weber, J.S. et al. Phase I/II Study of Ipilimumab for Patients With Metastatic Melanoma. *Journal of Clinical Oncology* **26**, 5950-5956 (2008).
16. Phan, G.Q. et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma.

- Proceedings of the National Academy of Sciences of the United States of America* **100**, 8372-8377 (2003).
17. NICE. Guidance on the use of trastuzumab for the treatment of advanced breast cancer. (National Institute for Clinical Excellence, 2002).
 18. Galon, J. et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960-1964 (2006).
 19. Mellman, I., Coukos, G. & Dranoff, G. Cancer immunotherapy comes of age. *Nature* **480**, 480-489 (2011).
 20. Higano, C.S. et al. Sipuleucel-T. *Nature Reviews Drug Discovery* **9**, 513-514 (2010).
 21. Funding boost for B-cell-based HIV vaccine research. *Nature News* **453**(2008).
 22. Harwood, N.E. & Batista, F.D. The Antigen Expressway: Follicular Conduits Carry Antigen to B Cells. *Immunity* **30**, 177-179 (2009).
 23. Phillips, T.M. Immune-Complex Assays - Diagnostic and Clinical-Application. *Critical Reviews in Clinical Laboratory Sciences* **27**, 237-264 (1989).
 24. Nezlin, R. A quantitative approach to the determination of antigen in immune complexes. *Journal of Immunological Methods* **237**, 1-17 (2000).
 25. Wykes, M., Pombo, A., Jenkins, C. & MacPherson, G.G. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *Journal of Immunology* **161**, 1313-1319 (1998).
 26. Carrasco, Y.R. & Batista, F.D. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity* **27**, 160-171 (2007).
 27. Carrasco, Y.R. & Batista, F.D. B cell recognition of membrane-bound antigen: an exquisite way of sensing ligands. *Current Opinion in Immunology* **18**, 286-291 (2006).
 28. Batista, F.D., Iber, D. & Neuberger, M.S. B cells acquire antigen from target cells after synapse formation. *Nature* **411**, 489-494 (2001).
 29. Batista, F.D. & Harwood, N.E. The who, how and where of antigen presentation to B cells. *Nature Reviews Immunology* **9**, 15-27 (2009).
 30. Siemasko, K. & Clark, M.R. The control and facilitation of MHC class II antigen processing by the BCR. *Current Opinion in Immunology* **13**, 32-36 (2001).
 31. Rodriguez-Pinto, D. B cells as antigen presenting cells. *Cellular Immunology* **238**, 67-75 (2005).
 32. Segura, E. & Villadangos, J.A. A Modular and Combinatorial View of the Antigen Cross-Presentation Pathway in Dendritic Cells. *Traffic* **12**, 1677-1685 (2011).
 33. Heath, W.R. et al. Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunological Reviews* **199**, 9-26 (2004).

34. Vyas, J.M., Van der Veen, A.G. & Ploegh, H.L. The known unknowns of antigen processing and presentation. *Nature Reviews Immunology* **8**, 607-618 (2008).
35. Craft, J.E. Follicular helper T cells in immunity and systemic autoimmunity. *Nature Reviews Rheumatology* **8**, 337-347 (2012).
36. Bachmann, M.F. & Zinkernagel, R.M. Neutralizing antiviral B cell responses. *Annual Review of Immunology* **15**, 235-270 (1997).
37. Janeway, C.A., Travers, P., Walport, M. & Shlomchik, M.J. *Immunobiology, Fifth Edition: The immune system in health and disease.*, (Garland Science, New York, 2001).
38. Gourley, T.S., Wherry, E.J., Masopust, D. & Ahmed, R. Generation and maintenance of immunological memory. *Seminars in Immunology* **16**, 323-333 (2004).
39. Stavnezer, J. Immunoglobulin class switching. *Current Opinion in Immunology* **8**, 199-205 (1996).
40. Hussain, R. et al. Selective Increases in Antibody Isotypes and Immunoglobulin-G Subclass Responses to Secreted Antigens in Tuberculosis Patients and Healthy Household Contacts of the Patients. *Clinical and Diagnostic Laboratory Immunology* **2**, 726-732 (1995).
41. Salfeld, J.G. Isotype selection in antibody engineering. *Nature Biotechnology* **25**, 1369-1372 (2007).
42. McHeyzer-Williams, M., Okitsu, S., Wang, N. & McHeyzer-Williams, L. Molecular programming of B cell memory. *Nature Reviews Immunology* **12**, 24-34 (2012).
43. Parker, D.C. T-Cell - Dependent B-Cell Activation. *Annual Review of Immunology* **11**, 331-360 (1993).
44. Milich, D.R., McLachlan, A., Thornton, G.B. & Hughes, J.L. Antibody-Production to the Nucleocapsid and Envelope of the Hepatitis-B Virus Primed by a Single Synthetic T-Cell Site. *Nature* **329**, 547-549 (1987).
45. Russell, S.M. & Liew, F.Y. T-Cells Primed by Influenza Virion Internal Components Can Cooperate in the Antibody-Response to Hemagglutinin. *Nature* **280**, 147-148 (1979).
46. Yin, L.S. et al. CD4(+) T Cells Provide Intermolecular Help To Generate Robust Antibody Responses in Vaccinia Virus-Vaccinated Humans. *Journal of Immunology* **190**, 6023-6033 (2013).
47. Schreiber, R.D., Old, L.J. & Smyth, M.J. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. *Science* **331**, 1565-1570 (2011).
48. Wardemann, H. et al. Predominant autoantibody production by early human B cell precursors. *Science* **301**, 1374-1377 (2003).
49. Miller, J. Principles of immunological tolerance. *Transfusion Medicine and Hemotherapy* **32**, 322-331 (2005).
50. Goodnow, C.C. B-Cell Tolerance. *Current Opinion in Immunology* **4**, 703-710 (1992).

51. Ferry, H. et al. B-cell tolerance. *Transplantation* **81**, 308-315 (2006).
52. Dunn, G.P., Old, L.J. & Schreiber, R.D. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* **21**, 137-148 (2004).
53. Emens, L.A. Cancer vaccines: on the threshold of success. *Expert Opinion on Emerging Drugs* **13**, 295-308 (2008).
54. Plotkin, S.A. Correlates of Protection Induced by Vaccination. *Clinical and Vaccine Immunology* **17**, 1055-1065 (2010).
55. Strassburg, M.A. The Global Eradication of Smallpox. *American Journal of Infection Control* **10**, 53-59 (1982).
56. Klebanoff, C.A., Acquavella, N., Yu, Z.Y. & Restifo, N.P. Therapeutic cancer vaccines: are we there yet? *Immunological Reviews* **239**, 27-44 (2010).
57. Cawood, R. et al. Recombinant viral vaccines for cancer. *Trends in Molecular Medicine* **18**, 564-574 (2012).
58. Rollier, C.S., Reyes-Sandoval, A., Cottingham, M.G., Ewer, K. & Hill, A.V.S. Viral vectors as vaccine platforms: deployment in sight. *Current Opinion in Immunology* **23**, 377-382 (2011).
59. Grgacic, E.V.L. & Anderson, D.A. Virus-like particles: Passport to immune recognition. *Methods* **40**, 60-65 (2006).
60. Bachmann, M.F. et al. The Influence of Antigen Organization on B-Cell Responsiveness. *Science* **262**, 1448-1451 (1993).
61. Kushnir, N., Streatfield, S.J. & Yusibov, V. Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development. *Vaccine* **31**, 58-83 (2012).
62. Schiller, J.T. & Lowy, D.R. Understanding and learning from the success of prophylactic human papillomavirus vaccines. *Nature Reviews Microbiology* **10**, 681-692 (2012).
63. EMEA. Gardasil, INN-Human Papillomavirus Vaccine. (2006).
64. Kirnbauer, R., Booy, F., Cheng, N., Lowy, D.R. & Schiller, J.T. Papillomavirus L1 Major Capsid Protein Self-Assembles into Virus-Like Particles That Are Highly Immunogenic. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 12180-12184 (1992).
65. Lowy, D.R. & Schiller, J.T. Prophylactic human papillomavirus vaccines. *Journal of Clinical Investigation* **116**, 1167-1173 (2006).
66. Bryan, J.T. Developing an HPV vaccine to prevent cervical cancer and genital warts. *Vaccine* **25**, 3001-3006 (2007).
67. Stanley, M., Pinto, L.A. & Trimble, C. Human Papillomavirus Vaccines - Immune Responses. *Vaccine* **30**, F83-F87 (2012).

68. Tegerstedt, K. et al. A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors. *Cancer Research* **65**, 5953-5957 (2005).
69. Klamp, T. et al. Highly Specific Auto-Antibodies against Claudin-18 Isoform 2 Induced by a Chimeric HBcAg Virus-Like Particle Vaccine Kill Tumor Cells and Inhibit the Growth of Lung Metastases. *Cancer Research* **71**, 516-527 (2011).
70. Wiedermann, U. et al. A virosomal formulated Her-2/neu multi-peptide vaccine induces Her-2/neu-specific immune responses in patients with metastatic breast cancer: a phase I study. *Breast Cancer Research and Treatment* **119**, 673-683 (2010).
71. Moloney, J.B. Biological Studies on a Lymphoid-Leukemia Virus Extracted from Sarcoma-37 .1. Origin and Introductory Investigations. *Journal of the National Cancer Institute* **24**, 933-951 (1960).
72. Forster, F., Medalia, O., Zauberman, N., Baumeister, W. & Fass, D. Retrovirus envelope protein complex structure in situ studied by cryo-electron tomography. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4729-4734 (2005).
73. Rein, A. Murine leukemia viruses: objects and organisms. *Advances in virology* **2011**, 403419-403419 (2011).
74. Miller, D.G., Edwards, R.H. & Miller, A.D. Cloning of the Cellular Receptor for Amphotropic Murine Retroviruses Reveals Homology to That for Gibbon Ape Leukemia-Virus. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 78-82 (1994).
75. Kozak, S.L., Siess, D.C., Kavanaugh, M.P., Miller, A.D. & Kabat, D. The Envelope Glycoprotein of an Amphotropic Murine Retrovirus Binds Specifically to the Cellular Receptor Phosphate Transporter of Susceptible Species. *Journal of Virology* **69**, 3433-3440 (1995).
76. Kavanaugh, M.P. et al. Cell-Surface Receptors for Gibbon Ape Leukemia-Virus and Amphotropic Murine Retrovirus Are Inducible Sodium-Dependent Phosphate Symporters. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 7071-7075 (1994).
77. Maetzig, T., Galla, M., Baum, C. & Schambach, A. Gammaretroviral Vectors: Biology, Technology and Application. *Viruses-Basel* **3**, 677-713 (2011).
78. Hindmarsh, P. & Leis, J. Retroviral DNA integration. *Microbiology and Molecular Biology Reviews* **63**, 836-843 (1999).
79. Perez, O.D. et al. Design and Selection of Toca 511 for Clinical Use: Modified Retroviral Replicating Vector With Improved Stability and Gene Expression. *Molecular Therapy* **20**, 1689-1698 (2013).
80. Tai, C.K. & Kasahara, N. Replication-competent retrovirus vectors for cancer gene therapy. *Frontiers in Bioscience* **13**, 3083-3095 (2008).

81. Dalba, C., Bellier, B., Kasahara, N. & Klatzmann, D. Replication-competent vectors and empty virus-like particles: New retroviral vector designs for cancer gene therapy or vaccines. *Molecular Therapy* **15**, 457-466 (2007).
82. Huret, C. et al. Recombinant retrovirus-derived virus-like particle-based vaccines induce hepatitis C virus-specific cellular and neutralizing immune responses in mice. *Vaccine* **31**, 1540-1547 (2013).
83. Ichim, C.V. & Wells, R.A. Generation of high-titer viral preparations by concentration using successive rounds of ultracentrifugation. *Journal of Translational Medicine* **9**(2011).
84. Erlwein, O., Buchholz, C.J. & Schnierle, B.S. The proline-rich region of the ecotropic Moloney murine leukaemia virus envelope protein tolerates the insertion of the green fluorescent protein and allows the generation of replication-competent virus. *Journal of General Virology* **84**, 369-373 (2003).
85. Sliva, K., Erlwein, O., Bittner, A. & Schnierle, B.S. Murine leukemia virus (MLV) replication monitored with fluorescent proteins. *Viral J* **1**, 14 (2004).
86. Kircheis, R. et al. Immunization of Rhesus monkeys with the conjugate vaccine IGN402 induces an IgG immune response against carbohydrate and protein antigens, and cancer cells. *Vaccine* **24**, 2349-2357 (2006).
87. Diaz-Montero, C.M. et al. Phase 1 studies of the safety and immunogenicity of electroporated HER2/CEA DNA vaccine followed by adenoviral boost immunization in patients with solid tumors. *Journal of Translational Medicine* **11**(2013).
88. Hayashi, S. & Hogg, J.C. Adenovirus infections and lung disease. *Current Opinion in Pharmacology* **7**, 237-243 (2007).
89. Carlisle, R.C. et al. Human erythrocytes bind and inactivate type 5 adenovirus by presenting Coxsackie virus-adenovirus receptor and complement receptor 1. *Blood* **113**, 1909-1918 (2009).
90. Marttila, M. et al. CD46 is a cellular receptor for all species B adenoviruses except types 3 and 7. *Journal of Virology* **79**, 14429-14436 (2005).
91. Gomez-Navarro, J. & Curiel, D.T. Conditionally replicative adenoviral vectors for cancer gene therapy. *The lancet oncology* **1**, 148-58 (2000).
92. Goncalves, M. & de Vries, A.A.F. Adenovirus: from foe to friend. *Reviews in Medical Virology* **16**, 167-186 (2006).
93. Tatsis, N. & Ertl, H.C.J. Adenoviruses as vaccine vectors. *Molecular Therapy* **10**, 616-629 (2004).
94. Seregin, S.S. & Amalfitano, A. Overcoming pre-existing adenovirus immunity by genetic engineering of adenovirus-based vectors. *Expert Opinion on Biological Therapy* **9**, 1521-1531 (2009).

95. Yang, Y.P., Ertl, H.C.J. & Wilson, J.M. Mhc Class I-Restricted Cytotoxic T-Lymphocytes to Viral-Antigens Destroy Hepatocytes in Mice Infected with E1-Deleted Recombinant Adenoviruses. *Immunity* **1**, 433-442 (1994).
96. Barouch, D.H. et al. Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *Journal of Immunology* **172**, 6290-6297 (2004).
97. Bangari, D.S. & Mittal, S.K. Development of nonhuman adenoviruses as vaccine vectors. *Vaccine* **24**, 849-862 (2006).
98. Siemens, D.R. et al. Cutting edge: Restoration of the ability to generate CTL in mice immune to adenovirus by delivery of virus in a collagen-based matrix. *Journal of Immunology* **166**, 731-735 (2001).
99. Naslund, T.I. et al. Comparative prime-boost vaccinations using Semliki Forest virus, adenovirus, and ALVAC vectors demonstrate differences in the generation of a protective central memory CTL response against the P815 tumor. *Journal of Immunology* **178**, 6761-6769 (2007).
100. Hole, N. & Stern, P.L. Isolation and Characterization of 5t4, a Tumor-Associated Antigen. *International Journal of Cancer* **45**, 179-184 (1990).
101. Zhao, Y. & Wang, Y.X. 5T4 oncotrophoblast glycoprotein: Janus molecule in life and a novel potential target against tumors. *Cellular & Molecular Immunology* **4**, 99-104 (2007).
102. Southall, P.J. et al. Immunohistological Distribution of 5t4 Antigen in Normal and Malignant-Tissues. *British Journal of Cancer* **61**, 89-95 (1990).
103. Starzynska, T. et al. Prognostic-Significance of 5t4 Oncofetal Antigen Expression in Colorectal-Carcinoma. *British Journal of Cancer* **69**, 899-902 (1994).
104. Ali, S., Mulryan, K., Taher, T. & Stern, P.L. Immunotherapy success in prophylaxis cannot predict therapy: prime-boost vaccination against the 5T4 oncofoetal antigen. *Cancer Immunology Immunotherapy* **56**, 165-180 (2007).
105. Harrop, R. et al. Active treatment of murine tumors with a highly attenuated vaccinia virus expressing the tumor associated antigen 5T4 (TroVax) is CD4(+) T cell dependent and antibody mediated. *Cancer Immunology Immunotherapy* **55**, 1081-1090 (2006).
106. Amato, R.J. et al. Vaccination of Renal Cell Cancer Patients With Modified Vaccinia Ankara Delivering the Tumor Antigen 5T4 (TroVax) Alone or Administered in Combination With Interferon-alpha (IFN-alpha) A Phase 2 Trial. *Journal of Immunotherapy* **32**, 765-772 (2009).
107. Amato, R.J. & Stepanskiw, M. Clinical Efficacy of TroVax in the Treatment of Progressive Castration-resistant Prostate Cancer. *Clinical Medicine Insights. Oncology* **6**, 67-73 (2012).
108. Amato, R.J. et al. Vaccination of Metastatic Renal Cancer Patients with MVA-5T4: A Randomized, Double-Blind, Placebo-Controlled Phase III Study. *Clinical Cancer Research* **16**, 5539-5547 (2010).

109. Yarden, Y. The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. *European Journal of Cancer* **37**, S3-S8 (2001).
110. Nahta, R., Yuan, L.Y.H., Zhang, B., Kobayashi, R. & Esteva, F.J. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Research* **65**, 11118-11128 (2005).
111. Slamon, D.J. et al. Human-Breast Cancer - Correlation of Relapse and Survival with Amplification of the Her-2 Neu Oncogene. *Science* **235**, 177-182 (1987).
112. Gutierrez, C. & Schiff, R. HER2 Biology, Detection, and Clinical Implications. *Archives of Pathology & Laboratory Medicine* **135**, 55-62 (2011).
113. Tookman, L. & Roylance, R. New drugs for breast cancer. *British Medical Bulletin* **96**, 111-129 (2010).
114. Mitra, D. et al. An oncogenic isoform of HER2 associated with locally disseminated breast cancer and trastuzumab resistance. *Molecular Cancer Therapeutics* **8**, 2152-2162 (2009).
115. Slamon, D.J. et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New England Journal of Medicine* **344**, 783-792 (2001).
116. Arteaga, C.L. et al. Treatment of HER2-positive breast cancer: current status and future perspectives. *Nature Reviews Clinical Oncology* **9**, 16-32 (2012).
117. Pazdur, R. FDA Approval for Pertuzumab. in *Cancer Drug Information (National Cancer Institute at the National Institute for Health)* (2012).
118. Scheuer, W. et al. Strongly Enhanced Antitumor Activity of Trastuzumab and Pertuzumab Combination Treatment on HER2-Positive Human Xenograft Tumor Models. *Cancer Research* **69**, 9330-9336 (2009).
119. Baselga, J. et al. Phase II Trial of Pertuzumab and Trastuzumab in Patients With Human Epidermal Growth Factor Receptor 2-Positive Metastatic Breast Cancer That Progressed During Prior Trastuzumab Therapy. *Journal of Clinical Oncology* **28**, 1138-1144 (2010).
120. Milani, A., Sangiolo, D., Montemurro, F., Aglietta, M. & Valabrega, G. Active immunotherapy in HER2 overexpressing breast cancer: current status and future perspectives. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **24**, 1740-8 (2013).
121. Ladjemi, M.Z., Jacot, W., Chardes, T., Pelegrin, A. & Navarro-Teulon, I. Anti-HER2 vaccines: new prospects for breast cancer therapy. *Cancer Immunology Immunotherapy* **59**, 1295-1312 (2010).
122. Stanton, R.J., McSharry, B.P., Armstrong, M., Tomasec, P. & Wilkinson, G.W.G. Re-engineering adenovirus vector systems to enable high-throughput analyses of gene function. *Biotechniques* **45**, 659-668 (2008).

123. Singer, V.L., Jones, L.J., Yue, S.T. & Haugland, R.P. Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. *Analytical Biochemistry* **249**, 228-238 (1997).
124. Koh, Y.T., Higgins, S.A., Weber, J.S. & Kast, W.M. Immunological consequences of using three different clinical/laboratory techniques of emulsifying peptide-based vaccines in incomplete Freund's adjuvant. *Journal of Translational Medicine* **4**(2006).
125. Rux, J.J. & Burnett, R.M. Adenovirus structure. *Human Gene Therapy* **15**, 1167-1176 (2005).
126. Pear, W. Transient transfection methods for preparation of high-titer retroviral supernatants. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.] Chapter 9*, Unit9.11-Unit9.11 (2001).
127. Panchuk-Voloshina, N. et al. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *Journal of Histochemistry & Cytochemistry* **47**, 1179-1188 (1999).
128. Stills, H.F. Adjuvants and antibody production: Dispelling the myths associated with Freund's complete and other adjuvants. *Ilar Journal* **46**, 280-293 (2005).
129. Stewart-Tull, D.E.S. Freund's Complete and Incomplete Adjuvants, Preparation, and Quality Control Standards for Experimental Laboratory Animals Use. in *Vaccine Adjuvants: Methods and Protocols*, Vol. 626 59-72 (2010).
130. Bennett, B., Check, I.J., Olsen, M.R. & Hunter, R.L. A Comparison of Commercially Available Adjuvants for Use in Research. *Journal of Immunological Methods* **153**, 31-40 (1992).
131. Petrovsky, N. & Aguilar, J.C. Vaccine adjuvants: Current state and future trends. *Immunology and Cell Biology* **82**, 488-496 (2004).
132. Woods, A.M. et al. Characterization of the murine 5T4 oncofoetal antigen: a target for immunotherapy in cancer. *Biochemical Journal* **366**, 353-65 (2002).
133. Mackler, B.F., Dumonde, D.C. & Wolstenc.Ra. Concanavalin-a as an Inducer of Human Lymphocyte Mitogenic Factor. *Nature-New Biology* **239**, 139-142 (1972).
134. Bear, A.S., Cruz, C.R. & Foster, A.E. T Cells as Vehicles for Cancer Vaccination. *Journal of Biomedicine and Biotechnology* (2011).
135. Zikherman, J., Parameswaran, R. & Weiss, A. Endogenous antigen tunes the responsiveness of naive B cells but not T cells. *Nature* **489**, 160-164 (2012).
136. Scott, A.M., Allison, J.P. & Wolchok, J.D. Monoclonal antibodies in cancer therapy. *Cancer immunity* **12**, 14 (2012).
137. Saxena, M., Thi Thu Hao, V., Baird, F.J., Coloe, P.J. & Smooker, P.M. Pre-existing immunity against vaccine vectors - friend or foe? *Microbiology-Sgm* **159**, 1-11 (2012).
138. Koga, K. et al. Purification, characterization and biological significance of tumor-derived exosomes. *Anticancer Research* **25**, 3703-3707 (2005).

139. Voelkel, C. et al. Pseudotype-Independent Nonspecific Uptake of Gammaretroviral and Lentiviral Particles in Human Cells. *Human Gene Therapy* **23**, 274-286 (2012).
140. Stoute, J.A. et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. *New England Journal of Medicine* **336**, 86-91 (1997).
141. Malaria vaccine candidate reduces disease over 18 months of follow-up in late-stage study of more than 15,000 infants and young children. in *GlaxoSmithKline Press Release* (2013).

10 Appendix

10.1 Publications

10.1.1 Publications in peer-reviewed journals

For full text papers, please see following pages.

Cawood R, Wong SL, Di Y, Baban DF, Seymour LW. MicroRNA Controlled Adenovirus Mediates Anti-Cancer Efficacy without Affecting Endogenous MicroRNA Activity. *Plos One* **6** (2011)

Part of this paper was from work carried out as a part of a rotation in the first year DPhil programme.

Cawood R, Hills T, Wong SL, Alamoudi AA, Beadle S, Fisher KD, Seymour LW. Recombinant viral vaccines for cancer. *Trends in Molecular Medicine* **18**, 564 – 574 (2012).

10.1.2 Abstracts presented at scientific conferences

Underlined names represent presenting author.

Wong SL, Cawood R, Seymour LW. MicroRNA regulation of adenovirus E1A viral protein. *Human Gene Therapy* 21(4) 507-525

Cawood R, Seymour L, Wong SL. MicroRNA regulation of adenovirus afford control of oncolytic activity without changing endogenous microRNA levels. *Human Gene Therapy* 21: 1358-1396

Cawood R, Wong SL, Seymour LW. MicroRNA control of Adenovirus Serotype 5 does not affect endogenous levels. *Abstract from 6th International Conference on Oncolytic Viruses as Cancer Therapeutics 2011*