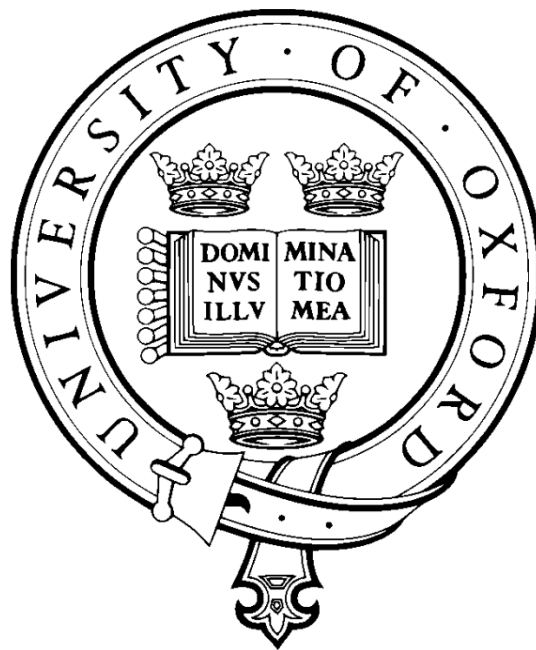


Formulation and testing of a liposomal-based vaccine for cancer and influenza

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A thesis presented for the degree of
Doctor of Philosophy



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Abstract

Vaccines are arguably the most important medical technology developed to date, and have provided dramatic reductions in disease morbidity and mortality over the last 200 years. Although vaccines have been undeniably successful, improvements in vector production, delivery and ease of use would be of great benefit. Historically, vaccine development has been based on the “three Is” paradigm of Louis Pasteur (isolate, inactivate, inject). However, an improved understanding of immunology, pathology and microbiology is now helping vaccine development adopt a more ‘rational design’ approach. This thesis builds on previous work in the development of a plug-and-play universal vaccination platform. Previously, this platform has been reported to evoke a rapid, high titre, isotype switched, epitope-specific antibody response against an epitope of the circumsporozoite protein from the malaria parasite. Here we describe repurposing the platform to vaccinate against ErbB-2 overexpressing tumours and H3N2 influenza viruses. Cancer is among the leading causes of morbidity and mortality today. ErbB-2 is a popular target for cancer treatment as it is localised to the cell surface and has raised expression in 15 - 30% of all breast cancers, as well as in some ovarian, renal, colon and lung cancers. Influenza is a common pathogen that annually infects 5 - 10% of adults and 20 - 30% of children worldwide. Despite influenza (flu) vaccines being available since the 1940s’ they have had restricted success due to flu proteins being subject to a

high rate of antigenic drift and shift. However, many groups have reported conserved regions of the viral proteins with low immunogenicity. If such low immunogenicity can be overcome then this would pave the way for a flu vaccine with long lasting efficacy. This thesis begins by first giving an overview of novel vaccination technologies before describing the formulation methods of the vaccine platform developed herein. The encapsulation efficiency of liposomal-vaccine particles after all purification steps was found to be approximately 0.1, 0.3, and 0.5% for the three formulation methods used. This low percentage is due to dilution factors in the purification steps and inherent poor passive loading efficiency of liposomes. The surface conjugation efficiency of the target peptide was demonstrated to increase from 0.4% in the first two formulation methods to 92.8% in the third method. The immunogenicity of this vaccination platform, after subcutaneous injection, is then demonstrated by the elicitation of a rapid (within 1 week) raised (10.5-fold, $p < 0.001$) humoral immune response specific for the ErbB-2 peptide. This process is shown to be driven by non-cognate T helper cells specific for an ovalbumin peptide, OVA_{323–339}. Lastly, to demonstrate the plug-and-play adaptability of the platform the formulation is repurposed to show raising of a class-switched, high-avidity, antibody response to a H3N2 influenza virus epitope, with titres reaching 98.7-fold higher than control groups ($p < 0.001$) just one week after initial vaccination.

Statement of Originality

I hereby declare that this submission is my own work and, to the best of my knowledge, it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at the University of Oxford or any other educational institution, except where due acknowledgement is made in the thesis.

Any contribution made to the research by others, with whom I have worked at the University of Oxford or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Research Dissemination

Peer-Reviewed Journal Publications

J. Wallis, A. Martin, P. Katti, T. Hills, L. W. Seymor, D. P. Shenton, R. C. Carlisle, “*A liposome-based cancer vaccine for a rapid and high-titre anti-ErbB-2 antibody response*” (in preparation)

J. Wallis, D. P. Shenton, R. C. Carlisle, “*Novel approaches for the design, delivery and administration of vaccine technologies*”, Clinical and Experimental Immunology, 196, 189 - 204 (2019)

Acknowledgements

This thesis would not have been possible without the support and kindness of so many people.

I would like to thank all those in the Synthetic Biology CDT team for giving me the opportunity to make the move from a mathematical background, and for giving me the training I needed to become a capable biological scientist. As such, I would also like to thank the Biotechnology and Biological Sciences Research Council, the Defence Science and Technology Laboratory, the Engineering and Physical Sciences Research Council, and the Oxford Centre for Drug Delivery Devices for sponsoring both me and my doctoral project.

I am eternally grateful to Prof. Robert Carlisle, my supervisor, for his patience and guidance throughout the course of my DPhil. I would also like to thank Dr Daniel Shenton for his contributions to my project.

I must also thank Dr Pramila Rijal for her advice and help with chapter 4 of this thesis. Without her, the influenza virus neutralisation studies would not have been possible.

A huge thank you goes to all of those in the BUBBL group who have made the past few years a sheer pleasure with frequent pub trips, coffee or, in my case, water breaks and aisle chats. Particular mention must be given to Erasmia Lyka, Alex Martin, and Catherine Paverd who made each day more entertaining and joyful.

I must also send my heartfelt thanks to my family: my parents, Tina and Keith, my sister Kirsty, my sister Natalie, her partner Andy, my new baby nephew Alfie, and my two dogs, Poppy and Molly. It is to them that I dedicate this thesis. Their unconditional love and support has helped me through the difficult times and has encouraged me to always give it my all.

Finally, words cannot explain how grateful I am to my better half, Rebecca Leigh, for her love, support, and patience. Her willingness to read and correct my thesis was unwavering. Having her with me along this journey has made every day easier and more enjoyable.

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Acronyms

5T4	A tumour-associated antigen
A(H1N1)	Influenza A, H1N1 virus
A(H3N2)	Influenza A, H3N2 virus
A(H5N1)	Influenza A, H5N1 virus
A549	Human lung carcinoma cell line
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADXS11-001	Recombinant live-attenuated bacterial cancer vaccine candidate expressing E7 protein from HPV virus
AF488	Alexa Fluor 488 fluorescence dye
AFP1	Alpha-Fetoprotein 1
ANOVA	Analysis of Variance
AP205	A tumour-associated antigen
APC	Allophycocyanin fluorescence dye
APC-Cy7	Tandem fluorochrome consisting of APC coupled with cyanine 7
B/Yamagata	One of the main lineages of influenza type B
B/Victoria	One of the main lineages of influenza type B
B16	Murine melanoma cell line
B16-F10	Strain of the B16 cell line
B16-M30	A tumour-associated antigen
B16-pIR-HH	B16 tumour cells transfected to express the human leukocyte antigen, HLA-A2/Kb, and epitopes of hepatitis B virus core antigen
B16F10EGFRvIII	B16-F10 cells stably expressing EGFRvIII
bFGF	Basic fibroblast growth factor
BALB/c	An inbred laboratory mouse strain
C57Bl/6	An inbred laboratory mouse strain
CD3e	Region of the cluster of differentiation 3 glycoprotein, which is a marker for T cells
CD4	Cluster of differentiation 4 glycoprotein, which is a marker for helper T cells
CD40	Cluster of differentiation 40 glycoprotein, which is a marker for antigen presenting cells
CD45.2	Isoform of the cluster of differentiation 45 glycoprotein, which is a marker for leukocytes

CD8	Cluster of differentiation 8 glycoprotein, which is a marker for cytotoxic T cells
CDC	Complement-Dependent Cytotoxicity
CpG	Short single-stranded DNA molecule which contain a cytosine followed by a guanine
CSP	Malaria circumsporozoite protein
DLS	Dynamic Light Scattering
DMPC	1,2 - dimyristoyl - sn - glycerol - 3 - phosphocholine
DMPG	1,2 - dimyristoyl - sn - glycerol - 3 - phospho - (1' - rac - glycerol)
DNA	Deoxyribonucleic acid
DSPE-PEG-mal	1,2 - distearoyl - sn - glycerol - 3 - phosphoethanolamine - N - [maleimide(polyethylene glycol) - 2000]
E	Free ErbB-2 _{266–296} peptide in PBS
E()	Empty liposomes with ErbB-2 _{266–296} peptide conjugated to the outer surface
E(O)	Liposomes encapsulating OVA _{323–339} peptide with ErbB-2 _{266–296} peptide conjugated to the outer surface
E6	Oncoprotein of the HPV virus
E7	Oncoprotein of the HPV virus
EGF	Epidermal growth factor
EGFRvIII	Epidermal growth factor receptor 1, variant 3
ELISA	Enzyme-linked immunosorbant assay
ErbB-2	Epidermal growth factor receptor 2
ErbB-2_{266–296}	Peptide of the ErbB-2 protein consisting of residues 266–296 with linker amino acids CGPSL added to the amino end
ErbB-3	Epidermal growth factor receptor 3
EUR	Euro
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
FVB/n	An inbred laboratory mouse strain
Globo-H	A tumour-associated carbohydrate antigen
GM-CSF	Granulocyte macrophage colony stimulating factor
GMT	Geometric mean titre
gp100	A tumour-associated antigen
GVAX	A whole-cell cancer vaccine candidate consisting of irradiated, genetically modified whole tumour cells
H1	Hemagglutinin glycoprotein, subtype 1, of influenza A viruses
H1N1	Influenza A virus with Hemagglutinin 1 and Neuraminidase 1 proteins
H3	Hemagglutinin glycoprotein, subtype 3, of influenza A viruses
H3N2	Influenza A virus with Hemagglutinin 3 and Neuraminidase

	2 proteins
H5	Hemagglutinin glycoprotein, subtype 5, of influenza A viruses
H5N1	Influenza A virus with Hemagglutinin 5 and Neuraminidase
	1 proteins
H5N2	Influenza A virus with Hemagglutinin 5 and Neuraminidase
	2 proteins
H7	Hemagglutinin glycoprotein, subtype 7, of influenza A viruses
H7N9	Influenza A virus with Hemagglutinin 7 and Neuraminidase
	9 proteins
H9	Hemagglutinin glycoprotein, subtype 9, of influenza A viruses
H9N2	Influenza A virus with Hemagglutinin 5 and Neuraminidase
	2 proteins
HA	Hemagglutinin, expressed on influenza viruses
HA₉₁₋₁₀₈	Peptide of the HA protein consisting of residues 91-108 with linker amino acids CGPSL added to the amino end
HA1	Globular region of the hemagglutinin protein, expressed on influenza viruses
HA2	Stem region of the hemagglutinin protein, expressed on influenza viruses
Her-2	Epidermal growth factor receptor 2
HI	Hemagglutination inhibition
Hib	Haemophilus Influenzae type B vaccine
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HPV	Human Papillomavirus
HRP	Horseradish Peroxidase
IgA	Immunoglobulin A
IgG1	Immunoglobulin G isotype 1
IgG2a	Immunoglobulin G isotype 2a
IgG2b	Immunoglobulin G isotype 2b
IgG2c	Immunoglobulin G isotype 2c
IgG3	Immunoglobulin G isotype 3
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-2	Interleukin-2
ISA-51	A water-in-oil emulsion
ISCOM	Immunostimulating complex
ISCOMATRIX	Improved form of an ISCOM
L1	Protein of the HPV virus
LCMV	Lymphocytic Choriomeningitis Virus
LL/2	Murine Lewis lung carcinoma cell line
M-001	Peptide-based influenza vaccine candidate consisting of nine conserved epitopes of multiple influenza strains
M1	Matrix 1 protein of the influenza virus

M2e	Extracellular domain of the matrix 2 protein of the influenza virus
MAGE-1	Melanoma-associated antigen 1
MAGE-3	Melanoma-associated antigen 3
MAMBO89	Cell line established from a mammary carcinoma of a human ErbB-2 transgenic FVB mouse
MC38	Murine colon adenocarcinoma cell line
Melan-A/MART-1	Melanoma antigen
MelQbG10	Conjugate cancer vaccine candidate consisting of Melan-A/MART-1 conjugated to a bacteriophage Q β VLP.
MenACWY	Meningococcal group A, C, W-135 and Y conjugate vaccine
MF59	A squalene-based immunogenic adjuvant
MHC	Major Histocompatibility Complex
MPLA	Monophosphoryl lipid A
mRNA	Messenger RNA
MTS	3 - (4,5 - dimethylthiazol - 2 - yl) - 5 - (3 - carboxymethoxyphenyl) - 2 - (4 - sulfophenyl) - 2H - tetrazolium
MUC-1	A tumour-associated antigen
N1	Neuraminidase glycoprotein, subtype 1, of influenza A viruses
N2	Neuraminidase glycoprotein, subtype 2, of influenza A viruses
N9	Neuraminidase glycoprotein, subtype 9, of influenza A viruses
NA	Neuraminidase, expressed on influenza viruses
NAI	Neuraminidase inhibition
Neu	Epidermal growth factor receptor 2
NHS	N-hydroxysuccinimide
NP	Nucleoprotein, of the influenza virus
NY-ESO-1	A tumour-associated antigen
OPT-822/821	Conjugate cancer vaccine candidate consisting of a Globo-H molecule conjugated to a keyhole limpet hemocyanin carrier protein
OVA	Ovalbumin
OVA₃₂₃₋₃₃₉	Peptide of the OVA protein consisting of residues 323-339
PAMP	Pathogen-associated molecular pattern
PB1	Component of influenza RNA polymerase
PBS	Phosphate-buffered saline
PC7A	A pH sensitive polymeric nanoparticle candidate
PCV	Pneumococcal conjugate vaccine
PE-Cy7	Tandem fluorochrome consisting of phycoerythrin coupled with cyanine 7
PEG	Polyethylene glycol
PerCp-Cy5.5	Tandem fluorochrome consisting of peridinin chlorophyll coupled with cyanine 5.5
PLA	Poly(lactic acid)
PLG	Poly(D,L-lactide-co-glycolide)

PLGA	Poly(D,L-lactic-coglycolic acid)
PLHMGA	Poly(D,L lactic-co-hydroxymethyl glycolic acid)
pM1	Plasmid encoding influenza M1 protein
pNP	Plasmid encoding influenza NP protein
pPB1	Plasmid encoding influenza PB1 protein
PPD	Purified Protein Derivative
PPD-MS	PPD in combination with mesoporous silica particles
PPD-MS/CaP	PPD in combination with both mesoporous silica particles and calcium phosphate particles
PSA	Prostate-specific antigen
PSA-MPyVLP	Murine polyomavirus like particles carrying the entire human PSA
QS-21	A purified plant extract that acts as an adjuvant
RNA	Ribonucleic acid
RTS,S/AS01	Recombinant protein-based malaria vaccine (Mosquirix)
rVV-M1	Recombinant vaccinia virus expressing influenza M1 protein
rVV-NP	Recombinant vaccinia virus expressing influenza NP protein
rVV-PB1	Recombinant vaccinia virus expressing influenza PB1 protein
SSEA3	A tumour-associated carbohydrate antigen
SSEA4	A tumour-associated carbohydrate antigen
TAA	Tumour-associated antigen
TACA	Tumour-associated carbohydrate antigen
TC-1	Murine transfected, immortalised, lung epithelial cell line expressing E6 and E7 oncoproteins from HPV virus
TLR	Toll-like receptor
TUBO	Murine mammary gland carcinoma cell line
UK	United Kingdom
USA	United States of America
USD	USA dollar
VLP	Virus Like Particle
VGX-3100	Therapeutic DNA-based cancer vaccine candidate
WHO	World Health Organisation

Chapter 1

Literature Review

1.1 Introduction

Vaccines are arguably the most important medical technology developed to date, and have provided dramatic reductions in disease morbidity and mortality since Edward Jenner first tested the principle in 1798 [1]. The World Health Organization (WHO) estimates that vaccinations for diphtheria, tetanus, whooping cough, and measles currently prevent 2 - 3 million deaths per year [2]. Smallpox was once one of the most feared diseases in the world until the implementation of global vaccination programmes led to it being declared eradicated in 1979 [3]. This eradication cost approximately 100 million USA dollars (USD), but is estimated to generate annual savings of 1.35 billion USD [4].

Cancer is among the leading causes of morbidity and mortality today. By 2030 the number of new cancer diagnoses is predicted to be 70% higher than that of 2010 [5]. WHO reported that in 2012 there were approximately 14.1 million new cancer diagnoses and 8.2 million cancer-related deaths worldwide [6]. An updated report states that in 2018 the number of new cancer diagnoses rose to 18.1 million, while the number of worldwide cancer deaths rose to 9.6 million [7]. In the UK, it is currently estimated that over half of the people born since 1960 will be diagnosed with some form of cancer in their lifetime [8]. In 2012, lung, breast, colorectal and stomach cancers accounted for over 40% of all reported cancers. With a few notable exceptions (Recombivax HB, Gardasil, and Provenge), effective vaccines for cancer have, for the most part,

not been provided by standard vaccination technologies [9–11].

Influenza is a common pathogen that annually infects 5 - 10% of adults and 20 - 30% of children worldwide [12]. There are four types of influenza virus (A, B, C, and D), three of which have been known to infect humans (A, B, and C) [13]. The most prevalent types of influenza virus are the A and B subtypes which cause annual seasonal epidemics and thus are the main targets for influenza vaccines [14]. While the majority of infected people experience mild illness, seasonal influenza epidemics, globally, lead to about 3 - 5 million severe illnesses and 290,000 - 650,000 deaths every year [14]. Despite influenza vaccines being available since the 1940s they have had fleeting, restricted success due to the high mutation rate of the flu genome which leads to antigenic drift and shift of the viral proteins commonly targeted by the vaccines [15]. Influenza A viruses are classified according to which of the 18 hemagglutinin (HA) subtypes (H1-18) and 9 neuraminidase (NA) subtypes (N1-N9) they contain, while influenza B viruses can be broken down into lineages, with the main ones being B/Yamagata and B/Victoria [14]. Systems are in place to predict forthcoming strains of seasonal influenza, however, due to extended production and distribution timelines there are often mismatches between the predicted and actual circulating strains. In 2014/15, due to the emergence of drifted H3 strains, seasonal influenza vaccines were only 19% effective in the USA [16, 17]. While in 2010/11, when circulating influenza strains closely matched predicted strains, seasonal vaccines were found to be 60% effective [17, 18].

Although vaccines have been undeniably successful, improvements in vector production, delivery and ease of use would be of great benefit. Historically, vaccine development has been based on the “three Is” paradigm of Louis Pasteur (isolate, inactivate, inject) [19]. However, an improved understanding of immunology, pathology and microbiology is now helping vaccine develop-

ment adopt a more ‘rational design’ approach [19, 20]. A large portion of these rationally designed vaccines consist of a ‘minimalist’ composition (i.e. they are subunit or peptide-based), and whilst this provides safety and cost-of-production benefits, they are typically less immunogenic [21]. However, it is hoped that the optimisation and combination of rational design approaches and the use of novel dosing and adjuvanting strategies can help close this efficacy gap. This literature review will delve into these approaches and strategies and describe how they are being used to help develop improved vaccines by building on a review paper by the author, with particular focus on cancer and influenza [22].

1.2 Vaccine Designs

1.2.1 Virus-like particles

Virus-like particles (VLPs) are highly ordered, repetitive structures that contain a high density of viral capsid proteins. This high density of capsid proteins provides copious amounts of conformational viral epitopes, capable of eliciting strong immune responses [23]. Crucially, VLPs are formed by the self-assembly of viral capsid proteins in the absence of any of the infectious nucleic acids from the virus. Thus, they are a potentially safer alternative to the attenuated viruses commonly used for vaccination due to their absolute inability to replicate. VLPs have been shown to be capable of generating strong immune responses, even in the absence of an adjuvant [24].

Being simpler in composition, VLPs also allow faster production of vaccine than traditional methods, which is especially useful for treatment of highly mutating pathogens such as influenza. Traditional production of an influenza vaccine takes 9 months after a new annual strain has been sequenced, but

VLP production takes only 3-12 weeks [25, 26]. The first VLP vaccine to be brought to market was the vaccine for hepatitis B (Recombivax HB), in 1986, which consists of self-assembled particles made from the virus' capsid protein, hepatitis B surface antigen [9]. Since then, VLP vaccines for human papillomavirus (HPV) (Gardasil) and hepatitis E (Hecolin) have also made it to market in 2006 and 2011, respectively [10, 27], with many more undergoing evaluation in clinical trials [28].

Both hepatitis B and HPV are oncoviruses with VLP-based vaccines clinically available. Hepatitis B has been associated with 55% of liver cancers, while HPV has been associated with almost 100% of cervical cancers worldwide [29]. The introduction of vaccines for these diseases has reduced the incidence rates of their respective cancers [30, 31]. The hepatitis B and HPV vaccines are preventative cancer vaccines; attempts have also been made in developing therapeutic VLP-based cancer vaccines [32]. Studies have shown VLPs are capable of overcoming the immunosuppressive state of the tumour microenvironment by decreasing the number of immunosuppressive leukocytes [33, 34].

Zhang, et al. inserted plasmids encoding chimeric proteins consisting of the hepatitis B virus core antigen fused to one of MAGE-1, MAGE-3 or AFP1 cancer epitopes into *E. coli* [35]. These chimeric proteins then self-assembled into VLPs displaying cancer epitopes, which were subsequently used to pulse dendritic cells. Vaccination of mice with these pulsed dendritic cells significantly increased the survival rate of mice subsequently challenged with B16-pIR-HH tumour cells [35].

The current HPV vaccines act as prophylactic cervical cancer vaccines, and there is yet to be a commercialised therapeutic vaccine for cervical cancer. Two popular target oncoproteins for established HPV-associated cervical cancers

are E6 and E7 [36]. One study used chimeric proteins consisting of HPV-16 L1 protein and epitopes of the E6 and E7 proteins that self-assembled into VLPs [37]. They demonstrated the ability of the chimeric VLPs to act as both a prophylactic and therapeutic vaccine by inducing the inhibition of TC-1 mouse tumour cells, which express E6 and E7, reducing tumour growth by 85% when given as a prophylactic vaccine and 57% when given as a therapeutic vaccine [37].

Prostate-specific antigen (PSA) is a commonly-used biomarker for the detection of prostate cancer due to its high overexpression in these cancerous cells [38]. Eriksson et al. formulated a murine polyomavirus VLP incorporating the entire human PSA (PSA-MPyVLPs) [39]. Immunisation with PSA-MPyVLPs loaded onto dendritic cells, with CpG present as an adjuvant, significantly increased the proportion of tumour-free mice (15% compared to 40-100%) [39]. Upon vaccination, increased CD4+ and CD8+ immune responses were observed, however no humoral antibody responses were detected. This was hypothesised to be due to PSA proteins being encapsulated inside of the VLPs and thus being shielded from B cell receptor binding and activation [39].

There is great concern about avian influenza A H5N1 viruses due to their potential to develop into a pandemic with a high mortality rate (60%) [40–42]. Smith, et al. developed a recombinant VLP-based vaccine with viral H5, N1 and Matrix 1 (M1) proteins that was able to protect ferrets from lethal challenge with the A/Indonesia/05/2005 (H5N1) virus [40]. The vaccine also reduced the shedding of infectious virus by 99.9% when compared to control groups vaccinated with PBS or control VLPs presenting viral H3, N2 and M1 [40].

The development of plant virus-derived transient expression systems has significantly improved the production speed and yield of VLPs [43, 44]. In-

influenza VLP vaccines formulated by this method would significantly reduce the long production time for current influenza vaccines which was an important factor in the 2009 A(H1N1) pandemic [45]. Medicago currently has a plant virus-derived VLP for seasonal influenza in a phase III clinical trial (NCT03301051: clinicaltrials.gov) and another with which they have completed a phase II trial for pandemic influenza (NCT01991561: clinicaltrials.gov) [46]. The seasonal vaccine is a quadrivalent vaccine that expresses HA from circulating H1 and H3 influenza A strains and the two circulating influenza B strains on *Nicotiana benthamiana* derived VLPs [47]. While the pandemic influenza vaccine is a similar VLP but with the target antigen being HA from A/Indonesia/05/2005 H5N1 virus [41]. Both of these formulations led to high hemagglutination inhibition (HI) titres, meeting the requirements for European licensure [41, 47]. The HI titres for most of the seasonal influenza vaccine strains were maintained for at least 6 months post-vaccination [47]. The pandemic vaccine was also able to induce a sustained HA-specific CD4+ T cell response up to 6 months post-vaccination [47]. These VLPs can be formulated within 19 days after circulating strains have been sequenced [48].

1.2.2 Conjugate vaccines

Vaccines that use either live-attenuated or inactivated pathogens contain a wide array of different antigens, both polysaccharide- and protein-based. However, only a small number of these may be required to induce protective immunity [49, 50]. This logic has been further extended for proteins by the realisation that each protein contains hundreds of possible immunogenic epitopes, not all of which are necessary for generation of a robust immune response. This has led to interest in peptide-based vaccines [49]. However, antigenic epitopes on a protein are not simply a sequence of amino acids, as the pep-

tides used must mimic the conformation of the immunogenic epitope within the native protein. Computational modelling has provided a powerful tool for locating and mapping the conformation of immunogenic epitopes within proteins [51, 52].

Peptide- or polysaccharide-based vaccines tend to be less immunogenic than when they are present on the surface of a pathogen, and thus they require the inclusion of an adjuvant when being administered [21, 53]. Another possibility is to conjugate the antigen to a second “helper” protein or polysaccharide that is known to increase immunogenicity; however, this can lead to the immune response being redirected towards the helper molecule [50, 54, 55]. Careful pairing and orientation of target and helper portions of the vaccine or spatial segregation of the two sub-units by use of carrier systems such as liposomes are approaches to overcome this issue (figure 1.1) [56, 57]. Peptides and polysaccharides are relatively cheap and simple to manufacture synthetically, which also removes the risk of contamination with infectious material as is possible with traditional live-attenuated or inactivated vaccines. Conjugate vaccines for haemophilus influenzae type b (Hib), pneumococcus (PCV), meningococcus (MenACWY) and malaria (Mosquirix) have been approved for use in humans. The RTS,S/AS01 (Mosquirix) vaccine, in particular, is a conjugate vaccine of a repeated region of the circumsporozoite protein from *Plasmodium* sporozoites conjugated to the hepatitis B surface antigen. This conjugate vaccine subsequently assembles into a VLP.

One promising conjugate cancer vaccine (MelQbG10) consists of an epitope of the melanoma antigen, Melan-A/MART-1, covalently conjugated to a bacteriophage Q β VLP [58, 59]. It has completed two phase II trials which recruited 21 patients with stage III-IV melanoma and 6 HLA-A*0201-positive patients with malignant stage I-II melanoma [58, 59]. Melan-A/MART-1 spe-

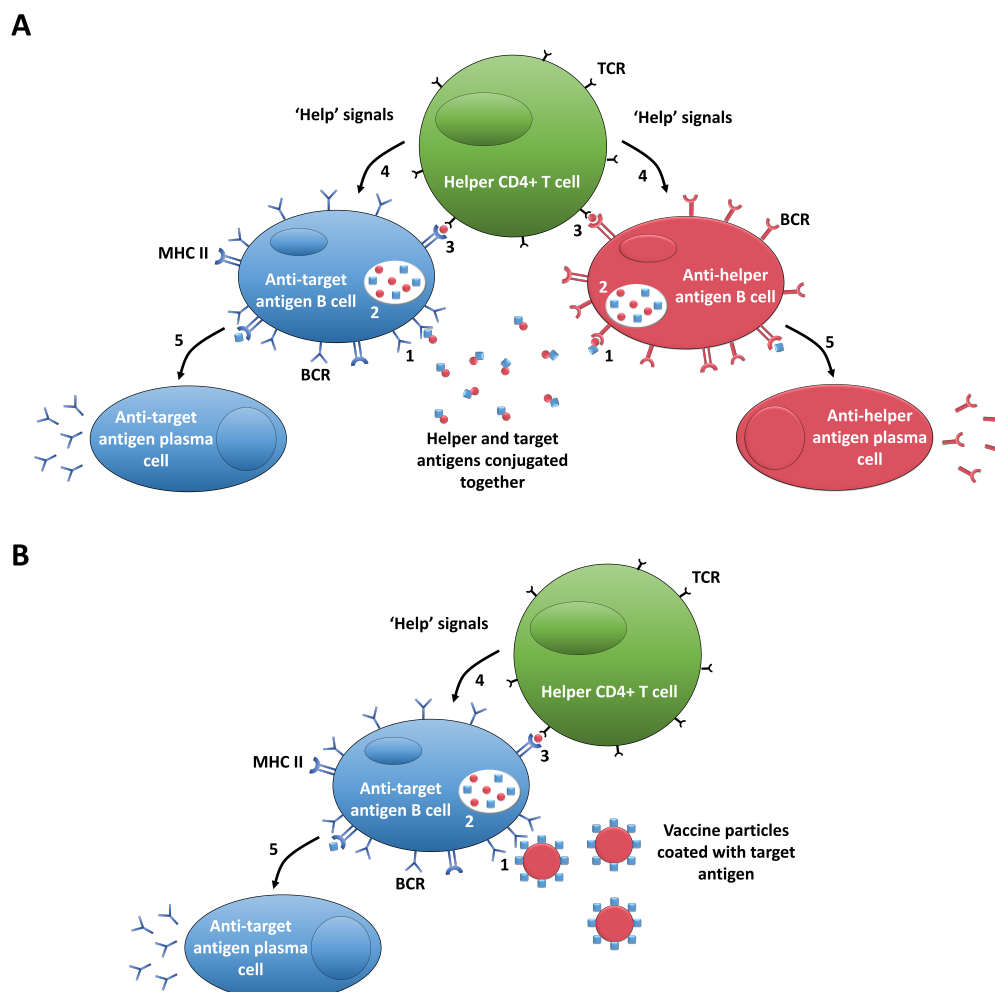


Figure 1.1: Mechanism of action for initiation of humoral immune responses to a target antigen aided by a secondary helper antigen when they are conjugated together (Panel A) and spatially segregated by use of a liposome (Panel B). (1) B cell with specific B cell receptor (BCR) for target/helper antigen binds to vaccine particle; (2) B cells engulf and digest vaccine particles; (3) vaccine particle antigens are presented on B cells class II major histocompatibility complexes (MHC II) for helper T cell recognition with specific T cell receptor (TCR) for presented antigen; (4) upon TCR binding, T cells produce 'help' signals to stimulate B cell differentiation; (5) B cells differentiate into plasma cells that secrete antibodies exclusively targeting antigen recognised by the BCR in step 1.

cific T cell responses were detected *ex vivo* in 76% of patients [58]. While strong VLP-specific antibody and CD4+ T cell responses were also detected [59].

ErbB-2 is overexpressed in 15-30% of all breast cancers as well as some ovarian, renal, colon and lung cancers. Palladini, et al. used a SpyTag/SpyCatcher

coupling to conjugate the extracellular region of ErbB-2 to an *Acinetobacter phage*, AP205 based VLP [60, 61]. Prophylactic vaccination with this construct reduced spontaneous development of mammary carcinomas by 50-100% in human ErbB-2 transgenic mice [61]. Growth of ErbB-2 overexpressing mammary carcinoma cells, MAMBO89, was also inhibited in wild-type mice [61].

Trastuzumab and Pertuzumab are two clinically-approved chemotherapy monoclonal antibodies used to treat ErbB-2 overexpressing cancers [62]. A phase I trial using a combination of two peptides, mimicking the Trastuzumab- and Pertuzumab-binding regions of ErbB-2, each conjugated to independent measles virus fusion protein helper epitopes has recently completed its first stages [63–66]. Each peptide was individually optimised pre-clinically and both were shown to generate strong humoral responses [63, 64]. The objective of the first stage of the trial was to establish the safety profile and discover any dose-limiting toxicities of the vaccine. As such it was open to all patients with metastatic, incurable solid tumour malignancies, regardless of ErbB-2 expression [66]. The second stage of the trial, which is currently ongoing, uses the optimised vaccine dose to vaccinate patients with ErbB-2 overexpressing tumours (NCT01376505: clinicaltrials.gov). The first stage of the trial found the vaccine elicited sustained ErbB-2 specific humoral responses in a majority of the patients across the different dose levels [66]. Of the 35 evaluable patients, 19 had progressive disease, 14 had stable disease, and 2 showed partial responses, though this limited response may be due to a proportion of enrolled patients having non-ErbB-2 overexpressing tumours [66]. One of the patients with a partial response continued to receive 6 month booster vaccines for 3.5 years [66].

Globo-H is a tumour-associated carbohydrate antigen (TACA) that is only detectable on cancer cells and not on non-cancerous cells [67]. At least 16

different epithelial cancers have been found to be Globo-H+, including 60% of breast cancers and 38% of non-small cell lung cancers [68, 69]. A Globo-H molecule conjugated to a keyhole limpet hemocyanin carrier protein (OPT-822/821) has been developed and has completed a randomised phase II/III trial in metastatic breast cancer patients [70]. On the whole, the vaccine did not improve the progression-free survival, however in those patients that did develop an immune response to the vaccine, progression-free survival and overall survival rate was significantly improved [70]. Inclusion of two additional TACAs (SSEA3 and SSEA4) into the vaccine is also being investigated [67]. These glycans are exclusively present on tumour cells, with over 98% of breast cancer patients being positive for at least one of these TACAs [67]. Therefore, inclusion of these glycans into the vaccine would remove the need to screen the patient’s tumour first.

Zhao, et al. developed a tetra-branched multiple antigenic peptide using the 24 amino acid extracellular domain of the Matrix 2 protein (M2e) from an influenza A/H5N1 virus [71]. The construct was able to induce strong M2e-specific IgG responses regardless of the adjuvant used and significantly reduced tissue damage within the lungs of mice challenged with two H5N1 strains (A/VN/1194 and A/SZ/406H) [71]. Vaccinated mice were also protected from challenge with 10x the 50% lethal dose of these H5N1 viruses, also showing significantly less weight loss upon challenge [71].

Biondavax currently have a peptide-based ‘universal influenza vaccine’, M-001, in a phase III trial (NCT03450915: clinicaltrials.gov). This vaccine is a single polypeptide expressed in *E.coli* consisting of three repetitions of nine linear epitopes (table 1.1) [72]. The peptides used are highly conserved epitopes from the HA, nucleoprotein (NP), and M1 proteins that are known to induce both humoral and cellular immunity (table 1.1). In a phase II trial, M-001

Protein	Residues	Epitope type	Conserved on
HA	354-372	B cell	Influenza B
HA	91-108	B cell	Influenza A H3
M1	2-12	B cell & CD8+ T cell	Influenza A
HA	150-159	B cell	Influenza A H1
HA	143-149	B cell	Influenza A H3
NP	206-229	CD4+ T cell	Influenza A
HA	307-319	CD4+ T cell	Influenza A H3
NP	335-350	CD8+ T cell	Influenza A
NP	380-393	CD8+ T cell	Influenza A

Table 1.1: List of conserved peptides used in the Multimeric-001 (M-001) influenza vaccine by Biondvax, currently in phase III trial. HA: Hemagglutinin, NP: Nucleoprotein, M1: Matrix 1

was used to prime elderly participants (≥ 65) before being given a commercial trivalent influenza vaccine to see if responses to the trivalent vaccine could be improved [73]. Priming with M-001 led to increased seroconversion towards all three strains used in the trivalent influenza vaccine compared to priming with a placebo [73]. Further, serum taken from participants when this study was conducted in 2011 was later tested against the A/Switzerland/9715293/2013 (H3N2-like) strain that caused the 2014/15 influenza epidemic [74]. Serum from participants primed with M-001 had improved seroprotection and seroconversion against this strain which was not known to circulate in 2011/12 [74].

1.2.3 Nucleic acid vaccines

Long-term gene expression from plasmid DNA has been demonstrated to be achieved by intramuscular injection in mice [75]. Such plasmids can be used to encode a viral antigen, and can thereby generate both humoral and cellular antigen-specific immune responses [76]. These studies led to a huge amount of research into DNA-based vaccines against a multitude of diseases such as influenza, human immunodeficiency virus (HIV) and lymphocytic choriomeningitis virus (LCMV) [77–79]. Practically, DNA vaccines are more cost-effective than protein, whole cell or viral vectors, as DNA can be synthesised by simple scalable chemistry or produced at scale in bacteria. However, the main drawback of DNA vaccines is their low immunogenicity due to the very low transfection rate they achieve. Non-condensed plasmid is a highly distended and negatively charged structure, which is prone to degradation in the extracellular compartment and lacks a mechanism to achieve cell entry. Even if cell entry can be achieved, localisation to and entry into the nucleus, steps which are required to achieve transcription, are extremely inefficient [80]. To increase immunogenicity, plasmids can be designed to encode multiple antigens as well as other immunostimulatory molecules to induce an adjuvanted immune response [81]. While no DNA vaccine has yet been licensed for use in humans, there have been several licensed for veterinary use, including West Nile (West Nile Innovator DNA) and salmon pancreas disease (Clynav) [82, 83].

Due to the low immunogenicity of both DNA vaccines and tumour-associated antigens (TAA), production of a therapeutic DNA-based cancer vaccine has been very limited [84]. The most successful DNA-based cancer vaccines have been those that target TAAs with a viral origin e.g. E6 and E7 from HPV infection [84]. One such vaccine, currently in phase III trials (NCT03721978 and

NCT03185013: clinicaltrials.gov) is VGX-3100, a DNA-based vaccine targeting the HPV 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3 [85]. It is injected intramuscularly followed by electroporation to aid the uptake of the DNA plasmid into the cells [85, 86]. A randomised, double-blind, phase IIb trial found that 49.5% of patients had regression of the virus-caused lesions compared to 30.6% in the placebo group [85].

Despite the ability of influenza DNA-based vaccines to induce protective immunity in small animals [87, 88], they have yet to consistently demonstrate protection in larger animals or in a clinical setting when administered alone [89]. Subsequently, a method of using a prime-boost approach, with the DNA vaccine as the prime and a conventional influenza vaccine as the boost, has been applied in an attempt to improve the efficacy of the conventional vaccine [90, 91]. However, this again has provided inconsistent results in the clinical setting [92–94].

The limitations of DNA vectors has resulted in mRNA-based vaccines gaining momentum in recent years [95]. Like DNA-based vaccines, they are low-cost and can be manufactured rapidly on a large scale. However, their application has previously been restricted by the instability of RNA and inefficient *in vivo* delivery [95]. Several methods of structural modification have been employed to increase the intracellular stability of the RNA molecules [96]. Crucially, in contrast to DNA, RNA does not require targeting to and entry into the nucleus, and so the main barrier RNA vaccines face is cell entry [97]. This can be addressed by formulation with poly-cationic carrier molecules that can condense and protect the RNA and aid its rapid cellular uptake [98].

The main disease focus of RNA-based vaccine development has been cancer, with numerous phase I-III clinical trials in progress [95, 99]. For infectious pathogens, two major types of RNA vaccine have been utilised: non-replicating

and self-amplifying. Non-replicating RNA vaccines are simpler and less expensive to manufacture, but may be limited in the duration and level of expression they can achieve. Self-amplifying RNA systems can be based on sequences and principles borrowed from ‘positive’ single-strand RNA viruses, such as alphaviruses (Alphavax). These vectors encode the non-structural genes and the immunogen, but no structural genes, so, in theory, can achieve a single replication cycle without the risk of infectious virus production. They, therefore, enable a small dose of vaccine to rapidly produce a large amount of antigen due to intracellular amplification of the antigen-encoding RNA. Several clinical trials using RNA-based vaccination have been undertaken for infectious pathogens such as HIV, rabies, and Zika [100–102]. Whilst RNA may currently seem like the more attractive of the nucleotide-based options, it should be noted that DNA potentially provides advantages in terms of coding capacity and the level and duration of immunogenic protein production. Should the delivery barrier faced by DNA be overcome, a resurgence in the interest in its use may follow. The recent development of scalable, cell-free, enzyme-driven DNA production technologies, strengthens the case for the translatability of DNA vaccines [103]

Weide et al. performed two phase I/II trials to treat stage III-IV metastatic melanoma, the first of which extracted RNA from the patient’s own metastasised tumour before amplifying it and injecting it back into the patient in combination with granulocyte macrophage colony stimulating factor (GM-CSF) as an adjuvant [104]. They found the treatment could induce anti-tumour humoral immune response in 9/13 patients. While no statistically significant clinical effectiveness was shown, they hypothesised this may be due to low sample size and thus should be looked at in future trials [104]. A subsequent trial used protamine-protected mRNA encoding six TAAs (Melan-A, Tyrosinase,

gp100, MAGE-A1, MAGE-A3, and Survivin) [105]. All patients were injected with mRNA adjuvanted with GM-CSF, and half of them also received keyhole limpet hemocyanin as an extra adjuvant. A significant decrease in the number of regulatory T cells was seen in the patients receiving the extra keyhole limpet hemocyanin adjuvant, while a significant decrease in myeloid suppressor cells was seen in the those that did not [105]. Of the 21 enrolled patients, 7 had measurable disease, only one of which showed a partial response [105].

Vogel, et al. compared non-replicating and self-amplifying RNA vaccines for influenza [106]. RNA-encoding HA from A/Puerto Rico/8/1934 (H1N1) was used to find the minimum dose needed to induce protection upon challenge with a lethal dose of the virus. Mice were immunised intramuscularly with the RNA constructs mixed in RNase-free PBS. The self-amplifying RNA vaccine could induce complete protection from lethal challenge with a 64-fold lower dose of 1.25 μ g compared to 80 μ g of the non-replicating vaccine [106]. Next, self-amplifying RNA vaccines encoding HA from the three strains of influenza used in the trivalent vaccine, A/H1N1, A/H3N2, and B/Yamagata, were shown to provide protection from viral challenge with mice showing less than 5% weight loss compared to unvaccinated mice which lost 10-15% of their weight 6 days after challenge [106].

1.2.4 Cellular vaccines

Due to the history of success of vaccination using live-attenuated viruses, inactivated viruses or bacteria, attempts have been made to apply a similar approach to vaccinate against cancer. Attenuated tumour cells have been administered to induce an immune response against specific types of cancer. Two types of whole cell vaccines have been used: autologous and allogenic. Autologous cell vaccines have been tested on a variety of cancers including lung, col-

orectal, melanoma, renal and prostate cancer [107–111]. However, autologous cell vaccines are limited to only certain types and stages of tumours as they require sufficient amount of the patient’s tumour for preparation. In contrast, allogenic cell vaccines typically contain a combination of established human tumour cell lines and so, while not being patient-specific, they do not have the production limitations of autologous cell vaccines. Many whole cell vaccines have been genetically modified to increase immune stimulation by inducing expression of cytokines, chemokines and co-stimulatory molecules [110, 112, 113]. To date, their clinical impact has been demonstrated by GVAX, a vaccine for the treatment of pancreatic cancer, currently in phase II clinical trials [114].

Another cellular-based vaccination strategy exploits a patient’s own immune cells, specifically their dendritic cells. Dendritic cell vaccines can be formulated by loading a patient’s autologous dendritic cells, that are simultaneously treated with immune-adjuvants, with either tumour-associated antigens or nucleic material encoding for tumour-associated antigens *ex vivo*. The newly-matured, antigen-loaded dendritic cells are then administered back to the patient to induce anti-tumour immunity. Dendritic cell vaccines have been tested against prostate, melanoma, renal and glioma tumours in clinical trials [115–118]. The first therapeutic cancer vaccine to receive FDA approval was a dendritic cell-based vaccine for prostate cancer, Sipuleucel-T (Provenge) [11]. This was on the basis of a phase III trial in 2010 which found that patients receiving Sipuleucel-T had a 4.1 month median improvement in overall survival [119, 120]. This approval represented a great success for this approach, but the development and approval rate of similar strategies has since been unremarkable. This may reflect factors relating to intellectual property, or may be a consequence of the expense of the treatment (93,000 USD for three infusions of the treatment). This vaccine regimen requires the isolation of peripheral

blood mononuclear cells from the patient, followed by cell culture processing and re-infusion, all of which makes for a costly and laborious process. Indeed, while these cell-based approaches are an interesting development, they do not obviously contribute to the continuum of the journey away from live and attenuated vaccines, into an era of vaccines with lower complexity and cost of production that are more suited to the treatment of large populations within the confines of reducing healthcare resources.

Immunocompromised patients, such as cancer patients with lymphoma or leukaemia or those treated with chemotherapy, have very limited responses to prophylactic vaccinations, such as influenza vaccinations. To address this issue, Kounduri, et al. pulsed dendritic cells from human cord blood with recombinant HA which were able to generate *de novo* T cell responses specific for recombinant HA [121]. Intraperitoneal injection with pulsed dendritic cells from mouse splenocytes led to high titres of anti-HA antibodies [121]. All mice which generated HA-specific titres survived viral challenge [121].

1.3 Vaccine Delivery Platforms

1.3.1 Liposomes

Liposomes are spherical vesicles with a lipid bilayer formed of biocompatible phospholipids. Their main use in vaccinology is either as a delivery vehicle or as an adjuvant [122, 123]. A key advantage of liposomes is their plasticity and versatility; the choice of lipids and their formulation method allows control over their charge, size and location of antigen incorporation [124]. Liposomes are often made up of 4 key components: a charged lipid, which affects how liposomes behave *in vivo*; a lipid-linked polyethylene glycol (PEG), to increase *in vivo* stability; cholesterol, to increase structural stability; and a phospho-

lipid, which supports the formation of a lipid bilayer. Some cationic liposomes, unlike anionic liposomes, are able to bypass the endosomal-lysosomal route of degradation in cells [125]. Furthermore, their net positive charge provides a means of condensing nucleic acid constructs (DNA or RNA) into discrete structures capable of achieving charge-mediated entry into target cells [125].

Antigens can be encapsulated within, conjugated to the surface of, or embedded within the lipid bilayer of liposomes [57, 126]. The location of an antigen in or on liposomes influences the type of immune response generated towards the vaccine. T cell responses are induced by both encapsulated and surface-conjugated antigens, while B cell responses are exclusively induced by surface-conjugated antigens [122]. Incorporation of CD4 T cell helper epitopes can aid in generating a stronger antibody response to a B cell target antigen [57, 127], and complete spatial segregation of the two antigens by the liposomal bilayer minimises the degree to which the immune response is focused on the T cell epitope (figure 1.1) [57]. The carrying capacity of liposomes allows immunostimulatory molecules, such as cytokines or toll-like receptor agonists, to be co-delivered to target immune cells, thereby reducing systemic exposure to these adjuvant molecules [57, 122]. Liposomes were first used as part of a vaccine for diphtheria toxin in 1974 [128]. Since then, liposome-based vaccines for hepatitis A (Epaxal) and influenza (Inflexal V) have been approved for use in humans [129, 130].

Inflexal V was introduced to market in 1997 and consisted of a mixture of three liposomal constructs, each containing the HA and NA from the three circulating strains, as recommended by WHO [130, 131]. When compared to conventional influenza vaccines, it was estimated that Inflexal V would save approximately 15 euro (EUR) per patient [130]. Several studies compared the efficacy of Inflexal V to competitor vaccines [130]. While the majority of stud-

ies found the different vaccines comparable, one study found that Inflexal V, compared to a split influenza vaccine (Fluarix), significantly improved seroconversion (88.8% vs 77.5%) and seroprotection (88.8% vs 78.3%) in children aged 6 - 71 months old [132]. However, production of Inflexal V was discontinued shortly after the originating company, Crucell, was taken over by Johnson & Johnson. A 2013 press release explained that this discontinuation was due to an ongoing evaluation of their global business priorities and product portfolio [133].

Tecemotide, formally known as Stimuvax or BLP25, is a liposomal cancer vaccine that targets the TAA, MUC-1, which is overexpressed on several cancers including breast, prostate, colorectal, and non-small-cell lung cancer [134]. Tecemotide is comprised of lipopeptide antigens encapsulated with monophosphoryl lipid A (MPLA) within multilamellar liposomes [135]. In a phase II trial, it was found that Tecemotide plus best supportive care was able to increase the median life expectancy of patients with stage IIIB locoregional non-small-cell lung cancer from 13.3 to 30.6 months compared to patients who did not receive Tecemotide [136, 137]. However, when taken to phase III trials the vaccine did not meet its primary or secondary end points and thus was terminated [138]. In 2014, Merck, the company owning Tecemotide, announced that it would discontinue worldwide clinical trials associated with the vaccine [138].

Neelapu, et al. used liposomes to encapsulate autologous idiotypes of immunoglobulins from patients with advanced stage follicular lymphoma in a phase I trial [139]. Patients chosen for the study had previously entered chemotherapy-induced clinical remission. The vaccine, which also included encapsulated IL-2 as an adjuvant, was able to generate both CD4+ and CD8+ T cell responses for at least 18 months post-vaccination [139]. After a median

follow-up of 50 months 6/10 patients remained in continuous first complete remission [139].

Basic fibroblast growth factor (bFGF) is an angiogenic stimulator that aids tumour growth and development [140]. Zhong, et al. encapsulated an immunogenic peptide of bFGF inside of a liposome containing MPLA as an adjuvant [141]. Murine total IgG levels were found to be significantly higher in the treatment group compared to control groups [141]. Treatment with the full construct led to inhibition of lung metastasis following challenge with mouse Lewis lung carcinoma, LL/2 cells, with a significant difference in the total lung weight three weeks post tumour challenge [141].

1.3.2 Polymeric particles

Polymeric particles have been increasingly researched in the field of vaccine delivery due to their potentially advantageous biocompatibility and biodegradability [142]. A wide range of both natural and synthetic polymers have been used to make particles for vaccine delivery, such as polysaccharides [143], poly(D,L-lactic-co-glycolic acid) (PLGA) [144], poly(lactic acid) (PLA) [144], and poly(D,L-lactide-co-glycolide) (PLG) [145]. These particles are able to either entrap or adsorb antigen for delivery to specific cells or allow for sustained antigen release over time because of their slow biodegradation rate [146, 147]. Polymeric particles are the main focus for development of a single-dose delayed-release vaccine that could replace the need for booster doses of many current vaccines [147]. The antigen release profile of a PLGA particle can be modified from a couple of days to over a year [148, 149]. Many studies have also used polymeric particles for their ability to act as an adjuvant, rather than their antigen release profile. Advax, an insulin-derived microparticle, has been used in clinical trials as an adjuvant for hepatitis B, influenza, and insect-sting

allergy vaccines [150–152].

Gordon, et al. conducted a phase I trial to compare the immunogenicity and tolerability of Advax mixed with a reduced (1/10th) dose of a trivalent influenza vaccine compared to the non-adjuvanted full dose of trivalent influenza vaccine [151]. Both treatments showed equivalent immunogenicity as measured by HI responses, and furthermore both groups were well tolerated with no safety issues being identified [151]. Most influenza vaccines are not used with adjuvants, however, successful adjuvantation has the potential to be dose-sparing and thus would easily provide an increased number of doses in the event of a pandemic.

Rahimian, et al. loaded synthetic long peptides containing CD8+ and CD4+ epitopes of the HPV16 E7 protein into poly(D,L lactic-co-hydroxymethyl glycolic acid) (PLHMGA) particles, both with and without a TLR3 agonist (poly IC) [153]. The peptide-loaded particles were capable of inhibiting tumour progression and generating high levels of HPV-specific CD8+ T cells in the presence of poly IC adjuvant, regardless of whether it was loaded onto the particles or free in solution [153]. The polymeric particle had similar adjuvanting efficacy when compared to incomplete Freund's adjuvant. The polymeric particles were reported to be free of adverse effects, unlike the incomplete Freund's adjuvant which has a high rate of adverse effects associated with it at the site of injection [153].

Luo, et al. developed a library of ultra pH sensitive polymeric nanoparticles that could escape endosomal degradation to release loaded antigen into the cytosol [154]. The size of the polymeric nanoparticles aids in targeting their delivery to lymph nodes [154]. One of the nanoparticles in their library, PC7A, achieved efficient cytosolic delivery of antigen to antigen-presenting cells in draining lymph nodes which led to both surface presentation of antigen and

activation of type 1 interferon genes [154]. Four different tumour models were used to test the ability of these particles to inhibit tumour growth. First, the particles were loaded with a model antigen, OVA, then mice were vaccinated with either the OVA-loaded particles, free OVA peptide or OVA peptide in combination with one of three adjuvants. Forty days post-challenge with OVA expressing melanoma cells, 50% of mice vaccinated with the OVA-loaded particles were still alive compared to 0% of all other mice [154]. Next, particles were loaded with cocktails of TAA peptides and challenged with either B16-F10 melanoma cells or MC38 colon adenocarcinoma cells, in both cases the loading of peptide into the PC7A particles was essential for tumour growth inhibition [154]. Finally, they compared the efficacy of PC7A particles loaded with E7 peptide to that of an anti-PD-1 antibody and the combination of the two. Mice were challenged with TC-1, lung carcinoma cells, and after 30 days all mice receiving only anti-PD-1 had died due to tumour burden while 100% of those receiving the particles both with and without anti-PD-1 were still alive. After 60 days, 60% of mice treated in the absence of anti-PD-1 were still alive, while 100% of those that were co-treated with anti-PD-1 were still alive with 9/10 mice tumour free [154]. Rechallenge of these tumour-free mice, 82 days post-first tumour challenge, led to complete tumour inhibition suggesting generation of long-term anti-tumour memory [154].

1.3.3 Inorganic particles

Many inorganic particle-based vaccines have been studied, despite their low biodegradability. Their main advantage lies in how much control can be achieved over their synthesis [155, 156]. Inorganic particles have been used as both adjuvants and as antigen delivery vehicles in order to enhance an immune response [157]. The four most commonly used types of inorganic par-

ticles deployed in vaccines are gold, aluminium, calcium phosphate, and silica. Structures made from pure carbon have also been investigated.

Gold particles are highly stable, and can be easily synthesised in a variety of different shapes and sizes [155]. Their surface is highly modifiable, making antigen conjugation straightforward in practice [158, 159]. However, there is often limited control over the orientation of antigen, which can be suboptimal [160]. Gold particles have been used as carriers in several clinical trials for a range of diseases, including melanoma, influenza and hepatitis B [161–163].

Ginsberg, et al. performed a clinical trial to compare the immunogenicity of a gold nanoparticles coated with a DNA-based vaccine encoding mouse gp100 when delivered by either intramuscular injection or particle-mediated epidermal delivery in patients with stage IIB-IV malignant melanoma [161]. Particle-mediated epidermal delivery is a method of non-viral gene transfer and was shown here to have comparable efficacy and safety profile to intramuscular injection despite only using a 500th of the dose of DNA [161].

Tao, et al. conjugated M2e onto gold nanoparticles and subsequently used this construct to vaccinate mice intranasally [164]. Moderate anti-M2e IgG titres were subsequently generated, however, the response was significantly improved by inclusion of CpG molecules [164]. After challenge with a lethal dose of A/PR/8/34 (H1N1), all mice vaccinated with M2e alone died, while 100% or 60% of those conjugated to gold nanoparticles with or without CpG survived, respectively [164].

Aluminium is a commonly-used adjuvant in vaccines [156, 165–167]. This stimulated some studies into the conjugation of antigen to aluminium nanoparticles [166, 167]. These studies showed that the aluminium particles are able to play the role of both carrier and adjuvant to stimulate the immune system, although it has also been shown that aluminium particles are capable of

adsorbing antigen so tightly that antigen structure is altered, which reduces vaccine efficacy [167, 168].

Sun, et al. compared aluminium oxide nanoparticles and conventional alum when co-delivered with an allogenic tumour cell vaccine in their ability to adjuvant the subsequent immune response [169]. Only mice treated with the vaccine and nanoparticles showed significant decrease in average tumour size compared to mice which received no vaccine or adjuvant [169]. Lymphocytes rescued from mice 30 days post tumour challenge were tested for their ability to kill tumour cells *in vitro*. Only lymphocytes from mice treated with vaccine and nanoparticles showed significant tumour lysis [169].

Orr, et al. produced stable aluminium oxyhydroxide nanoparticles (nanoalum) that demonstrated improved adjuvanting ability compared to conventional Al-hydrogel [170]. Mice vaccinated with recombinant HA adjuvanted with the nanoalum showed increased cytokine and antibody production as well as increased survival upon lethal challenge with A/PR/8/34 (H1N1) virus [170].

Calcium phosphate particles are a promising candidate for vaccine applications as they are bioresorbable, non-toxic, have adjuvanting properties and can easily be loaded with antigen [171]. Previously, calcium phosphate was used as an adjuvant for a commercialised diphtheria, tetanus, pertussis, and poliomyelitis vaccine but was replaced by aluminium salts in the 1980s [172]. It has been suggested that calcium phosphate should replace aluminium as an adjuvant in currently commercialised vaccines due to toxicity and side-effect concerns with aluminium adjuvants [160, 172, 173].

Morçöl, et al. formulated an inactivated whole virus influenza vaccine with calcium phosphate particles to test the particles ability to induce a dose-sparing, comparable response to the non-adjuvanted or alum-adjuvanted vaccine [174]. The calcium phosphate adjuvanted vaccine generated similar HI

titres, virus neutralisation and IgG titres to the other groups of the same doses. However, the calcium phosphate vaccine provided equal levels of protection to the non-adjuvanted and alum-adjuvanted vaccine after challenge with A/CA/04/2009 (H1N1) at a third of the dose [174].

Silica-base particles are a popular form of inorganic particle in vaccine research, as they are biocompatible and their interactions with cells can be modified by altering their size and shape [175]. Their surface can also be modified to allow for improved cellular targeting and cellular uptake [176, 177]. Mesoporous silica particles have been shown to be effective antigen carriers for sustained antigen release *in vivo* [178].

Li, et al. compared the adjuvanting ability of tuberculin purified protein derivative (PPD) on its own, in combination with mesoporous silica nanoparticles (PPD-MS), and in combination with both mesoporous silica particles and calcium phosphate particles (PPD-MS/CaP) [179]. Mice were initially injected with live Lewis lung carcinoma cells, which were allowed to grow for 10 days before being surgically removed and immersed in liquid nitrogen for 30 minutes before being homogenized. The autologous tumour sample was then mixed with one of the three adjuvants and injected back into the mouse at the site of excision 3, 7 and 14 days post tumour removal and recurrence rates were measured for 28 days [179]. No significant difference in recurrence rates was observed. Mice were then rechallenged with tumour cells on the opposite flank and tumour presence was monitored for 35 days. On day 35, 50%, 75% and 80% of mice vaccinated with PPD, PPD-MS and PPD-MS/CaP, respectively, were still alive [179].

Newborns are a high risk group for influenza infection due to the ineffectiveness of vaccines in the first few months of life. Russell, et al. used a silicon dioxide-based adjuvant to try to bypass the restrictive immune response in

neonatal mice by stimulation of inflammasomes [180]. Mice were vaccinated intramuscularly with 0.1 μg purified surface antigen from A/California/7/2009 (H1N1) with or without 50 μg silica dioxide microparticles. Both adult and neonatal mice receiving the silica dioxide adjuvant lost significantly less weight and recovered earlier after challenge [180].

Carbon nanoparticles have also been studied for drug and vaccine delivery [142]. They can be synthesised into a variety of different nanotubes and mesoporous spheres [181, 182]. Carbon nanotubes are potentially capable of carrying multiple antigens and are rapidly taken up by antigen-presenting cells [183]. Carbon nanoparticles have also been synthesised to be responsive to magnetic force, and with a model antigen attached, were used to track, target and manipulate dendritic cells [184]. Carbon mesoporous spheres, encapsulating antigen, have been utilised as an oral vaccination method with BSA as a model antigen [185].

Hassan, et al. loaded multi-walled carbon nanotubes with the model antigen ovalbumin conjugated to a CpG molecule with an anti-CD40 antibody present as a second immunoadjuvant and studied the particle's ability to induce an immune response capable of inhibiting growth of OVA expressing tumours [186]. The presence of anti-CD40 was necessary to delay the growth of tumours, though significant delays in tumour growth were seen only when anti-CD40 was conjugated to the carbon nanotubes, thus allowing co-delivery of every component to individual immune cells [186]. Sixty days post injection of tumour cells, 50% of mice which received the nanotubes conjugated to both the ovalbumin-CpG molecule and anti-CD40 were still alive, compared to 0% in all other groups [186].

1.3.4 Plant-like material

Plant cells are an attractive vaccine delivery platform for oral administration because of the ease and low-cost with which large populations could be vaccinated. Furthermore, their tough cell wall is able to protect intracellular material from harsh environments encountered within the stomach [187]. The rationale driving this approach is that once within the gastro-intestinal tract, the cell wall is then broken down by microbes and the intracellular material is released [187]. Transgenic plant cells have, therefore, been produced to express antigenic material for use in vaccination [188]. These transgenic cells may then be able to deliver antigenic material to the intestines where it is free to interact with the gut-associated lymphoid tissue. Crops such as rice and maize have been utilised as expression vectors. These crops are staple foods in target vaccination areas, are inexpensive to produce, and easy to grow in large quantities [189, 190]. Plant cell-based vaccines have been developed for a wide range of pathogens, including influenza, hepatitis B, and anthrax [190–192]. A potential limitation of this approach is how the complexity and diversity of the microbiome will impact on the reliability of the response.

Similar to plant cells, single-cellular algae also have a tough cell wall, making them another attractive delivery platform. Algae-based vaccines have many advantages over plant cell-based vaccines: they are easier to genetically modify, they can be grown in bioreactors, and do not require large areas of land, seasonal conditions or extended durations to grow [193]. Several pathogens have been targeted pre-clinically by algae-based vaccines such as foot-and-mouth disease, malaria, and staphylococcus [194–196].

Pollen grains are naturally occurring plant-based materials that have also recently been investigated as a vaccine delivery platform [197]. They consist

of a tough outer shell that is used to protect the male gamete of the plant for pollination. The outer shell has been shown to be able to survive the harsh conditions of the stomach, thus making them a possible delivery platform for oral vaccination [198]. Recently, it has been shown that *Lycopodium clavatum* (clubmoss) and *Ambrosia elatior* (ragweed) spores can be chemically cleaned to remove any potentially allergenic native proteins present, before being refilled with proteins of interest for vaccination [197, 199]. These studies demonstrated that systemic and mucosal antibody responses could be generated against the model antigen, ovalbumin, when encapsulated within the pollen grains. Questions remain about the complexity and scalability of this approach.

Reports of the use of plant-like material in cancer vaccines is very limited, although a lot of work has been done to develop the use of plant cells as bioreactors to produce antigenic material [200]. The use of plant-like materials as antigen delivery platforms for cancer vaccines has mainly been focused on those cancers which are related to infection by pathogens such as hepatitis B, HPV, and *Helicobacter pylori* [191, 200–202].

Lee, et al. transformed *Arabidopsis* plants to express HA from A/Hong Kong/213/03 (H5N1) as an oral influenza vaccine [203]. HA accumulated within the endoplasmic reticulum of the cells, allowing for protection from proteolytic degradation and thus providing long-term stability. Leaves of transfected plants were picked, freeze dried, ground into a powder and suspended in PBS, with or without saponin as an adjuvant, before oral administration to mice [203]. The adjuvanted vaccine induced high levels of HA-specific serum IgG and mucosal IgA, while the non-adjuvanted vaccine generated comparable levels to the PBS control [203]. Mice were then challenged with a lethal dose of A/Korea/w81/2005 (H5N2) with HA sharing 93% homology with that in

the vaccine. All mice lost approximately 30% of their body weight, but only those receiving the adjuvanted vaccine were able to recover, with 80% alive 28 days after challenge [203]. The discovery of an oral influenza vaccine would be of great use in vaccinating poultry and thus reducing the risk of zoonotic transfer.

1.3.5 Infectious material

Bacteria and viruses can be genetically modified to produce antigenic material from another pathogen [204, 205]. The bacterial or viral strains used for this method of vaccination are generally considered to be safe, either through natural lack of pathogenicity or through attenuation, but can still closely mimic a natural infection, and therefore can stimulate an immune response. However, the immune response generated is often dominated by a response against the carrier vector and not the desired target antigen [206].

Many viruses have been engineered to express tumour antigen transgenes [207]. Some of these specifically infect professional antigen presenting cells, thus enhancing presentation of tumour antigens to the immune system [207]. One such virus that has undergone several clinical trials is an attenuated recombinant vaccinia virus expressing the TAA 5T4, named TroVax [207]. A phase II clinical trial looking at the efficacy of TroVax in patients with colorectal cancer with resectable liver metastases found that 18/19 patients generated 5T4-specific cellular and/or humoural responses [208]. In those who received at least 4 doses of TroVax and potentially curative surgery (n=15), patients with above median 5T4 specific proliferative responses or T cell infiltration into resected tumour tissue had significantly improved survival compared to those below the median [208]. TroVax is also currently undergoing a placebo-controlled phase II trial in patients with ovarian, fallopian tube, and peritoneal

cancer. A phase III, placebo-controlled trial comparing TroVax with and without cytokines and sunitinib in metastatic renal cell cancer patients found no significant difference in overall survival between the treatment and placebo groups (median survival of 20.1 months on the treatment arm vs 19.2 months on placebo arm) [209]. However, 56% of patients in the treatment arm had detectable anti-5T4 antibody levels compared to 6% in the placebo arm, the magnitude of anti-5T4 antibody response induced by vaccination was associated with enhanced patient survival [209].

Wang, et al. developed a recombinant vaccinia viral-based influenza vaccine which expressed PB1 (rVV-PB1), NP (rVV-NP) or M1 (rVV-M1) from the A/Beijing/30/95 (H3N2) virus [210]. Their recombinant virus was used both in comparison to and in combination with a DNA-based vaccine encoding for the same antigen as the corresponding recombinant virus (pPB1, pNP, or pM1). While intramuscular vaccination with pNP or rVV-NP induced a high titre IgG response, the prime - boost regime of pNP and rVV-NP led to 100-fold increased titre levels. Vaccines encoding PB1 were not able to induce detectable IgG levels and those with M1 generated moderate IgG titres [210]. Mice were then challenged with varying doses of A/PR/8/34 (H1N1) virus. At 1.7x the 50% lethal challenge dose, mice vaccinated with rVV-NP alone, or the prime - boost regimes pNP and rVV-NP or pNP+pPB1+pM1 and rVV-NP+rVV-PB1+rVV-M1 were completely protected from challenge, while all the other vaccine regimes led to only partial or no protection [210]. Only mice vaccinated with the prime - boost regimes pNP and rVV-NP or pNP+pPB1+pM1 and rVV-NP+rVV-PB1+rVV-M1 were completely protected from 5x the 50% lethal challenge dose [210]. At 10x, 100% of the pNP and rVV-NP vaccinated mice were alive 18 days post-challenge, while only 80% of pNP+pPB1+pM1 and rVV-NP+rVV-PB1+rVV-M1 vaccinated mice survived the challenge [210].

This vaccine approach was, therefore, able to use a conserved protein from a H3N2 virus to induce 100% protection at 10x the 50% lethal challenge dose of a H1N1 virus, thus providing a useful candidate for a universal influenza vaccine. However, its use in the clinic would likely be limited to younger patients as the strain of vaccinia virus used was developed as a smallpox vaccine in China and used for approximately 50 years prior to its eradication in 1980 [210].

In common with viruses, bacteria have also been engineered to express tumour antigens [211]. One bacterium that has been extensively explored is *Listeria monocytogenes*, which has been used to deliver antigenic material pre-clinically to treat a range of cancers, including cervical, breast, melanoma, colon and prostate cancers [212]. *Listeria monocytogenes* has recently been used in a phase II clinical trial which compared the overall survival of patients receiving the vaccine, ADXS11-001, with and without cisplatin in patients with cervical cancer [213]. ADXS11-001 is a live, attenuated *Listeria monocytogenes*-based vaccine engineered to secrete an antigen-adjuvant fusion protein consisting of human HPV16 E7 protein fused to a listeriolysin O protein [213]. The phase II trial found comparable overall survival rates between the two groups with median progression-free survival of 6.1 vs 6.08 months in patients with recurrent/refractory cervical cancer following prior chemotherapy and/or radiotherapy [213].

Sounders, et al. were the first to demonstrate *in vivo* efficacy using bacteria to deliver DNA into mammalian cells to treat a solid tumour by utilising a plasmid-release mechanism involving the suicide of the carrier bacteria [214]. Their vaccine inhibited the growth of E7-expressing tumours by inducing a strong CD8+ T cell response [214]. However, although the vector was more effective than administering plasmid alone, it was not as effective as utilising

recombinant bacteria to deliver E7 in protein form [214].

The HA protein of influenza virus has two main regions, the globular region (HA1) and the stem region (HA2) [13]. Yang, et al. used a recombinant *Lactobacillus plantarum* bacterium displaying the HA2 region from the A/Anthropoides virgo/Baicheng/219/2013 (H9N2) virus to create an oral vaccine for poultry [215]. Chickens were either orally vaccinated with PBS, non-recombinant bacteria, or recombinant bacteria, or intramuscularly with inactivated H9N2 virus. While anti-HA2 serum IgG and lung-wash IgA titres generated by the recombinant bacteria were comparable to those generated by the inactivated H9N2 virus 2 weeks post-vaccination, the recombinant bacteria was able to generate increased faecal IgA levels [215]. Chickens were then challenged, 2 weeks post-vaccination, with A/Anthropoides virgo/Baicheng/219/2013 (H9N2) virus. At days 3 and 5 post-challenge, viral load in the recombinant bacteria and inactivated H9N2 vaccinated groups were 99 and 99.9% less than in the control groups, respectively [215].

1.3.6 Outer membrane vesicles

Outer membrane vesicles are naturally occurring, non-replicating vesicles produced by gram-negative bacteria [216]. They consist of bacterial phospholipids, lipopolysaccharides, outer membrane proteins and entrapped periplasmic components [216, 217]. This gives them inherent immunostimulatory properties as they naturally contain several pathogen-associated molecular patterns (PAMP) on their surface, which makes them an adjuvanting particle. Antigens can be present either on the surface, inside the lumen of the vesicle or unbound in solution. Antigens within the lumen of the vesicles were believed to be hidden from B cell recognition. However, several groups have reported strong antibody responses to a luminal antigen [218, 219]. One of the difficul-

ties of using outer membrane vesicles to target an antigen non-native to the producing bacteria is that the vesicles naturally contain many immunogenic components, which could lead to an immune response predominantly targeting the vesicle instead of the antigen of interest. To date, two outer membrane vesicle vaccines for Meningitis B (Bexsero and Trumenba) have been licensed for use in humans [220, 221].

Grandi, et al. engineered outer membrane vesicles to present epitopes of two cancer antigens, EGFRvIII and B16-M30, either individually or in combination [222]. They found that vaccination with outer membrane vesicles engineered to present the EGFRvIII epitope on the surface led to inhibition of the growth of B16F10EGFRvIII tumours [222], while mice vaccinated with outer membrane vesicles presenting both EGFRvIII and B16-M30 epitopes on their surface, were completely protected from tumour challenge [222].

Watkins, et al. used recombinant outer membrane vesicles displaying the well-conserved M2e peptide from influenza A viruses, with or without encapsulation within PLGA microparticles for sustained antigenic release [223]. The PLGA microparticles were able to sustain release over a period of 30 days *in vitro*. Encapsulated outer membrane vesicles were given as a single dose, while non-encapsulated vesicles were given as a prime - boost regime, 4 weeks apart [223]. Four weeks post-vaccination, mice vaccinated with PLGA encapsulating microparticles generated equivalent anti-M2e specific IgG titre levels to those vaccinated with the prime - boost regime 8 weeks post-vaccination [223]. Mice were subsequently challenged with a lethal dose of A/Puerto Rico/8/1934 (H1N1) virus either 10 weeks or 6 months post-vaccination. All mice vaccinated with the vesicles, with or without the PLGA microparticles recovered from the infection. While similar weight loss profiles between the two vaccinated groups were seen with challenge at 10 weeks, those vaccinated with

PLGA particles lost significantly more weight at the 6 month challenge compared to those vaccinated with the prime - boost of the free vesicles [223].

1.3.7 Immunostimulating complexes

Immunostimulating complexes (ISCOMs) are spherical cage-like particles that are spontaneously formed by mixing phospholipids, cholesterol, saponin and protein antigens [224, 225]. ISCOM formulation requires the use of amphipathic proteins which restricts the type of antigens that can be included in the complex [226]. An alternative form of ISCOM is ISCOMATRIX which is formulated without antigen [226]. This approach allows for a more flexible application as almost any antigen can potentially be mixed with the ISCOMATRIX adjuvant. ISCOMATRIX adjuvant has been used for both prophylactic and therapeutic vaccines in clinical trials [227, 228].

Davis, et al. performed a placebo-controlled clinical trial with a vaccine consisting of the full protein of a cancer-testis antigen, NY-ESO-1, formulated with ISCOMATRIX [229]. The vaccine was tested in patients with resected NY-ESO-1+ tumours and was found to be well-tolerated. High-titre antibody responses and T cell responses for a broad range of NY-ESO-1 epitopes were subsequently generated [229]. A subsequent trial was performed to follow up persistent immunity in patients. The time for the patients between the two trials ranged from 336 to 1179 days [230]. The follow up trial found 10/14 patients who received the ISCOMATRIX adjuvant had persisting anti-NY-ESO-1 immunity in contrast to 3/14 who did not receive ISCOMATRIX [230]. Downregulation of NY-ESO-1 and HLA class I was observed on tumours from patients who had relapsed, suggesting the occurrence of immunoediting [230]. When tested in patients with advanced stage NY-ESO-1+ melanoma, no clinical response was observed. This was explained by the increased number of cir-

culating regulatory T cells in advanced stage patients compared to those with minimal residual disease [231]. A second cohort of patients with advanced stage NY-ESO-1+ melanoma was recruited and pre-treated with cyclophosphamide to deplete the number of regulatory T cells before administration of the vaccine [232]. This led to a significant increase in NY-ESO-1-specific CD4+ T cell responses compared to the first cohort's patients treated with vaccine and no cyclophosphamide [232]. No significant decline in circulating regulatory T cells was detected 14 days after cyclophosphamide treatment, though a decline at an earlier time point could not be excluded [232].

Fries, et al. performed a randomised, observer-blinded, placebo-controlled clinical trial of a VLP in combination with varying concentrations of ISCOMATRIX [233]. The VLP combined the A/Anhui/1/13 (H7N9) viruses, HA and NA proteins with the A/Indonesia/5/05 (H5N1) viruses M1 protein [233]. Participants received two identical intramuscular injections 21 days apart with blood samples taken at days 21 and 35 and tested for HI and NA inhibition (NAI) ability. At 21 days, patients vaccinated with the non-adjuvanted vaccine started to generate HI titres, while those vaccinated with adjuvanted vaccine had 1.5-fold higher NAI titres [233]. At 35 days, those vaccinated with adjuvanted vaccine had higher HI (5.4-fold) and NAI (2.5-fold) titres than their non-adjuvanted counterparts [233].

1.3.8 Emulsions

Emulsions are heterogeneous liquid systems commonly used in vaccines as adjuvants. Their simplest iteration is in the form of water-in-oil or oil-in-water, but they can be formulated in more complex multiple emulsion systems such as water-in-oil-in-water [234]. The antigen release characteristics of an emulsion are determined by a range of factors such as droplet size, viscosity, and the

oil-to-water ratio [235]. One emulsion frequently used in human vaccines today is an oil-in-water, squalene-based emulsion, MF59, which has been included in the inactivated flu vaccine, Flaud, since 1997 [235, 236].

Emulsions have been used as adjuvants in vaccines for a range of cancers in clinical trials including melanoma and non-small cell lung cancer [237, 238]. A phase I trial compared the immune responses of high-risk stage IIB-IV resected melanoma patients to a gp100 peptide-based vaccine in the presence of the water-in-oil emulsion ISA-51 or the purified plant extract QS-21 [237]. Both adjuvants produced comparable immune responses, though ISA-51 emulsion was associated with fewer toxicities than QS-21 [237]. A phase II trial used an epidermal growth factor (EGF) conjugate vaccine emulsified in ISA-51 to vaccinate patients with stage IIB-IV non-small cell lung cancer and found good anti-EGF antibody levels in 51.3% of patients in the treatment group compared to none in the control group [238]. In the treatment group, 64.3% of patients showed a major decline in serum EGF, with patients whose serum EGF concentration dropped below 168 pg/mL surviving significantly longer than all other patients (median survival 13 vs 5.6 months) [238]. The survival of the treatment group was statistically significant compared to controls in patients younger than 60 years of age (median survival 11.57 vs 5.33 months) [238].

1.4 Discussion

Vaccines are the world's most effective life-saving medical technology to date. The field of vaccinology continues to advance at an impressive rate with more effective new vectors and approaches reaching clinical practice. The traditional three Is paradigm (isolate, inactivate, inject) of vaccine development is

increasingly being phased out for a more rational design paradigm. Due to the low immunogenicity of cancer cells, their high mutation rate, and their inherent similarity to non-cancerous cells they are difficult to produce a vaccine for. However, with the introduction of these rationally designed vaccines, more and more cancer vaccines are progressing into the clinic and their future is very promising. As for influenza vaccination, these newly developed vaccines are providing methods for preparing influenza vaccines at a faster rate after the discovery of circulating strains to readily combat seasonal epidemics and emerging pandemics. These methods also open the doors to improved methods for vaccinating against conserved epitopes on influenza viruses and thus to the possibility of creating a universal influenza vaccine. The literature review presented here indicates that the range of complementary approaches emerging from research and clinical testing will allow the beneficial impact of vaccines to continue to grow. There are many interesting and exciting options for vaccine development being explored, with varying success. What is clear is that an ideal vaccine formulation needs to be highly immunogenic, target-specific, able to induce a rapid response, easy to produce and low-cost. This thesis discusses the formulation and testing of a synthetic vaccine platform against different types of disease (ErbB-2 overexpressing tumours and influenza viruses) by using the liposomal vaccine method described previously [57]. It is hypothesised that, as it has the potential to meet the above criteria, the formulation developed and tested here may be a strong vaccine candidate.

ErbB-2 is a popular target for cancer treatment as it is overexpressed on 15-30% of all breast cancers, as well as in some ovarian, renal, colon and lung cancers. Some cancer patients have shown the ability to mount weak immune responses against ErbB-2, indicating that it is possible to overcome the self-tolerance and initiate an immune response against these ErbB-2 overex-

pressing cancerous cells [239, 240]. The FDA-approved, humanised monoclonal antibodies, Trastuzumab and Pertuzumab, are currently used to give patients passive immunity to breast cancer. The efficacy of these biological agents and their relatively favourable toxicity profile compared to conventional chemotherapeutics means they are viewed as an important part of cancer therapy. However, whilst antibodies represent a growing class of therapeutics, even despite their great expense, they are rarely completely curative, meaning there is much contention regarding their impact on healthcare economics. Therefore, there is interest in developing vaccines that are both prophylactic and therapeutic, that could induce a sustainable ErbB-2 targeted immune response and have a lower comparative cost.

HA is a highly immunogenic influenza virus protein, however it is also subject to a high rate of mutation and thus yearly vaccination against the circulating virus strains is required to mitigate infection. Nevertheless, HA remains a popular vaccination target due to its crucial functional role in infection. HA binds to sialic acid on glycosylated proteins located on cell membranes, initiating cellular uptake and, therefore, cellular infection [13]. Anti-HA antibodies are capable of neutralising the virus by preventing the HA protein from binding to the cellular sialic acid and aiding in viral clearance by complement-mediated cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) [241]. Several groups have discovered well-conserved epitopes on HA that provide possibilities of developing a long-term vaccine which would not require annual administration due to changes in circulating strains [242].

Hills, et al. previously reported the development of a vaccine for malaria which makes use of peptides and liposomes [57]. Like traditional fusion peptide methods, this vaccine system works by boosting the immune response to the

target, weak antigen by taking advantage of pre-existing immunity to a second, strong antigen. However, Hills' approach differs from traditional peptide vaccines as the liposomes were formed by encapsulating the strong antigen within and presenting the weak antigen exclusively on the outside. Thus, spatially segregating the weak and strong antigens and focusing the immune response exclusively onto the external weak target antigen. The proposed mechanism of action is shown in the figure 1.1B, which represents how B cells with receptors specific for the liposomal surface antigen can, upon contact with the liposomes, engulf the vaccine particles. Endosomal conditions lead to degradation of the liposomes and the generation of peptide fragments from both the weak and strong antigens. These peptides are then presented on B cell MHC class II molecules on the cell surface ready for CD4+ helper T cell recognition. Helper CD4+ T cells can recognise the same epitope as the B cell (cognate), i.e. the external antigen, or another protein simultaneously taken up by the B cell (non-cognate). Pre-existing non-cognate CD4+ helper T cells that recognise the strong antigen are able to provide costimulatory signals to the B cell, resulting in confirmation for antibody production [243]. These signals in turn lead to B cell proliferation, differentiation, antibody production, somatic hypermutation and isotype switching. As the specificity of this antibody response was initiated at the point of B cell receptor-mediated recognition of the external weak antigen on the liposomes, the antibodies produced will exclusively be targeted against the weak antigen and not the strong internal antigen. Hills, et al. used malaria circumsporozoite (CSP) as the weak antigen and an ovalbumin (OVA) T cell epitope as the encapsulated strong antigen. They showed that in mice with pre-existing anti-OVA CD4+ T cell immunity the vaccine evokes a rapid, high titre, isotype switched CSP-specific antibody response. This was shown to be consistent with the involvement of the anti-OVA T helper

cells in confirming activation of anti-CSP B cells [57]. Some previous studies have taken advantage of strong T helper epitopes incorporated into liposomal vaccines [244, 245], but this method of spatially segregating the target and helper peptides focuses the immune response specifically onto the weak target antigen.

The proposed vaccination platform explored in this thesis provides a plug-and-play platform that has the potential to rapidly generate a dynamically class-switched, high-titre humoral immune response against any desired target, regardless of its inherent immunogenicity, be it a cancer epitope that has ‘self’-antigen resemblance or a well-conserved epitope of an influenza virus that intrinsically has low immunogenicity compared to the rest of its originating protein. The objectives of this thesis can be outlined as follows:

1. Improvement of liposomal formulation and characterisation methods, specifically:
 - (i) Improved scalability of the formulation method.
 - (ii) Improvement of the peptide incorporation efficiency.
 - (iii) Confirmation that the internal helper peptide is exclusively internal to the liposome.
2. Confirmation of the mechanism of action by demonstrating the requirement of helper peptide presentation for increased anti-target specific antibody production.
3. Determine the efficacy of the proposed mechanism of action targeted against a cancer epitope in inhibiting tumour growth.
4. Demonstrate the adaptability of the mechanism of action by repurposing the mechanism of action to target an epitope of viral origin.

The first research objective is explored in chapter 2 by iterative changes in the formulation method including changes from the thin-film hydration method used by Hills, et al. ([57]) to the more scalable lyophilization method before changing the extrusion method from repeat use extruders to single-use disposable extruders for efficiency and cleanliness. The second research objective is then explored in chapter 3 by testing the vaccine particles ability to induce a specific anti-target immune response in both BALB/c and FVB/n mice after subcutaneous injection. FVB/n mice provide a useful control as they lack the MHC II haplotype capable of presenting the OVA peptide and so this removes the possibility of non-cognate CD4+ T cell help [246, 247], providing an exquisite control to demonstrate the proposed mechanism of action. Chapter 3 also addresses the third research objective by measuring the vaccine’s immunogenicity and tumour inhibition ability in BALB/c mice. This is done by examining the antibody responses generated towards the target peptide to determine that a high-titre, isotype switch, anti-target specific antibody response is generated. The antibodies’ ability to bind to their target on cells and induce cell death *in vitro* is also examined, as well as their ability to inhibit tumour growth *in vivo*. Lastly, the fourth research objective is explored in chapter 4 by repurposing the vaccination platform to target an epitope of the influenza virus to test for the ability to induce an anti-HA antibody response.

The external, weak antigen used for the cancer studies discussed in chapter 3 was a slightly modified version of the immunogenic ErbB-2_{266–296} peptide described by Allen et al. currently used in a phase I trial [63, 65, 66]. For influenza studies, in chapter 4, a modified version of the HA_{91–108} peptide used in the M-001 vaccine currently in a phase III trial [72–74] was used. The data presented here shows that this vaccination system can successfully induce raised humoral immune responses specific for the target peptides by recruiting

the help of non-cognate T helper cells specific for OVA_{323–339}. These antibodies are subsequently demonstrated to be able to bind to their respective epitopes on the whole protein target, and in the case of cancer are able to induce cell death.

Chapter 2

Identification of Optimal Formulation Process

2.1 Introduction

This chapter describes formulation and subsequent characterisation methods of liposomal vaccine constructs that spatially segregate helper and target peptides to focus and enhance the immune response to the target peptide. Three variations of the formulation methods are described herein, as well as their subsequent characterisations. Liposome purity, size, polydispersity, surface charge, incorporated peptide yield and *in vitro* cytotoxicity are investigated. The effect of long term storage of liposomes is also explored.

2.2 Material & Methods

2.2.1 Epitopes

OVA_{323–339} peptide (ISQAVHAAHAEINEAGR) (Innovagen, Sweden: SP-O323-5) is a T cell epitope which was used as the strong internal antigen within liposomes. A control liposome with externally conjugated OVA peptide was also formulated for which a custom made OVA_{323–339} with a single cysteine residue added to the C-terminus (denoted Oc), was also synthesised (ThermoScientific, Germany). ErbB-2_{266–296} peptide (ThermoScientific, Germany) mimics the Pertuzumab binding region of the full ErbB-2 protein with sequence CGPSLLHCPALVTYNTDTFESMHNPEGRYTFGASCV. This sequence is a modified version of the immunogenic ErbB-2 peptide described by Allen, et al.

[63] in which the measles virus fusion helper peptide used is replaced with a single cysteine residue to provide a means of conjugation to the liposome surface via maleimide chemistry. HA_{91–108} peptide (ThermoScientific, Germany) is a well-conserved region of the H3 protein of influenza A viruses with sequence CGPSLSKAYSNCYPYDVPDYASL. This sequence is a modified version of the immunogenic HA_{91–108} peptide described by Müller, et al. [248] in which a single cysteine residue is added to the N-terminus to provide a means of conjugation to the liposome surface via maleimide chemistry.

2.2.2 Liposomal formulation

Liposomes were either formulated by the thin-film hydration method originally described by Bangham [249] or by lyophilisation, originally described by Szoka and Papahadjopoulos [250]. Liposomes were prepared to contain **43** mol% DMPC (1,2 - dimyristoyl - sn - glycerol - 3 - phosphocholine, Avanti Polar Lipids, USA: 850345P), **42** mol% Cholesterol (Sigma Aldrich, UK: C8667), **10** mol% DSPE-PEG-mal (1,2 - distearoyl - sn - glycerol - 3 - phosphoethanolamine - N - [maleimide(polyethylene glycol) - 2000], Avanti Polar Lipids, USA: 880126P), and **5** mol% DMPG (1,2 - dimyristoyl - sn - glycerol - 3 - phospho - (1' - rac - glycerol), Avanti Polar Lipids, USA: 840445P). The DSPE-PEG-mal was used to conjugate the peptide by maleimide-thiol crosslinking.

Thin-film hydration method

Lipids were dissolved in a 2:1 chloroform:methanol mix, to achieve a total lipid concentration of 20 - 40 mg/mL. A LABOROTOR 4011 rotary evaporator and water bath (Heidolph Instruments, Germany) set to 60 rpm, 45°C, and 5 - 10 inHg vacuum was used to remove the chloroform and methanol for approximately 30 minutes. The remaining trace solvent was then removed

using a high vacuum pump for at least two hours leaving a thin lipid film on the bottom of the round bottomed flask. This film was then hydrated with 1 mL citrate buffer (20 mM, pH 6.3) containing OVA_{323–339} at a concentration of 1 mg/mL using the rotary evaporator at 80 rpm and 45°C without a vacuum for 40 minutes. The resulting liposome and antigen mixture was then extruded in a water bath at 50°C with 11 passes through a 400 nm polycarbonate membrane (Avanti Polar Lipids, USA: 610007) using 1 mL glass syringes and a mini-extruder (Avanti Polar Lipids, USA: 610023). This was then repeated with another 11 passes through a 200 nm polycarbonate membrane (Avanti Polar Lipids, USA: 610006) before the resulting liposomes were purified using a PBS-washed Sephadex G-75 filled size exclusion column (Sigma Aldrich, UK: G75120, 17-0435-01) and collected into 1mL fractions. Visual inspection was used to identify liposome containing fractions due to the opacity of the liposomes. Purified liposomes, in PBS, were immediately added to 100 μ L of ErbB-2 peptide at a concentration of 10 mg/mL and left to react overnight at 4°C. Liposome solution (1-2 mL) was then injected into a 20K dialysis cassette (ThermoScientific, Germany: 66003) and immersed in 400mL PBS at room temperature for one hour to remove free unreacted ErbB-2 peptide. The PBS wash was then refreshed for another hour, before being refreshed a third time and left for 24 hours at 4°C.

Lyophilisation method

Lyophilisation is a more scalable approach for forming liposomes when compared to thin-film hydration [251]. It is capable of producing a large quantity of lipid cakes that can be easily and stably stored for long periods of time [252]. Lipids were dissolved in a 2:1 chloroform:methanol mix, to achieve a total lipid concentration of 20 - 40 mg/mL. Chloroform and methanol were then removed

using a high vacuum pump overnight, leaving a lipid film on the bottom of the vial. The dried lipids were redissolved in cyclohexane to a concentration of 20 - 40 mg/mL. The lipid-cyclohexane mixture was then flash frozen by dipping the vial into liquid nitrogen before being placed into a pre-cooled (-30°C) freeze dryer (FTS Lyostar I freeze drying system, SP Industries, USA). Primary drying was carried out at -50°C for 12 hours and secondary drying was performed at 20°C for 6 hours. Pressure was kept constant at 50 mTorr during both drying phases. Freeze dryer vials were then removed from the freeze dryer and the resulting lipid cakes were hydrated with 1 mL citrate buffer (20 mM, pH 6.3) containing 1 mg of OVA_{323–339}. The liposome-OVA mixture was then either extruded using a mini-extruder (Avanti Polar Lipids, USA: 610023), as for the thin-film hydration method, or extruded at 50°C on a hot plate 11 times through a 400nm Nanosizer MINI (t&tScientific, USA: TT-030-0004) before 11 further passes through a 200nm Nanosizer MINI (t&tScientific, USA: TT-003-0010). The t&t Scientific extruders are pre-assembled, sterile, disposable extruder cassettes, while the Avanti extruder kit is a reusable kit that requires cleaning before and after use. Liposomes were then purified by size-exclusion, as in the thin-film hydration, before the addition of 100 μL of ErbB-2_{266–296}, HA_{91–108}, or Oc peptide at a concentration of 10 mg/mL. Liposomes were then left to react for 24 hours at 4°C . Dialysis was used to purify away free ErbB-2, as described in the thin-film hydration method. Oc is a cysteine-modified OVA peptide that was used as a positive control for testing the level of OVA peptide associated with the outside of the liposomes.

2.2.3 Dynamic light scattering

Liposomal size was characterised by dynamic light scattering (DLS) using a Zetasizer Nano ZS instrument (Malvern, UK) with a 1 mL sample dilution of

1:100 in PBS in UV grade cuvettes (Fisher Scientific, UK: 759015). Data was analysed using Malvern Zetasizer software v6.32. Samples were analysed by 3 lots of 12 sub-runs and data is presented as intensity plots.

2.2.4 Zeta potential

Surface charge of the liposomes was characterised by zeta potential using the Nano ZS instrument (Malvern, UK) with a 1 mL dilution of 3:100 in PBS in disposable folded capillary cells (Malvern, UK: DTS1070). Data was analysed using Malvern Zetasizer software v6.32. Samples were analysed by 3 lots of 25-30 sub-runs.

2.2.5 Enzyme-linked immunosorbant assay (ELISA) for peptide concentrations

Antigen was plated onto Nunc Immuno MaxiSorp 96 well plates (Sigma Aldrich, UK: M9410), the plates were sealed and incubated overnight at 4°C. Wells were washed 6 times with 0.05% Tween-20 in PBS, blocked with 10% FCS in PBS and incubated at room temperature for 1 hour. Plates were again washed 6 times before addition of primary antibody diluted in PBS. After 2 hour incubation at room temperature plates were washed 6 times and secondary antibody conjugated to HRP was added at a 1:10000 dilution in PBS. Plates were then incubated for 1 hour at room temperature before being washed a final 6 times. Ultra-TMB HRP substrate (Sigma Aldrich, UK: 34029) was added to each sample well. After 30 minutes the reaction was stopped by the addition of 0.5 M sulfuric acid. Absorbance of the plate was then read at 450 nm using a BMG LABTECH FLUOstar Omega plate reader, data was analysed using Omega software version 3.00 R2.

ELISAs were used to confirm purification of non-encapsulated OVA pep-

tide away from liposome samples and to quantify peptide concentrations in liposome samples. To confirm purification of liposomes, fractions eluted from a size exclusion column were used as the initial antigen, anti-OVA_{323–339} antibody (Innovagen, Sweden: PA-O323-100) as the primary antibody and HRP conjugated anti-rabbit IgG antibody (Abcam, UK: A0545) as the secondary antibody. When quantifying liposomal peptide concentrations, liposome samples were used as the initial antigen and either anti-OVA_{323–339} antibody (Innovagen, Sweden: PA-O323-100) or plasma from pilot studies containing anti-ErbB-2_{266–296} antibodies were used as the primary antibodies. Secondary antibodies used were HRP conjugated anti-rabbit IgG antibody (Abcam, UK: A0545) for OVA_{323–339} peptide quantification or HRP conjugated anti-mouse IgG antibody (Sigma Aldrich, UK: A4416) for ErbB-2_{266–296} peptide quantification.

2.2.6 Transmission electron microscopy

Transmission electron microscopy was performed with negative staining by Mr Adam Costin at the Electron Microscopy Facility at the Dunn School Bioimaging Facility, University of Oxford.

2.2.7 Human plasma collection and liposome stability in plasma

Human blood plasma was obtained from a healthy volunteer. Blood was drawn into 8mL CPT vacutainer tubes coated with sodium heparin anticoagulant (BD, UK: 362753). CPT vacutainers allow for separation of blood components by centrifugation. After blood collection, vacutainers were inverted several times to ensure sufficient mixing of sodium heparin. Vacutainers were then centrifuged at 1500 xg for 15 minutes at 4°C before the removal of plasma.

Plasma was sized using DLS (see section 2.2.3) prior to the addition of liposomes at a 1:100 ratio. Plasma was then resized multiple times after incubation at room temperature.

2.2.8 Protein A purification

A protein A purification kit (ThermoFisher, UK: 89948) was used to confirm encapsulation of OVA peptide. Briefly, protein A spin columns were washed and reconstituted with anti-OVA_{323–339} antibody (Innovagen, Sweden: PA-O323-100). Excess unbound antibody was removed by washing 3 times with binding buffer before incubation with pre-diluted liposomal samples for 10 minutes with end-over-end mixing. Liposome samples were diluted to contain a calculated internal OVA concentration of 0.2 μg . Columns were then spun at 5000 xg and resulting flow-through was collected and assayed for OVA peptide concentration by ELISA. A positive control which was surface modified with OVA was included. As this control should be amenable to binding to the protein A-anti-OVA antibody construct there should be very poor recovery of these liposomes from the column. In contrast liposomes with internal OVA peptide should pass freely through the column. The MaxiSorp ELISA plates (Sigma Aldrich, UK: M9410) used strongly bind lipid and thus burst open liposomes enabling the internal peptide to be detected.

2.2.9 Cell lines

Human lung carcinoma cells, A549 were purchased from European Collection of Authenticated Cell Cultures, UK and maintained according to the suppliers' guidelines using high-glucose DMEM culture media.

2.2.10 Cytotoxicity

Liposomal cytotoxicity tests were performed on A549 cells. On day 1, cells were plated out onto a sterile 96 well plate (Sigma Aldrich, UK: CLS3595) at a density of 10,000 cells per well. The plate was then incubated at 37°C for 24 hours. On day 2, the media in the wells was removed and replenished with media mixed with a range of liposome concentrations (0, 0.2, 1, 5, 10 %), with n=5 repeats of each concentration. The plate was then placed back in the incubator for another 24 hours. On day 3, the media-liposome solution in the wells was removed and replaced with media containing 20% MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega, USA: G3580) per well. The plate was then incubated for approximately 15 minutes before reading absorbance at 490 nm using a BMG LABTECH FLUOstar Omega plate reader, data was analysed using Omega software version 3.00 R2.

2.2.11 Peptide loss with storage

Liposomes formulated by method B (table 2.1) were measured for loss of incorporated peptide after 15 months of being stored at 4°C. Samples of liposomes were passed through a 30 kDa Amicon Ultra filter (Sigma Aldrich, United Kingdom: UFC503008) by centrifugation at 5000xg for 40 minutes, at which point 2/3 of the fluid had passed through. Opacity of liposomes showed them to have not passed through the filter. Flow through was collected and analysed by ELISA for peptide content, as described in section 2.2.5.

	Method A	Method B	Method C
Formulation	Thin-film hydration	Lyophilisation	Lyophilisation
Extrusion	Avanti lipid extruder	Avanti lipid extruder	t&t Scientific Nanosizer

Table 2.1: Liposomes were formulated either by thin-film hydration or lyophilisation (section 2.2.2) before being extruded through an extrusion kit made by either Avanti (product codes: 610023, 610007, and 610006) or t&t Scientific (product codes: TT-030-0004 and TT-003-0010).

2.2.12 Statistics

Statistical analysis was performed using either Microsoft Excel or R. Cytotoxicity means were compared with one-way ANOVA followed by Tukey's post-test. Differences were considered to be significant when the p-value was less than 0.05. Indicators of statistical significance: ns, $p > 0.05$, *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$.

2.3 Results

Liposomes were formulated and extruded by one of three methods (table 2.1). Liposomes were originally formulated as in Hills, et al. by method A as the thin-film hydration formulation is simple for small scale laboratory testing [57]. Later formulations were made by lyophilisation (methods B and C), as this is more reproducible and scalable when compared to thin-film hydration. Methods B and C differ by the use of different extrusion cassettes.

During formulation, liposomes are subject to two purification processes to remove non-incorporated peptide. The first of which removes non-encapsulated

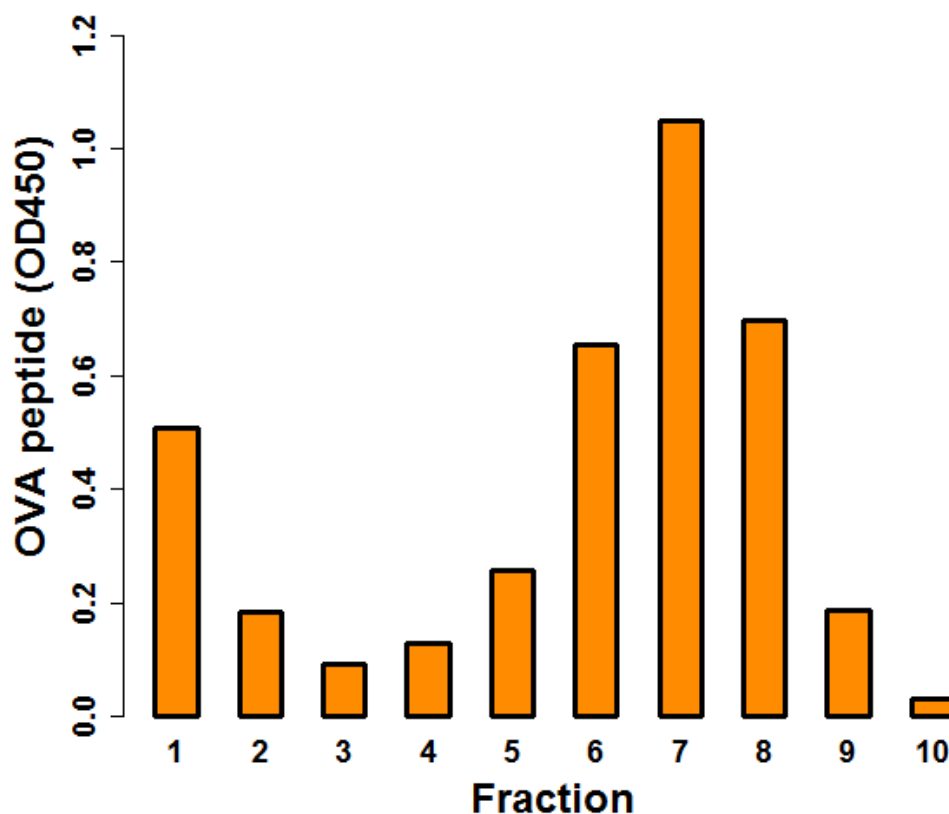


Figure 2.1: Purification of liposomes: liposomes were passed through size exclusion columns and collected into 1mL fractions to purify away non-encapsulated OVA peptide. Visual inspection showed fractions 1 and 2 to be opaque identifying them as liposome positive (data not shown). Fractions were assayed for OVA peptide content using an ELISA (see section 2.2.5). Each bar represents blank corrected mean absorbance of $n=2$ samples. Data shown for liposomes formulated by method B (table 2.1). Control liposomes containing no OVA gave no detectable absorbance by anti-OVA ELISA (data not shown).

OVA peptide using a size exclusion column. The first ten 1mL fractions which passed through the column, after the void volume was eluted, were collected. Figure 2.1 shows enzyme-linked immunosorbant assay (ELISA) results for OVA peptide presence in fractions collected from the size exclusion column. A clearly bimodal distribution to the eluted OVA is apparent with fractions 1-2 and 6-8 containing a substantial proportion of the total. It is clear from the profile that only a small proportion of the OVA was successfully loaded, but this is in accordance with the passive nature of the process used here [57]. Notably, visual inspection and dynamic light scattering (DLS)

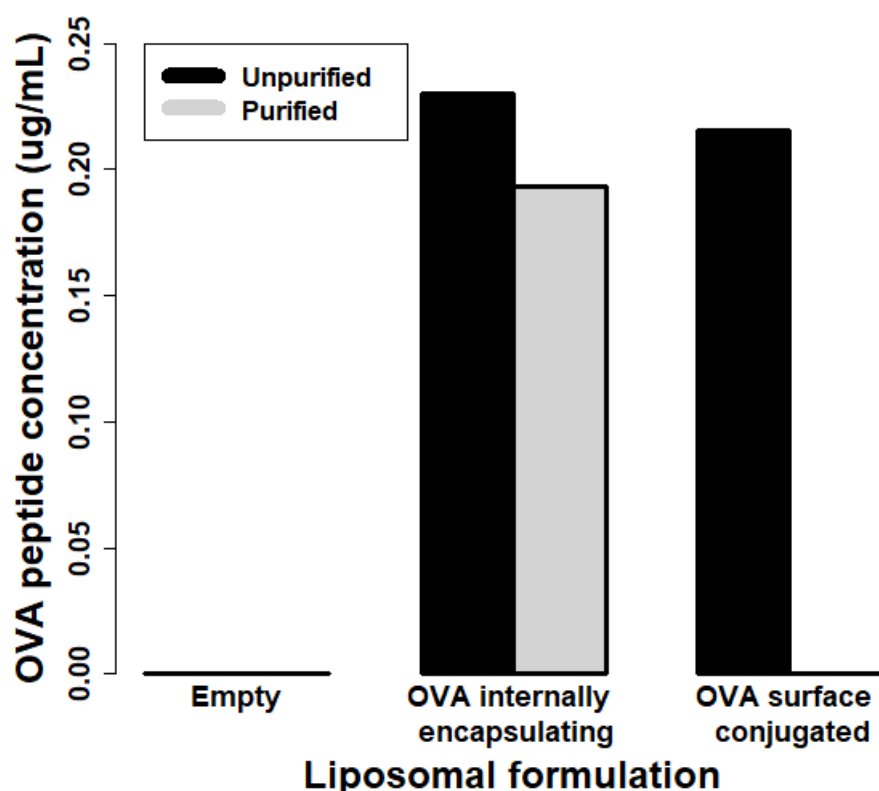


Figure 2.2: Protein A-anti-OVA antibody purification: samples of liposomal formulations were diluted and passed through a protein A column primed with anti-OVA_{323–339} antibody to capture available OVA peptide. Resultant spinout from the columns were then assayed by ELISA for OVA peptide concentration. Each bar represents blank corrected mean absorbance of $n=2$ sample wells. Data shown for liposomes formulated by method C (table 2.1).

analysis showed fractions 1 and 2 to be opaque identifying them as liposome containing, whilst no liposomes were detectable in later fractions (data not shown). The presence of liposomes and OVA peptide in fractions 1 and 2, but only free peptide in later fractions, indicated liposomes were successfully combined with OVA, which would otherwise have eluted in the later fractions. The data shown in figure 2.1 is for liposomes formulated by method B, those formulated by methods A and C show similar results (data not shown).

This data does not prove that the OVA peptide was successfully encapsulated as co-elution could be the result of association with the outside of the liposomes. The mechanism of action of the liposome vaccine platform requires

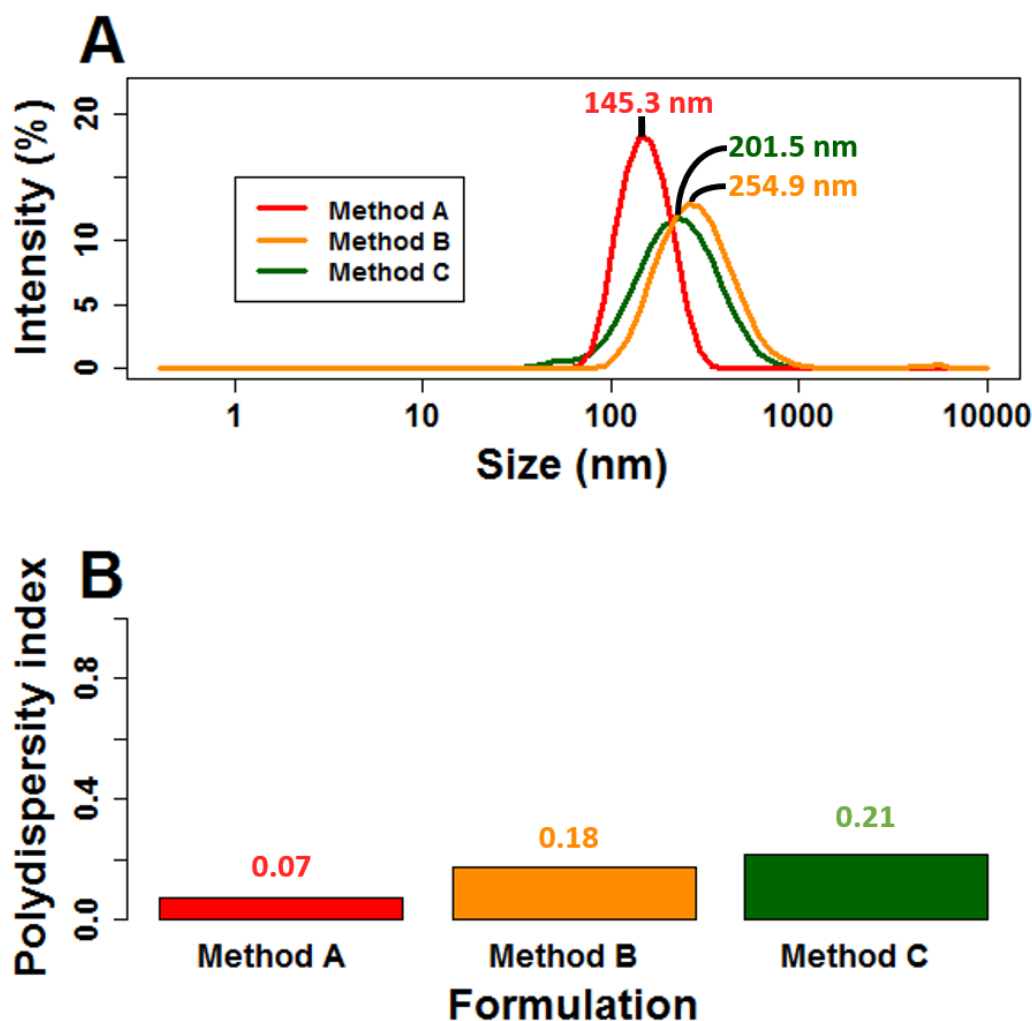


Figure 2.3: Liposome size: size was characterised by dynamic light scattering (DLS) as described in section 2.2.3. Panel A shows the intensity plot of sizes of liposome samples, while panel B shows the polydispersity index of samples. Results shown are averaged from $n=2$ samples each, measured 3 times with 12-14 sub-runs. Liposome samples were OVA_{323–339} encapsulating and surface decorated with ErbB-2_{266–296}.

spatial segregation of the OVA peptide from the external peptide. Hence, free OVA or OVA on the surface of the liposome surface would not only reduce the number of reactive groups available for attachment of weak target antigen it would potentially diminish B cell receptor binding of the external ErbB-2 or HA peptides. To test this, samples of liposome formulations were diluted and purified by protein A-anti-OVA antibody columns to confirm the OVA

peptide was located within liposomes and not conjugated externally (figure 2.2). No OVA peptide could be detected in the empty liposomes before or after purification. Control liposomes with an externally conjugated OVA peptide were unable to be detected in the eluate from the columns, indicating they were successfully bound to the anti-OVA antibody within the column. Conversely, the OVA encapsulating liposomes were shown to maintain 85% of their OVA content whilst still eluting freely from the column. This low level of decrease in OVA content may be caused by free peptide, peptide associated with the liposome surface or encapsulated peptide released due to disruption of the formulation during its passage through the column.

Liposomal size was measured by DLS. Liposomes were formulated to have an approximate size of 200nm so as to be similar in size to a viral particle (20 - 400 nm). Liposomes formulated by method A, B or C had a mean size of 145, 201, or 255 nm, respectively (figure 2.3A). All methods produced samples with a low polydispersity index showing the liposomes in the samples to be monodisperse (figure 2.3B). However, it is noted that method C produced less monodisperse samples (PDI = 0.21) than methods A or B (PDI = 0.07 and 0.18, respectively). This may be a consequence of the larger batch method of formulation. Liposomes formulated by method C were also imaged by transmission electron microscopy (figure 2.4).

Zeta Potential was used to characterise the surface charge of liposomes. Liposomes were designed to be anionic so as to prevent them from escaping endosomal degradation upon uptake by B cells [125]. All formulations produced liposomes with an average surface charge between -21 and -43 mV (figure 2.5). The zeta potential tending more towards neutral may explain the higher size and polydispersity of methods B and C which can result from aggregation as a result of insufficient opposing charge of adjacent liposomes. Lower surface

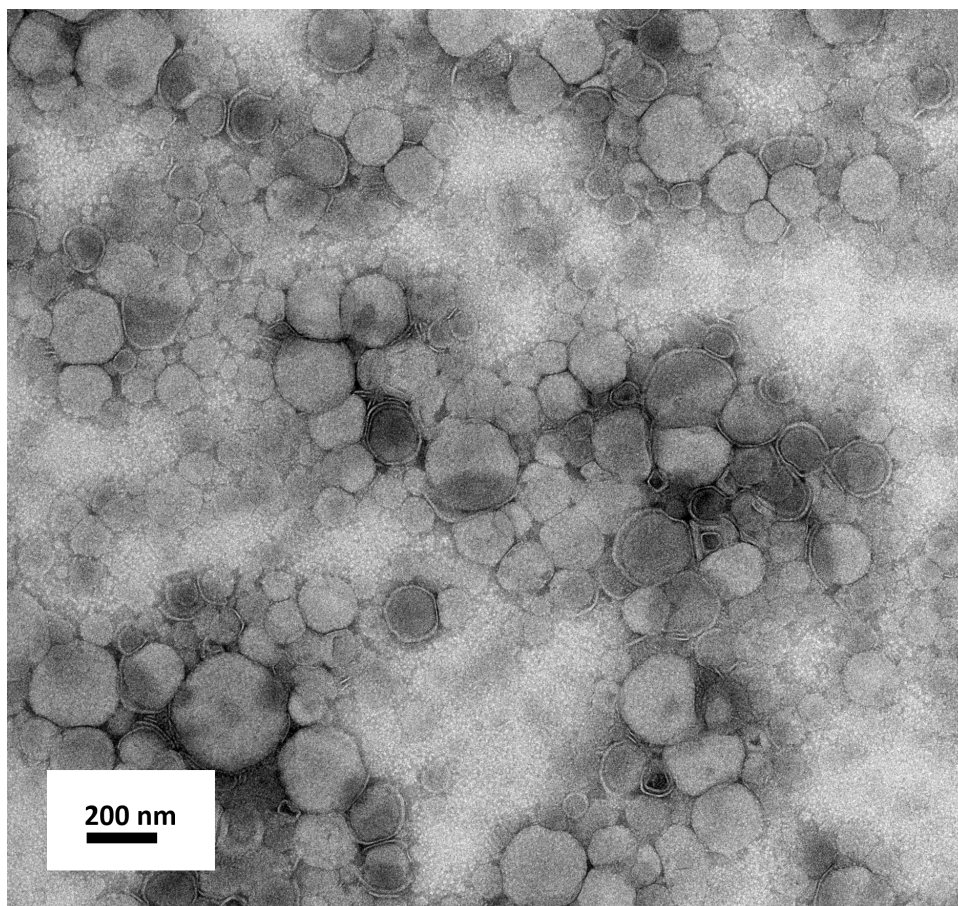


Figure 2.4: Transmission electron microscopy of liposomes formulated by method C was performed using negative staining. Liposome samples were OVA_{323–339} encapsulating and surface decorated with ErbB-2_{266–296}.

charges may be due to less incorporation of the DMPG negatively charged lipid or increased differences of shielding charge by different levels of PEG between formulations.

ELISAs were used to calculate concentrations of OVA_{323–339} and ErbB-2_{266–296} peptides formulated into and on liposomes (table 2.2). Lyophilisation led to an increased level of OVA peptide being encapsulated within the liposomes while extrusion with t&t Scientific Nanosizers led to a large increase (0.42% yield to 91% yield) in the amount of ErbB-2 attached to the liposome surface. This dramatic increase was confirmed by centrifuging samples through a 30 kDa filter and detecting free peptide in the flow-through (table 2.3). When

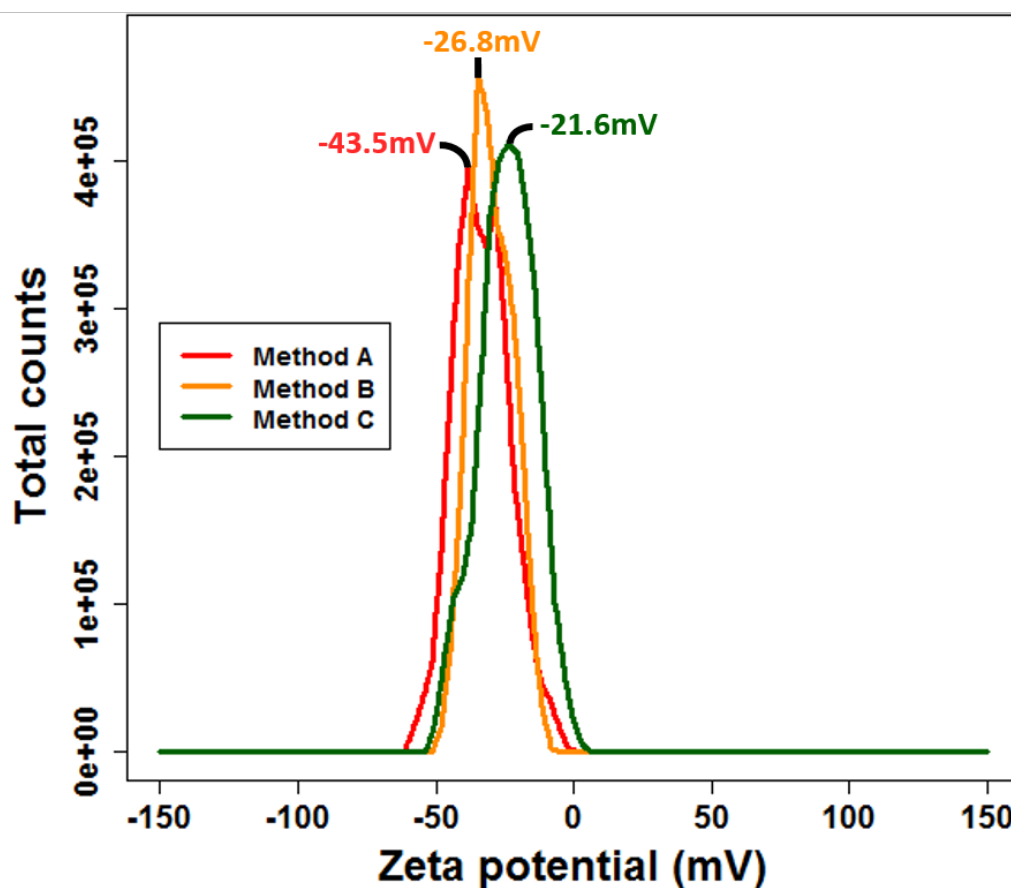


Figure 2.5: Liposome surface charge: surface charge was characterised by Zeta potential as described in section 2.2.4. Results shown are averaged from $n=2$ samples each, measured 3 times, each with 25-30 sub-runs. Liposome samples were OVA_{323–339} encapsulating and surface decorated with ErbB-2_{266–296}.

	OVA _{323–339}	ErbB-2 _{266–296}
Method A	1 (0.1%)	4 (0.4%)
Method B	3.2 (0.32%)	4.2 (0.42%)
Method C	5.2 (0.52%)	910 (91.0%)

Table 2.2: Peptide concentrations incorporated into liposomal formulations, as found by ELISA (see methods section 2.2.5), and their respective yields compared to the amount of peptide added. Concentrations are given as mean ($n=2$) and are presented as $\mu\text{g/mL}$ (% yield). Yield percentages do not factor in various sample dilutions after addition of peptide caused by purification processes.

	Free ErbB-2_{266–296} ($\mu\text{g/mL}$)	ErbB-2_{266–296} conjugated liposomes ($\mu\text{g/mL}$)
Pre-filter	100.0	100.0
Flow Through	97.4	0.0

Table 2.3: Confirmation of high peptide conjugation of liposomes formulated by method C (table 2.1), as measured by ELISA. ErbB-2_{266–296} surface conjugated liposomes and free ErbB-2_{266–296} were diluted to equal concentrations and passed through a 30 kDa filter by centrifugation at 5000xg for 40 minutes. Pre-filter and flow through samples were then analysed by ELISA (see methods section 2.2.5) for peptide content. Concentrations are given as mean (n=2).

non-conjugated peptide was tested, high recovery was achieved demonstrating free peptide could pass through the filter (table 2.3). In contrast, no peptide was detected following filtration of liposome formulation. The stability of the liposomes during this centrifugation purification was monitored by DLS. Figure 2.6 shows that the liposomes were impervious to this centrifugation. The polydispersity index appears to decrease after centrifugation. This is likely due to larger particles sedimenting against the filter and being removed from solution. DLS was also run on the flow-through, no particle could be detected (data not shown).

In order to generate a robust antigen-specific immune response, the formulation must be free of cytotoxicity, which may kill cells before peptides can be presented. Cytotoxicity of liposomes was, therefore, examined via an MTS assay on human lung carcinoma cells, A549. Cells were exposed to liposomes for 24 hours at varying liposome:media ratios to detect low level *in vitro* toxicities. One-way ANOVA tests at the $\alpha = 0.05$ confidence level found no statistically significant differences in viability for cells receiving any formulation of liposome for concentrations up to 10% liposome in media (figure 2.7).

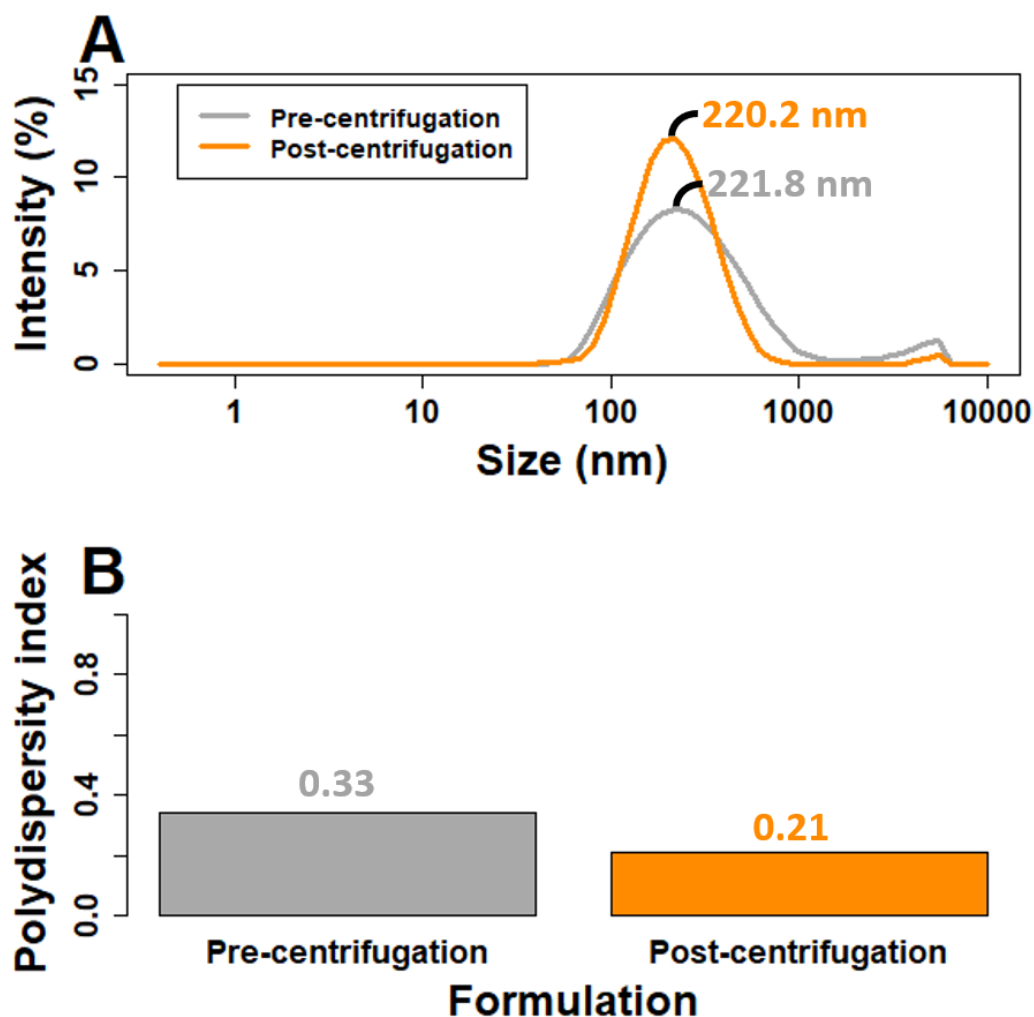


Figure 2.6: Liposome size: size was characterised by DLS as described in section 2.2.3. Liposomal size was measured both before and after centrifugation through a 30kDa filter at 5000 xg for 40 minutes at room temperature. Liposomes were unable to pass through the filter. Panel A shows the intensity plot of sizes of liposome samples, while panel B shows the polydispersity index of samples. Results show samples measured 3 times with 12-14 sub-runs.

In vivo stability of liposomes is essential for vaccination, therefore it is of interest to determine if liposomes can still be detected after incubation in plasma. Figure 2.8 shows the sizing distribution of liposomes in plasma over time. Immediately after the addition of liposomes, the intensity of the peak around 200 nm increases, indicating an increase in particles of this size. Subsequently the intensity of the smaller peaks decreases as they are now fewer

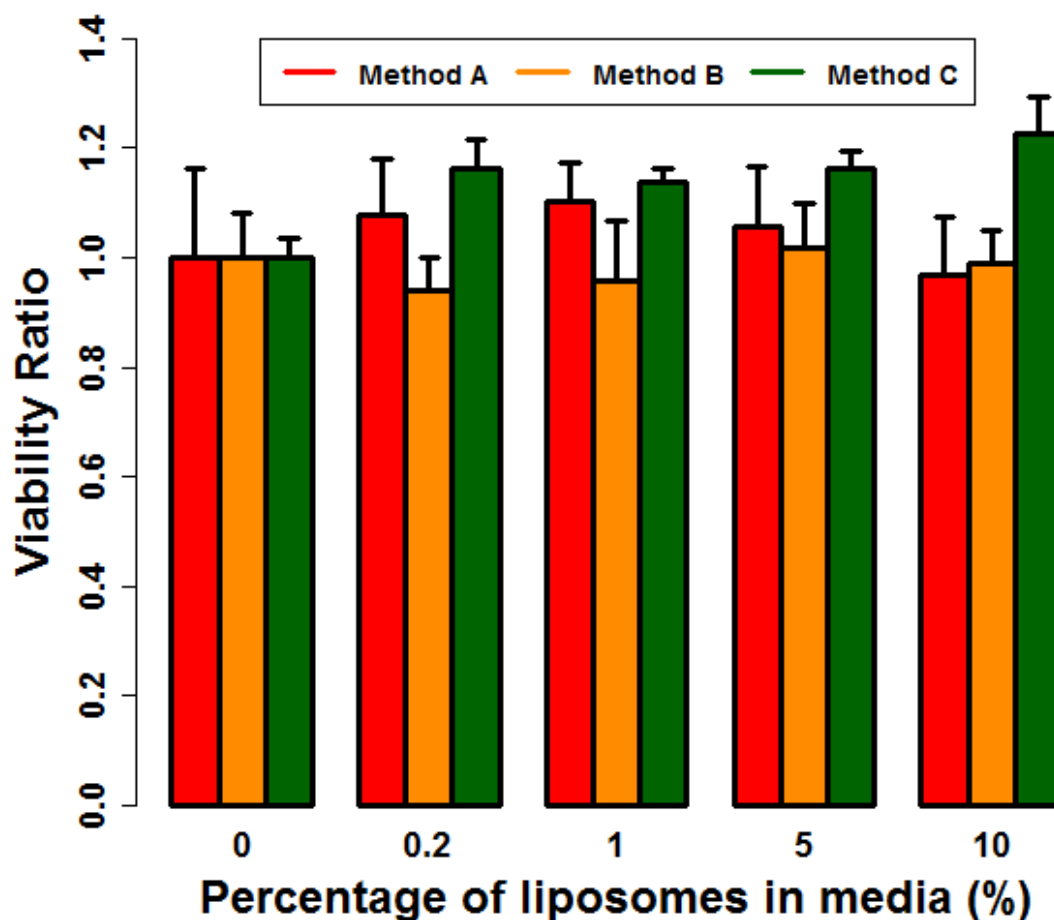


Figure 2.7: Cytotoxicity of liposomes: *in vitro* cytotoxicity of the liposomes was tested by an MTS assay on A549 cells, as described in section 2.2.10. Data is presented as a ratio of viability of sample wells compared to the average viability of untreated cells. Each bar represents the mean of $n=5$ wells, error bars represent standard deviation. Liposome samples were OVA_{323–339} encapsulating and surface decorated with ErbB-2_{266–296}.

in proportion. The peak around 200nm appears to gradually spread out in the direction of larger particles with increasing time. This may be the result of protein corona formation around the liposomes which is known to increase particle size over time [253, 254]. Notably, when complement activation was assessed by C3a release, post-incubation of liposomes with human plasma from the same donor, there was no detectable increase in C3a from background (Human C3a ELISA, Thermo Scientific: BMS2089, data not shown).

A unique selling point of this approach is that the liposome ‘platform’

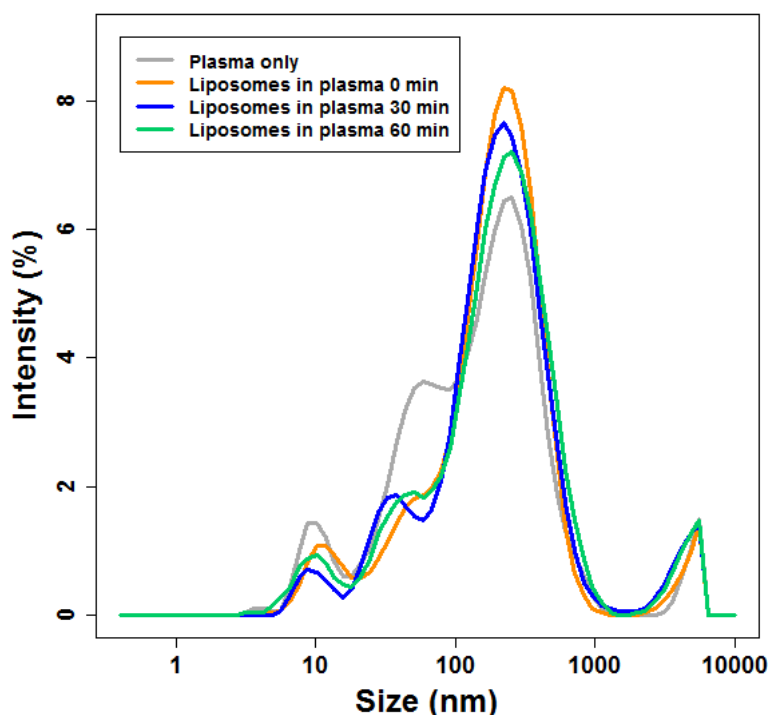


Figure 2.8: The size of plasma components was measured by DLS as described in section 2.2.3 both before and after the addition of liposomes at a 1:100 dilution and incubated for 30 minutes. Liposomes used were formulated by method C. Results show samples measured 3 times with 12-14 sub-runs. Liposome samples were OVA_{323–339} encapsulating and surface decorated with ErbB-2_{266–296}.

	OVA _{323–339}	HA _{91–108}
Method C	5.6 (0.56%)	928 (92.8%)

Table 2.4: Peptide concentrations incorporated into liposomes formulated by method C using HA_{91–108}, as found by ELISA (see methods section 2.2.5) and their respective yields compared to the amount of peptide added. Concentrations are given as mean (n=2) and are presented as $\mu\text{g/mL}$ (% yield). Yield percentages do not factor in various sample dilutions after addition of peptide caused by purification processes.

containing a ubiquitous strong antigen (OVA in these experiments, potentially an epitope of the human cytomegalovirus in humans) can be formulated and stored and then whichever required target antigen can be added in response to whichever pathogen. In contrast, a conjugate vaccine would have to be

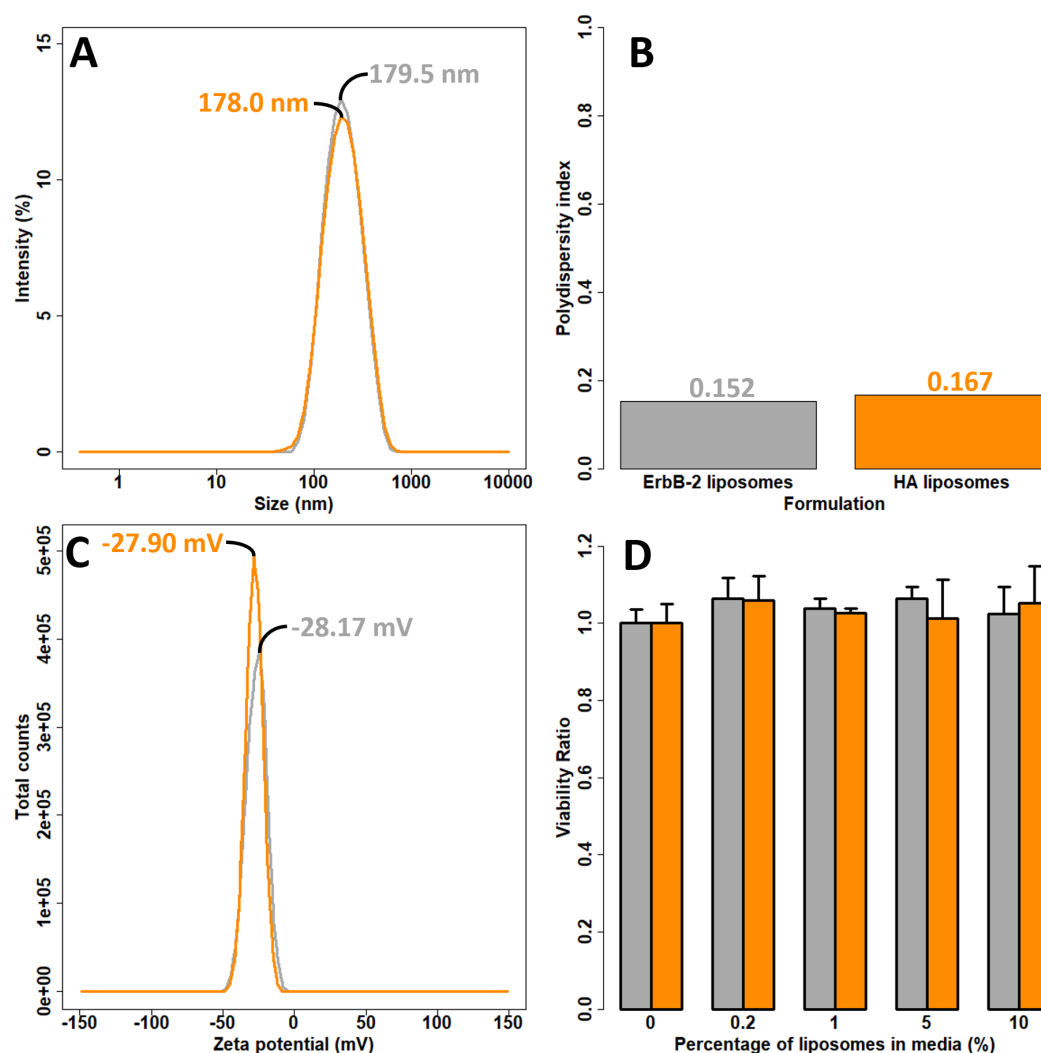


Figure 2.9: Comparison of characterisations of liposomes with different conjugated target peptides. Liposomes were formulated by method C (table 2.1) with one of two target peptides: ErbB-2_{266–296} or HA_{91–108}. Liposomal size (panel A) and polydispersity (panel B) were determined by DLS, while liposomal surface charge (panel C) was determined by Zeta Potential and Cytotoxicity (panel D) was determined by MTS assay. Data in grey represents ErbB-2_{266–296} conjugated liposomes, while data in orange represents HA_{91–108} conjugated liposomes. DLS results show samples measured 3 times with 12–14 sub-runs. Zeta Potential results show samples measured 3 times with 25–30 sub-runs. Cytotoxicity results are given as the mean of n=5 wells with error bars representing standard deviations.

completely resynthesised for each new target.

To validate the adaptability of the vaccination platform, liposomes were also formulated by method C (table 2.1) with an influenza epitope conjugated to the maleimide linker on the surface of the liposomes instead of the ErbB-2

	Pre-filter	Flow through
OVA_{323–339} ($\mu\text{g/mL}$)	3	0
ErbB-2_{266–296} ($\mu\text{g/mL}$)	4	0

Table 2.5: Peptide shedding and leakage after storage of liposomes, formulated by method B, at 4°C for 15 months. Liposomes were passed through a 30 kDa filter by centrifugation at 5000xg for 40 minutes, at which point 2/3 of the fluid had passed through (see methods section 2.2.11). Pre-filter and flow through samples were then analysed by ELISA (see methods section 2.2.5) for peptide content. Concentrations are given as mean of n=2 sample wells.

peptide. This change in peptide was demonstrated to not have an impact on the size or polydispersity of the liposomes, with ErbB-2_{266–296} and HA_{91–108} conjugated liposomes having sizes of 179.5 and 178.0 nm and polydispersity indexes of 0.152 and 0.167, respectively (figure 2.9 A and B). Similarly, the surface charge of the liposomes is relatively unperturbed by the change in target peptide, with ErbB-2_{266–296} and HA_{91–108} conjugated liposomes having surface charges of -28.17 and -27.90 mV, respectively (figure 2.9 C). Both liposome formulations were also determined to be non-cytotoxic (figure 2.9 D). The impressive conjugation efficiency of method C was again demonstrated by the HA_{91–108} conjugated liposomes, with ELISA giving a total target peptide conjugation yield of 92.8% (table 2.4).

Clinical utility of a vaccine is enhanced if it can be shown to maintain structural and physiochemical integrity over extended periods of time. Storage of liposomes in liquid form, at 4°C, after 15 months was measured by DLS to monitor change in size and polydispersity (figure 2.10), and by ELISA to monitor peptide shedding and leakage for liposomes (table 2.5) formulated by method B. Liposomal size marginally increased in the 15 months between measurements (205 vs 221 nm), while their polydispersity remained unchanged,

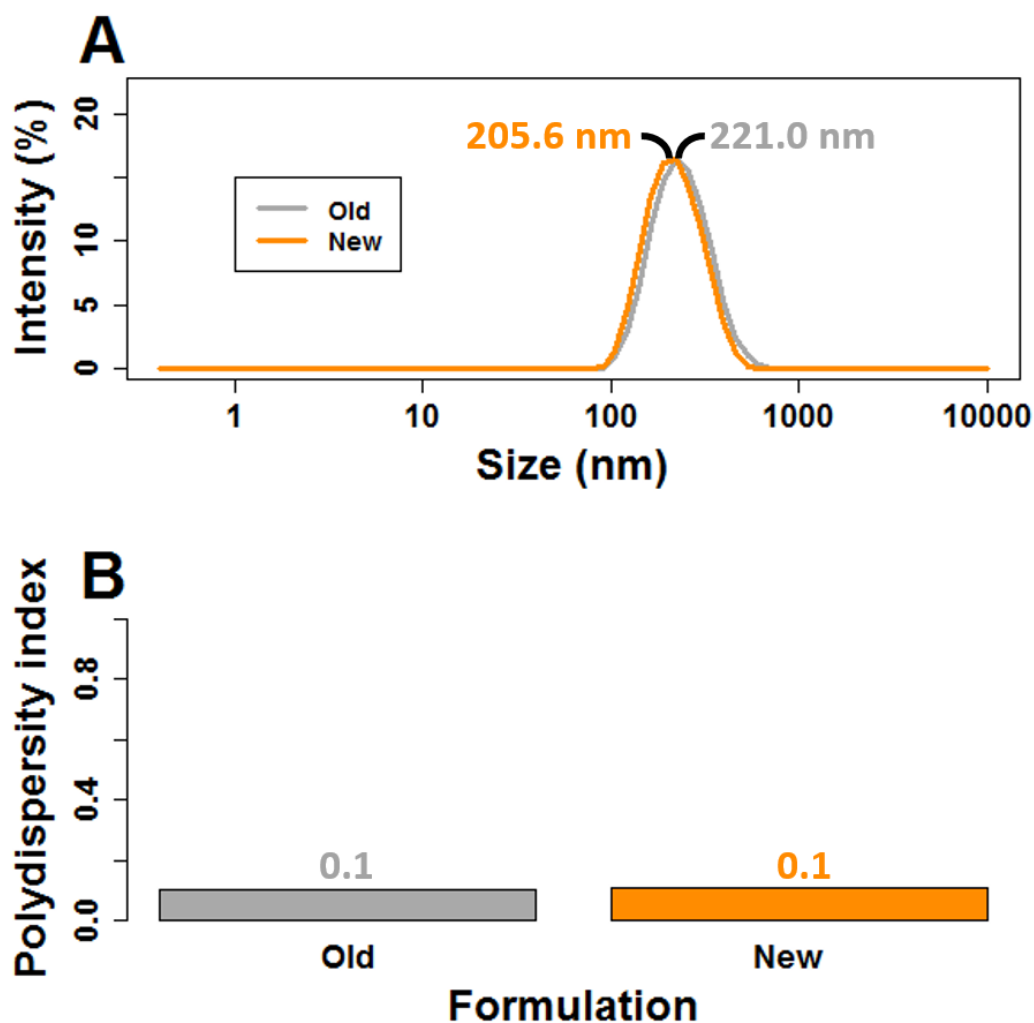


Figure 2.10: Liposome size: size was characterised by DLS as described in section 2.2.3. Liposomes were stored at 4°C for 15 months before being remeasured. Panel A shows the intensity plot of sizes of liposome samples, while panel B shows the polydispersity index of samples. Results show samples measured 3 times with 12-14 sub-runs.

indicating they have swelled slightly in size in a stable manner. Shedding of surface ErbB-2_{266–296} and leakage of internal OVA_{323–339} was measured by filtration of liposomes and analysing the filtrate (see methods section 2.2.11). Table 2.5 shows that there was no detectable peptide in the filtrate indicating that liposomes had no peptide leakage or shedding after being stored at 4°C for over a year.

2.4 Summary

This chapter describes the formulation methods of liposomal-based vaccines and their subsequent characterisations. Three variations of the formulation method were explored (table 2.1). Liposomes were initially formulated by thin-film hydration (method A) as in Hills, et al. [57] due to the simplicity of the method. However, for later studies liposomes were formulated by lyophilisation (method B and C) as this allowed for more reproducible formulations and is a more scalable method than the thin-film hydration method [251]. Methods B and C differed by the use of different extrusion cassettes.

Free unencapsulated OVA peptide was shown to be successfully separated from liposomes by size exclusion, an essential step in ensuring the desired mechanism of action. The concentration of OVA present in liposome samples after all purification steps was found to be approximately 1, 3.2, and 5.2 $\mu\text{g}/\text{mL}$ for methods A, B, and C respectively. This is much lower than the initial concentration of peptide added to the dried lipids (1mg/mL) due to dilution factors in the purification steps and inherent poor passive loading efficiency of liposomes. Method A was previously used by Hills, et al., who saw similarly low levels of OVA encapsulation [57]. The binding efficiency of the ErbB-2 peptide was dramatically (216-fold) improved when method C was used compared to methods A and B. This is likely to be due to the single-use kit utilised in method C, which offers a quicker and cleaner approach. This gives less opportunity for the maleimide linker on liposomes to react or be hydrolysed before it comes into contact with the ErbB-2 peptide. The change to single-use disposable extruders also minimises the risk of contamination of vaccine.

Liposomes were designed to be anionic to prevent them from escaping endosomal degradation once engulfed by B cells [125]. The mechanism of action

requires vaccine particles to be degraded once engulfed so that B cells can present peptide fragments on their MHC II proteins for helper T cell recognition. Zeta potential measurement showed them to have a net negative surface charge between -20 and -45 mV. DLS showed their size to be 145 - 255 nm in diameter, which falls within the expected range for a virus particle (20-400 nm). Larger liposomes (>500 nm) tend to have a much shorter *in vivo* half-life due to phagocytosis [255]. It has also been indicated that small liposomes (20-200 nm) can freely traffic into draining lymph nodes [256]. Carrasco, et al. showed that after administration, 200 nm particles coated with protein accumulated in the boundary of the lymph node follicles where B cells initially encounter their cognate antigen [257]. All formulation methods also produced liposomes that were non-cytotoxic *in vitro*. The liposomal characterisations were also unperturbed by changes in the target peptide, demonstrating the ease with which the formulation can be modified to be retargeted against a different epitope. In summary, the optimised processes here provided a formulation with the ease of manufacture, physicochemical characteristics, peptide loading efficiency, stability and toxicity profile to warrant further investigation.

In the next chapter, the mechanism of action of these liposomal-based vaccines is explored *in vivo*. The requirement of non-cognate T cell activation in the mechanism will be demonstrated by making use of FVB/n mice, which lack the MHC class II haplotype capable of presenting the OVA peptide for T cell recognition [246, 247]. The necessity of every vaccine component in generating a rapid, high-titre antibody response will then be demonstrated in a large cohort murine study followed by optimisation of the dosing regime.

Chapter 3

Cancer vaccination

3.1 Introduction

In this chapter, the vaccination platform first described by Hills, et al. is applied to target a cancer antigen. The chosen target is the cell surface receptor ErbB-2, which is overexpressed on 15-30% of all breast cancers, as well as in some other cancers, including ovarian, colon and lung [258]. The ErbB family consists of four structurally-related proteins that regulate cell growth, survival, differentiation and angiogenesis [259]. ErbB-2 is activated by the formation of either a homo- or heterodimer with a second protein of the ErbB family (figure 3.1). Pertuzumab is an FDA-approved, monoclonal antibody used in the clinic to treat patients with ErbB-2 overexpressing breast cancers. A phase III trial comparing the response of patients with ErbB-2+ metastatic breast cancer treated with either Trastuzumab and docetaxel alone or in combination with Pertuzumab found that, when combined with Pertuzumab, the median progression-free survival was increased from 12.4 months to 18.5 months without affecting the treatment safety profile [260]. A follow up to this study showed that the median overall survival of patients had increased from 40.8 months to 56.5 months for those with Pertuzumab included in their treatment [261]. Pertuzumab works by inhibiting ErbB-2/ErbB-3 dimerisation, which is the most potent dimerisation in the ErbB family [262]. The target epitope used in the cancer vaccination studies described here is a slightly modified version of the immunogenic ErbB-2_{266–296} peptide described by Allen et al. which is cur-

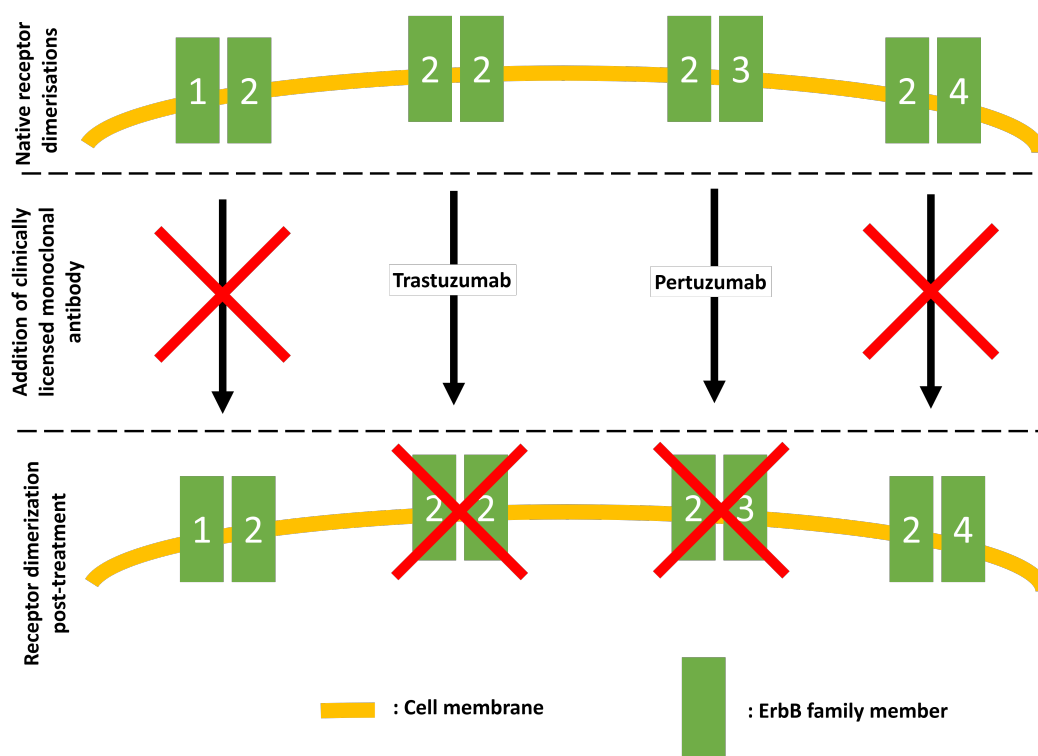


Figure 3.1: Schematic of ErbB-2 dimerisation before and after addition of clinically licensed monoclonal antibodies. Trastuzumab and Pertuzumab block ErbB-2 dimerisation with ErbB-2 or ErbB-3, respectively, whereas no currently licensed monoclonal antibody therapies block ErbB-2 dimerisation with either ErbB-1 or ErbB-4.

rently in an ongoing phase I trial (NCT01376505: clinicaltrials.gov) [63, 65, 66]. This peptide mimics the Pertuzumab binding region and thus should produce antibodies that inhibit ErbB-2/ErbB-3 dimerisation. The rat version of the ErbB-2 peptide and protein, which share 93.5 and 95.2% homology with the respective mouse peptide and protein, are used in these studies as a proof of concept.

The proposed mechanism of action of the vaccine developed here is explored first by comparing the anti-ErbB-2_{266–296} antibody profiles of vaccinated FVB/n and BALB/c mice. FVB/n mice, unlike BALB/c mice, lack the MHC class II haplotype, I-A(d), capable of presenting the internal OVA_{323–339} peptide for T cell receptor recognition [246, 247]. Thus, the proposed mechanism of action should break down for these mice.

Next, the requirement of each component of the vaccine to generate high-titre, isotype-switched antibody levels was tested. The ability of antibodies to bind to ErbB-2 in either recombinant or cancer cell derived form was also examined. Anti-ErbB-2_{266–296} antibodies were then demonstrated to be able to induce cell death *in vitro*. Tumour challenge of vaccinated mice with ErbB-2 overexpressing tumour cells was then tested. The dosing regime was then optimised and the optimal regime was used to repeat the large challenge study to see if the antibody responses can be increased further and the tumour response improved. Resected tumours were examined by flow cytometry for ErbB-2 expression on tumour cells and the number of tumour-infiltrating lymphocytes. The cellular immunological profiles of splenocytes was also explored.

3.2 Material & Methods

3.2.1 Cell lines

TUBO cells were gifted by Dr Natalia Saveyeva at the University of Southampton, UK and maintained in high glucose DMEM media supplemented with 20% FBS. TUBO cells are mouse mammary gland tumour cells spontaneously generated from a BALB-*neu*T mouse, which highly overexpress rat ErbB-2 protein on their cell surface [263]. TUBO cells were used to test the binding ability of generated antibodies to their target on tumour cells, to monitor *in vitro* cell death once incubated with plasma positive for anti-ErbB-2_{266–296} antibodies, and to challenge vaccinated mice. TUBO cell lysate was obtained using ice-cold NP40 lysis buffer made using 150 mM sodium chloride, 50mM Tris pH 8.0 and 1% Triton X-100.

Panel	Stain	Purpose	Dilution
Tumour Panel 1	Zombie Violet ¹	Viability stain	1:500
	ErbB-2 - AF488 ²	Target expression	1:1000
	CD45.2 - PE-Cy7 ³	Leukocyte population	1:1000
Tumour Panel 2	Zombie Violet ¹	Viability stain	1:500
	CD3e - AF488 ¹	T cell population	1:100
	CD4 - PerCp-Cy5.5 ⁴	CD4+ T cell subpopulation	1:1000
	CD8 - APC-Cy7 ⁴	CD8+ T cell subpopulation	1:1000
Spleen Panel 1	Zombie Violet ¹	Viability stain	1:1000
	CD3e - AF488 ¹	T cell population	1:100
	CD4 - PerCp-Cy5.5 ⁴	CD4+ T cell subpopulation	1:1000
	CD8 - APC-Cy7 ²	CD8+ T cell subpopulation	1:500
Spleen Panel 2	Zombie Violet ¹	Viability stain	1:1000
	B220 - PE-Cy7 ¹	B cell population	1:500
	CD19 - APC ¹	B cell population	1:1000

Table 3.1: List of staining panels used for flow cytometry of cells extracted from tissue samples. Dilutions were optimised for each tissue type in preliminary studies. ¹: Biolegend, USA (product codes: 423114, 100321, 115511, and 103221); ²: Novus Biologicals, USA (product code: NBP2-34641AF488); ³: Invitrogen, USA; ⁴: eBioscience, USA (product codes: 25-0451-82, 45-0042-82, and 47-0081-82)

3.2.2 Flow cytometry of TUBO cells

TUBO cells were suspended at 1×10^6 cells/mL in chilled media and centrifuged at 500 xg for 5 minutes. Cells were resuspended in chilled media with or without a 1:500 dilution of anti-rat ErbB-2 antibody conjugated to AF488 (Novus Biologicals, UK: NBP2-34641AF488) and left on ice in the dark for 30 minutes. Cells were washed and loaded onto a Becton Dickson FACSCalibur. Data was then analysed using the software FlowJo version 10 (FlowJo LLC, USA).

3.2.3 Flow cytometry of resected tissues

Tumours and spleens were resected, weighed, suspended in FACS buffer (5% FBS in PBS) and placed on ice during tissue collection. Tumour samples were then placed in 10mL tumour digestion media, consisting of high glucose DMEM media supplemented with 1.25 mg/mL collagenase (Sigma Aldrich, UK: C5138) and 0.3 mg/mL DNase I (Sigma Aldrich, UK: 10104159001) in a C-MACS tissue dissociator tube (Miltenyi Biotec, UK: 130-093-237). Tumours underwent mechanical disruption using a gentaMACS tissue dissociator (Miltenyi Biotec, UK: 130-093-235). Dissociated tumours were then passed through a 70 μ m cell strainer (Sigma Aldrich, UK: Z742103) with mashing to break up remaining clumps of tissue. Spleen samples were mashed straight onto a 70 μ m cell strainer (Sigma Aldrich, UK: Z742103) and flushed through with FACS buffer. Samples, once in single-cell suspensions, were centrifuged at 520 xg for 5 minutes at 4°C and the resulting supernatants were removed. Spleen sample pellets were resuspended in red cell lysis buffer (ThermoFisher, UK: 00-4333-57) to remove red blood cells. Spleen samples were incubated for 5 minutes at room temperature before being centrifuged as before. All tissue samples were then washed twice with PBS by centrifugation to remove free protein. After the last wash, tumour cells and splenocytes were resuspended in PBS to a concentration of 100 μ g of starting tissue/mL to adjust for approximate number of cells. Samples were then aliquoted into a round-bottomed 96 well plate, with 200 μ L of each sample. Plates were then centrifuged at 520 xg for 5 minutes at 4°C before removal of the supernatant. Cell pellets were resuspended in a given dilution of Zombie Violet viability stain (Biolegend, USA: 423114) in PBS (table 3.1) and incubated at room temperature in the dark for 20 minutes. Plates were then centrifuged as before, supernatant was re-

moved and cell pellets were resuspended in 1:40 dilution of purified anti-mouse CD16/32 antibody (Biolegend, USA: 101302) in FACS Buffer and incubated in the dark at 4°C for 15 minutes in order to block cellular Fc receptors. Plates were again centrifuged, and cell pellets were resuspended in a given antibody panel stain (table 3.1) diluted in FACS buffer. Plates were incubated for 30 minutes in the dark at 4°C, before being centrifuged, and washed once in FACS Buffer. Samples were then loaded onto and run on a Becton Dickson FAC-SCanto II until 500,000 non-debris events were collected (figure 3.16). Pooled single-cell suspensions of individual tissue types were used for single-marker staining and full-minus-one (FMO) staining to aid in gating strategies. Data was then analysed using the software FlowJo version 10 (FlowJo LLC, USA).

3.2.4 Mice

Female BALB/c and FVB/n mice were purchased from Charles Rivers at 4 - 6 weeks old and housed in specific pathogen-free conditions at the University of Oxford animal facility and used between 5 and 18 weeks of age. Animal weights were recorded twice a week from the start of treatment until cull. Animal experimentation was performed in accordance with institutional and UK Home Office guidelines. All mice showed consistent weight gain throughout the experiment and there was no evidence of toxicity (piloerection, lack of movement/activity, lack of socialisation) in any mouse. So as to reduce the number of mice used in the larger-cohort studies, a power analysis was conducted using R to find the minimum number of mice required.

3.2.5 Vaccination

All injections were performed subcutaneously under anaesthetic. Mice first received a pre-vaccination dose of either PBS or 10 μ g OVA_{323–339} peptide mixed

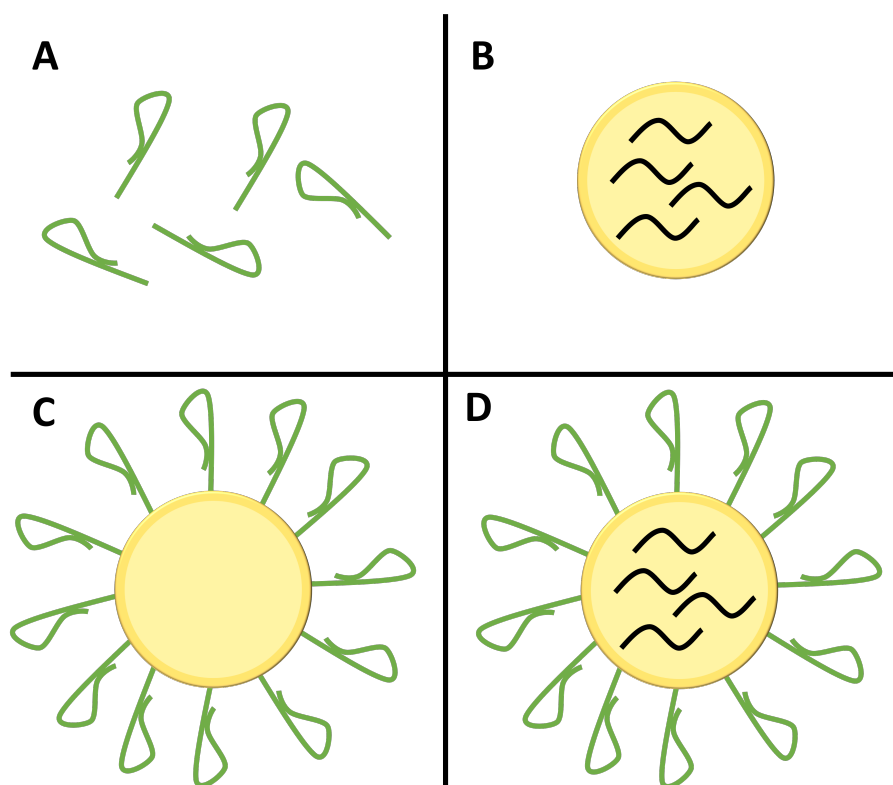


Figure 3.2: Diagrammatic representation of liposomes used in ErbB-2 vaccinations. Panel A represents free ErbB- $_{266-296}$, denoted ‘E’; panel B represents OVA $_{323-339}$ encapsulating liposomes, denoted ‘(O)’; panel C represents ErbB- $_{266-296}$ presenting, empty liposomes, denoted ‘E()’; panel D represents OVA $_{323-339}$ encapsulating, ErbB- $_{266-296}$ presenting liposomes, denoted ‘E(O)’.

with adjuvant in a 1:1 dilution. Adjuvant used was either TitreMax Gold (TMG) (Sigma Aldrich, UK: T2684) or Addavax (Invivogen, France: vac-adx-10) as specified in figure captions. An identical booster pre-vaccination was given two weeks later. After a further two weeks mice were vaccinated with either: PBS, free ErbB- $_{266-296}$ peptide, denoted ‘E’; OVA $_{323-339}$ encapsulating liposomes, denoted ‘(O)’; ErbB- $_{266-296}$ presenting empty liposomes, denoted ‘E()’; or OVA $_{323-339}$ encapsulating, ErbB- $_{266-296}$ presenting liposomes, denoted ‘E(O)’ (figure 3.2). Booster doses were given as described in each individual study.

3.2.6 Plasma collection

Weekly blood samples (40 μ L) were collected from the tail vein of mice after dosing with liposomal vaccines. Blood samples were immediately diluted 1:10 in PBS before being centrifuged at 1000 xg for 5 minutes. Supernatants were collected and frozen at -20°C until required.

3.2.7 ELISA for total anti-ErbB-2 IgG titre

ELISAs were performed as described in section 2.2.5 to quantify the anti-ErbB-2 total IgG titre in plasma samples using ErbB-2_{266–296} as the initial antigen at a concentration of 10 μ g/mL. Plasma from mice was used at varying dilutions as the primary antibody and a HRP conjugated anti-mouse IgG antibody was used as the secondary at a 1:10000 dilution (Sigma Aldrich, UK: A4416). The titre of plasma was defined to be the last dilution that gave an absorbance reading above 0.05 after subtraction of the average absorbance and 2 standard deviations of 16 blank wells treated with PBS instead of plasma as the primary.

3.2.8 Isotyping antibody responses

ELISAs were performed as described in section 2.2.5 to quantify anti-ErbB-2 IgM, IgG1, IgG2a, IgG2b, IgG3 concentrations in mouse blood using ErbB-2_{266–296} as the initial antigen at a concentration of 10 μ g/mL. Plasma from treated mice was used at varying dilutions as the primary antibody and a HRP conjugated anti-mouse IgM, IgG1, IgG2a, IgG2b or IgG3 antibody was used as the secondary (Abcam, UK: ab97240, ab97245, ab97250, ab97260, ab97230). The concentration of antibodies in plasma samples was calculated by using a standard curve of isotype controls (Biolegend, UK: 401401, 401501, 401201, 401301, 401601) as the initial antigen, with PBS replacing the pri-

mary antibody. Isotype plasma concentrations were then back calculated to find concentrations in whole blood. Calculated concentrations represent lower bounds of the actual blood concentration due to competitive binding between the different isotypes within samples.

3.2.9 Tumour inoculation

Mice were challenged with subcutaneous injections of either 1×10^5 or 3.4×10^4 TUBO cells, as stated in the text. Tumours were allowed to grow for 3-4 weeks and measured at least twice a week in three orthogonal axes (x, y and z). Tumour volumes were calculated as $xyz/2$.

3.2.10 Western blot

Samples were diluted in a 1:1 dilution with Laemmli sample buffer (Bio-Rad, USA: 1610747) with 5% β -mercaptoethanol before heating to 95°C for 5 minutes. Samples were then run on a pre-cast 4-20% polyacrylamide gel (Bio-Rad, USA: 4561096) with 1x Tris-Glycine-SDS running buffer (Bio-Rad, USA: 1610732) and 160V for 1 hour using a Mini-PROTEAN Tetra Vertical electrophoresis kit (Bio-Rad, USA). Gels were transferred onto a 0.2 μ m pore size nitrocellulose membrane (Bio-Rad, USA: 1620095) with 30V overnight using a Mini Trans-Blot western blotting system (Bio-Rad, USA). Membranes with transferred proteins were blocked with 5% skimmed milk in PBS for 1 hour before being washed with 0.05% Tween-20 in PBS and incubated with plasma from mice diluted 1:1000 in PBS 0.5% skimmed milk for 1 hour. Next, membranes were washed with 0.05% Tween-20 in PBS and incubated with a HRP conjugated anti-mouse IgG antibody raised in goat (R&D Systems, USA: HAF007) at a dilution of 1:1000 in PBS 0.5% skimmed milk for a further hour. Immobilon Western HRP Substrate (Merck Millipore, USA: WBKLS0500) was

used for chemiluminescent detection of protein. Membranes were washed with 0.05% Tween-20 in PBS before addition of the substrate for 3 minutes. Excess substrate was then drained before exposing the membranes to X-ray films (ThermoFisher, UK: 10137683) for approximately 10 seconds. Films were developed using an Agfa CP 1000 film processor.

3.2.11 Cell death

Ability of plasma samples, positive for anti-ErbB-2_{266–296} antibodies, to induce cell death was tested on TUBO cells. Cells were plated onto a 96 well plate at 10,000 cell/well and incubated for 24 hours. The media was then removed and replaced with fresh media containing different dilutions of either PBS, anti-ErbB-2_{266–296} negative plasma (from pre-treatment bleeds), or anti-ErbB-2_{266–296} positive plasma (from OVA+E(O) treated mice). Plasma samples started at a 1:10 dilution in PBS and were complement-deactivated by heating to 55°C for 30 minutes prior to dilution with media. Cells were then incubated for a further 24 hours before replacement of the media dilutions with fresh media. Cells were then imaged using brightfield microscopy on an Eclipse TI inverted microscope (Nikon Inc, USA) at 10x magnification.

Cells challenged with media only and the highest dilutions of PBS, and anti-ErbB-2_{266–296} negative and positive plasma were also washed with fresh media and analysed on a Becton Dickson FACSCalibur until 5000 events per sample were collected to measure the amount of live cells in the samples (n=3). Data was then analysed using the software FlowJo version 10 (FlowJo LLC, USA).

3.2.12 Statistics

Statistical analysis was performed using R. ELISA results were first tested for normality by Shapiro-Wilks test with a p-value less than 0.05 and thus were analysed using non-parametric tests. Kruskal-Wallis and Conover-Iman tests were used to find differences between antibody responses when more than two groups were compared. Meanwhile, Mann-Whitney U tests were used to find differences between IgM and IgG isotype concentrations when only two groups were being compared. To determine if group differences existed for tumour data, mixed-effect linear regression models with zero-inflation were used for tumour growth curves of groups; and multivariate ANOVA was used for comparison of resected tumour and spleen weights. Differences were considered to be significant when the p-value was less than 0.05. Indicators of statistical significance: ns, $p > 0.05$, *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$.

3.3 Results

3.3.1 Confirmation of the mechanism of action

The proposed mechanism of action for the liposomal vaccine was tested by comparing the ability of BALB/c and FVB/n mice to generate anti-ErbB-2_{266–296} antibodies after vaccination. Mice first received two identical pre-vaccinations of either PBS or 10 μ g of OVA peptide, emulsified with immunoadjuvant titreMax Gold (TMG), two weeks apart to generate pre-existing anti-OVA immunity in some of the mice. Two weeks after the second pre-vaccination, mice were injected with either OVA_{323–339} encapsulating liposomes, denoted ‘(O)’; ErbB-2_{266–296} presenting liposomes, denoted ‘E()’; or OVA_{323–339} encapsulating, ErbB-2_{266–296} presenting liposomes, denoted ‘E(O)’, formulated

Pre-vaccination	Liposomal vaccination	Group notation	Number of FVB/n mice	Number of BALB/c mice
PBS + TMG	(O)	PBS+(O)	5	5
	E(O)	PBS+E(O)	5	5
OVA + TMG	E()	OVA+E()	5	5
	E(O)	OVA+E(O)	5	5

Table 3.2: Number of FVB/n and BALB/c mice in each group for vaccination study confirming the proposed mechanism of action. TMG is an immunoadjuvant called TitreMax Gold; OVA is a peptide of Ovalbumin; ‘(O)’ denotes liposomes encapsulating OVA peptide; ‘E()’, empty liposomes surface decorated with ErbB-2 peptide; ‘E(O)’, OVA peptide encapsulating, ErbB-2 peptide surface decorated liposomes. All liposomes were formulated by method A (table 2.1) and mice received 0.4 μ g of ErbB-2 peptide.

by method A (table 2.1). Identical samples and treatment groups were used for both BALB/c and FVB/n mice (table 3.2). Mice in the PBS+E(O) group (table 3.2) lack the non-cognate CD4+ T cell populations capable of providing activating signals to B cells to initiate antibody responses [57], this should, therefore, limit the increase in the amount of antibody produced via this mechanism. Similarly, B cells should not receive activation signals when the pre-existing anti-OVA immunity is present and the OVA peptide is missing from the liposome construct, as is the case for the OVA+E() group.

Weekly plasma samples were collected from both FVB/n (figure 3.3A) and BALB/c (figure 3.3B) mice, which first showed an antibody response 7 days after vaccination. At 14 days post-vaccination, no statistically significant difference was found between the mean responses of any of the FVB/n mice exposed to ErbB-2_{266–296}. This indicates that anti-OVA_{323–339} non-cognate CD4+ T cells have not played a role in activation of antibody production. In contrast, BALB/c mice, at 14 days showed a significantly ($p < 0.01$) raised (4.2-fold)

Pre-vaccination	Liposomal vaccination	Group notation	Number of mice
PBS + Addavax	E	PBS+E	10
	(O)	PBS+(O)	10
	E()	PBS+E()	10
	E(O)	PBS+E(O)	10
OVA + Addavax	E	OVA+E	10
	(O)	OVA+(O)	10
	E()	OVA+E()	10
	E(O)	OVA+E(O)	10

Table 3.3: Number of mice in each group for vaccination study with a larger cohort. Addavax is an immunoadjuvant similar to MF59; OVA is a peptide of Ovalbumin; E denotes free ErbB-2 peptide; ‘(O)’, liposomes encapsulating OVA peptide; ‘E()’, empty liposomes surface-decorated with ErbB-2 peptide; ‘E(O)’, OVA peptide encapsulating, ErbB-2 peptide surface-decorated liposomes. All liposomes were formulated by method B (table 2.1). Liposomes were diluted prior to dosing (100 μ L) to have identical ErbB-2 concentrations (4 μ g/mL).

response in the OVA+E(O) group, which has pre-existing anti-OVA_{323–339} immunity and was vaccinated with E(O) liposomes. Notably, there was no difference between the PBS+E(O) group and the OVA+E() group, as in both cases non-cognate T cells were unable to produce helper signals to the B cells. Despite the mechanism being functional in only the BALB/c mice, FVB/n mice produced a globally higher anti-ErbB-2_{266–296} signal. This is due to intrinsic differences in the strain of mice. It has been observed in other vaccination studies that FVB/n mice produce globally higher levels of antibodies than BALB/c mice [63, 264].

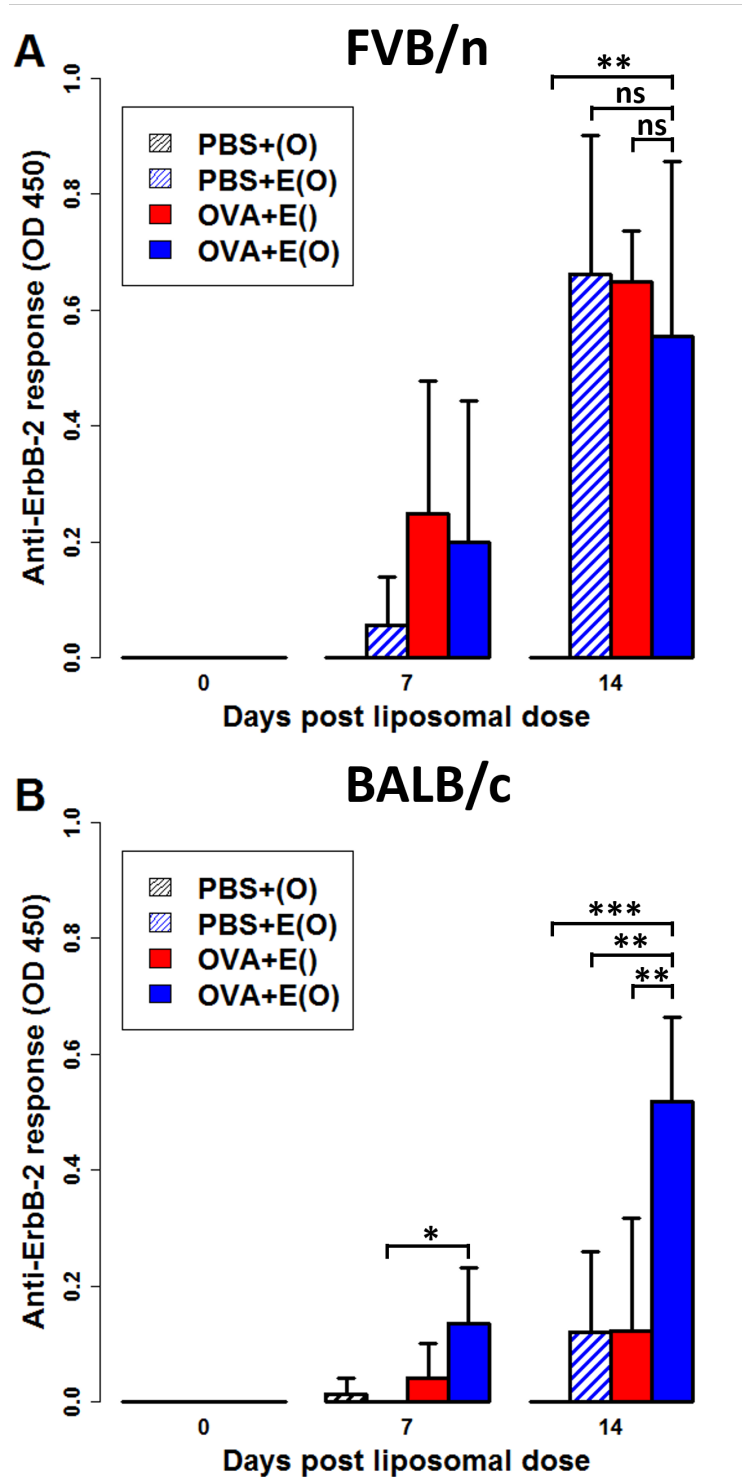


Figure 3.3: Anti-ErbB-2 IgG responses in FVB/n (Panel A) and BALB/c (Panel B) mice: plasma samples were taken from the mice on 0, 7, and 14 days post liposomal vaccination and tested by ELISA to detect if mice had generated an antibody response to the ErbB-2 peptide. Plasma samples were run using a 1:400 dilution. Mice pre-vaccinated with PBS or OVA peptide are shown with striped or solid bars, respectively. Mean of n=5 mice, error bars represent standard deviation. Kruskal-Wallis statistical tests were first used to find if differences existed between all groups, followed by Conover-Iman tests to find pairwise differences between groups. Indicators of statistical significance: ns, $p > 0.05$; *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$.

3.3.2 Larger-cohort tumour challenge study

Having confirmed the mechanism of action, the power of the response generated against ErbB-2 could be tested by challenging immunised mice with TUBO cells shown to overexpress the target (figure 3.4). Hence, the vaccination system was next tested on a larger cohort of BALB/c mice to show the necessity of each vaccination component (table 3.3).

Mice received two identical pre-vaccinations of either PBS or OVA peptide with immunoadjuvant, Addavax, on weeks -4 and -2 (figure 3.5A). The immunoadjuvant, Addavax, replaced the TMG used in the previous study as it was easier to accurately administer equal amounts between mice. Then on weeks 0 and 4, mice were injected with identical doses of free ErbB-2 peptide, denoted 'E'; or liposome formulations (O), E(), or E(O). Weekly plasma samples were collected from the day of the first liposomal vaccination, which were subsequently tested for anti-ErbB-2_{266–296} antibody responses (see methods sections 3.2.7 and 3.2.8). On week 5, mice were challenged with the TUBO tumour cell line. Tumours were allowed to grow for 3 weeks with monitoring of growth by calliper measurement (see methods section 3.2.9) before sacrifice and analysis.

Figure 3.5 B-H shows the anti-ErbB-2_{266–296} total IgG titres of individual mice for the duration of the experiment. At week 0 (figure 3.5 B), the day of initial liposomal dose, there was no detectable antibody response, showing that the mice do not have detectable levels of pre-existing IgG towards the peptide target in accordance with the rat origin [263] of the sequence. Just 1 week after vaccination (figure 3.5 C), 9/10 mice in the OVA+E(O) group generated detectable anti-ErbB-2_{266–296} antibody levels, with a geometric mean titre (GMT) at least 10-fold higher than any other group. At week 2 (figure 3.5

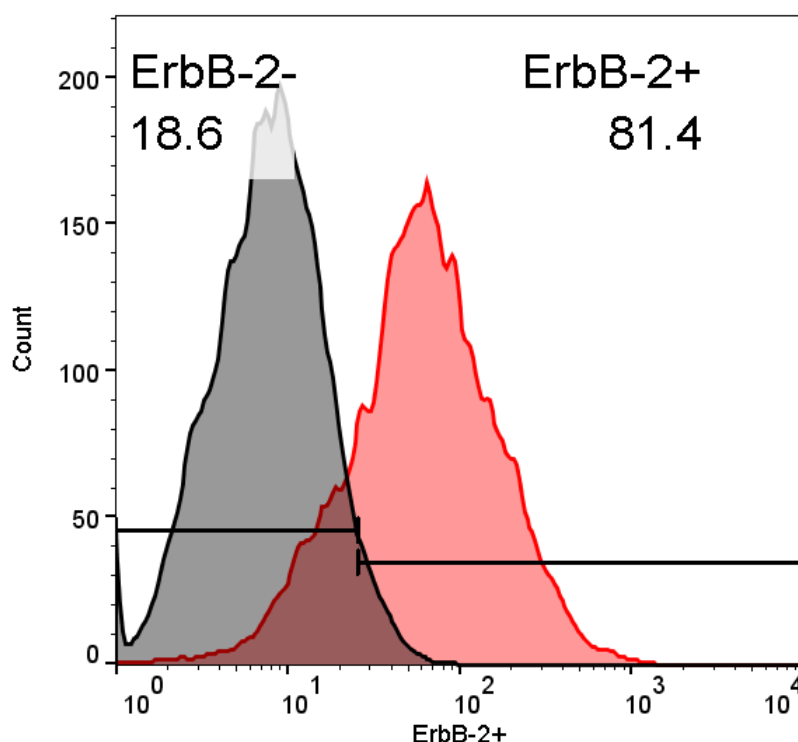


Figure 3.4: Flow cytometry of TUBO murine mammary cancer cells expression of rat ErbB-2 receptor. Grey histogram is unstained cells, while the red histogram is cells incubated with anti-rat ErbB-2 conjugated to AF488. Each histogram represents the results of 10,000 events. Gating was chosen so that 5% of unstained cells were ErbB-2 false-positive. Profile representative of $n=3$.

D), all mice in the PBS+E(O) groups had also generated detectable antibody levels, though these levels were substantially (7-fold) and significantly ($p<0.01$) lower than those achieved in the OVA+E(O) group. By week 3 (figure 3.5 E), the level of anti-ErbB-2_{266–296} antibodies in the OVA+E(O) group had reached a plateau, while those in the PBS+E(O) group continued to show week-on-week increases, although levels still did not surpass those of the OVA+E(O) group. Between weeks 3 and 4, all mice received a booster liposomal vaccine, identical to their first. At week 4, the OVA+E(O) groups antibody levels were maintained, while the PBS+E(O) groups levels continued to rise (figure 3.5 F). The effect of the booster vaccine was clearly shown at week 5, with the OVA+E(O) and PBS+E(O) groups GMT rising 7- and 4-fold, respectively,

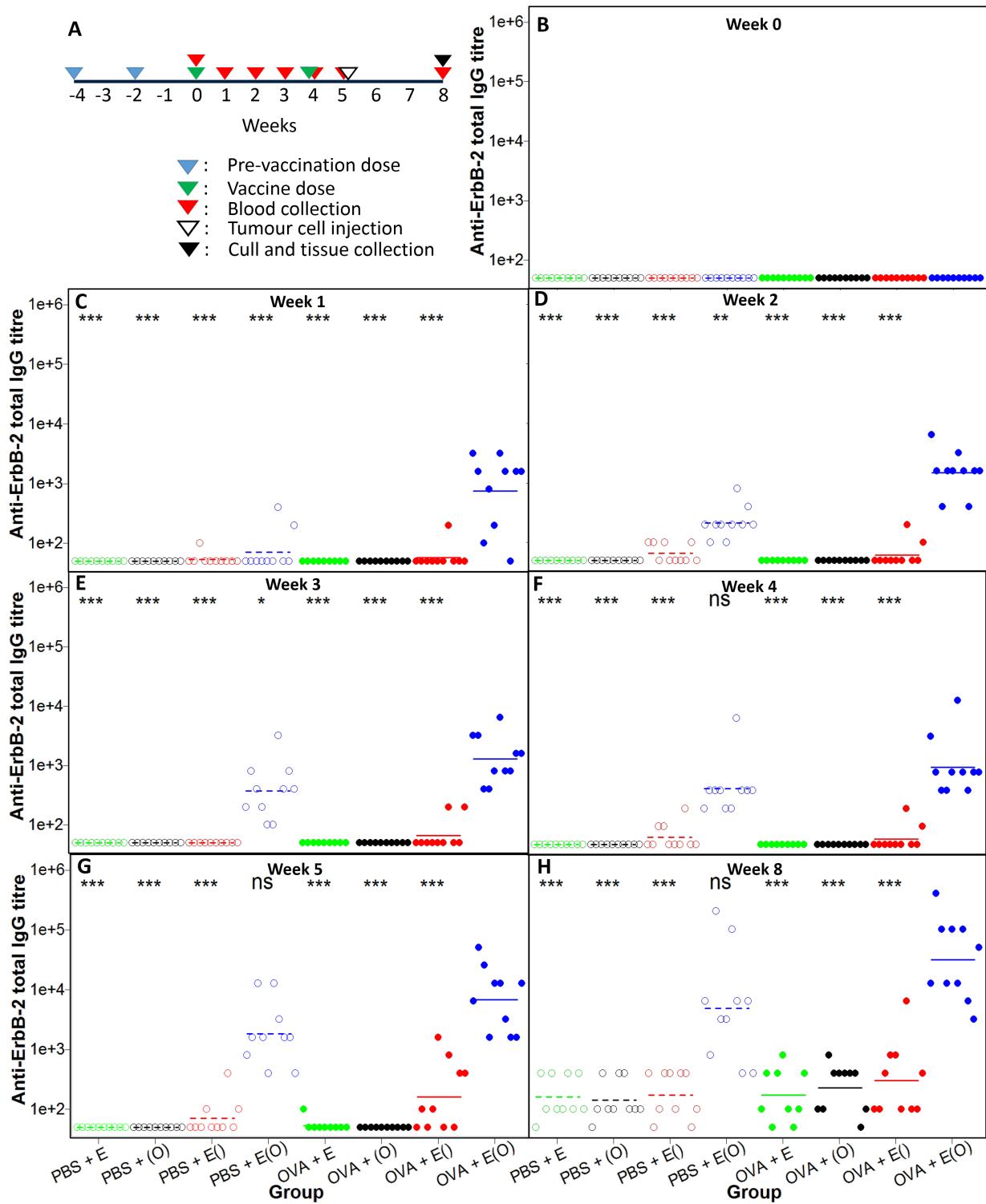


Figure 3.5: Anti-ErbB-2 IgG titres in BALB/c mice in larger-cohort study: weekly plasma samples were taken from mice and tested by ELISA to detect an antibody response to the ErbB-2 peptide. Panel A shows a timeline of the experiment and panels B-H show weekly titres of individual mice from the day of first liposomal vaccination (Panel B) to cull (Panel H). Kruskal-Wallis statistical tests were used to find if differences existed between all groups, followed by Conover-Iman tests to find pairwise differences between groups. Lines represent geometric means of $n=10$. Indicators of statistical significance are shown for comparisons between the OVA+E(O) group and all other groups: ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

from week 4 (figure 3.5 G). A rise in those vaccinated with E() liposomes can also be seen. At week 5, mice were challenged with TUBO tumour cells to monitor the ability of each group to reduce tumour take rate and growth. At the time of cull, OVA+E(O) and PBS+E(O) mice had antibody levels 104- and 16-fold greater than all remaining groups, respectively (figure 3.5 H). After tumour challenge, anti-ErbB-2_{266–296} antibodies could be detected in groups that had not previously been exposed to the ErbB-2 peptide, confirming the presence of the peptide sequence on the TUBO tumour cells post-implant and its immunogenicity.

From this basic analysis it was evident that the OVA+E(O) strategy provided substantial and significant advantage over all groups, including PBS+E(O), especially at earlier time points. The nature of the antibody response was then probed further. Specifically, antibody responses of PBS+E(O) and OVA+E(O) groups were further characterised by quantification of IgM and IgG isotype concentrations in whole blood (figure 3.6). Antibodies of different isotypes interact with the immune system differently [265–267], therefore class-switching provides a stimulation of multiple different branches of the immune system. For example, murine IgG1's main role is believed to be similar to that of the human IgG4 isotype in limiting inflammatory responses and neutralising target molecules from binding their receptor [266]. IgG2a and IgG2b both have similar functions in mice with both isotypes able to fix complement and having strong interactions with Fc γ R effector cells [266]. IgG2a levels linked to an increase in cytotoxic T cell levels and IgG2b levels linked to T cell independent B cell activation [266]. IgG3 is the earliest produced IgG isotype and is able to fix complement well which leads to a cascade of inflammatory responses [266]. IgM is the first antibody isotype produced after initial antigen exposure [268]. Both groups showed very low natural IgM and IgG3 antibody levels with ErbB-

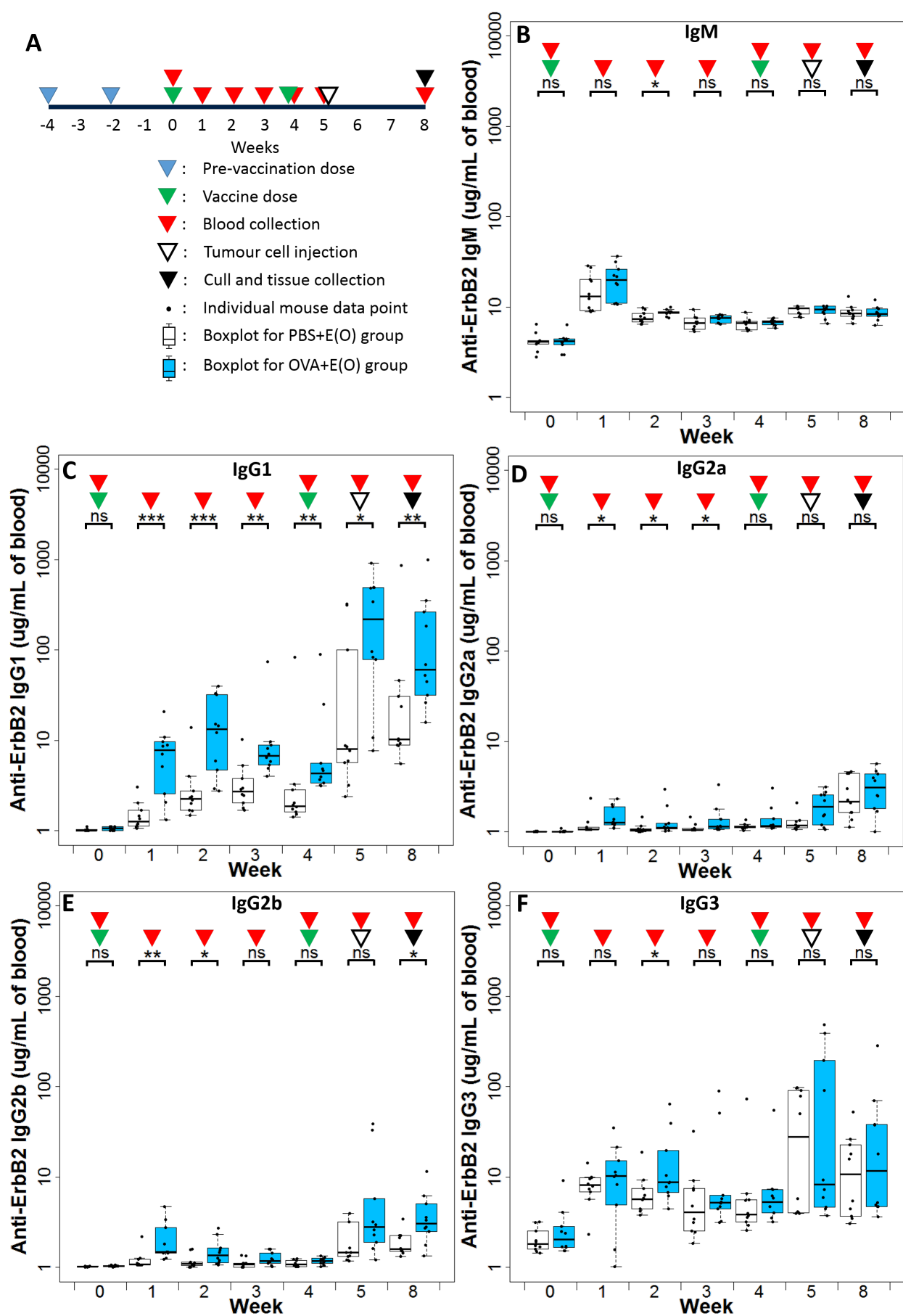


Figure 3.6: Caption located on following page.

Figure 3.6: IgM and IgG isotype concentrations in the blood of BALB/c mice in PBS+E(O) and OVA+E(O) groups were quantified by ELISA. Panel A shows experimental dosing regime and a key for the rest of the figure. Panels B, C, D, E, and F show the boxplots of whole blood concentrations of IgM, IgG1, IgG2a, IgG2b and IgG3, respectively. Each boxplot is made from n=10 data points. To avoid zero values for log-scaling on the y-axis, all data points had 1 added to them. Mann-Whitney U tests were used to compare isotype concentrations between groups for each weeks blood samples. Indicators of statistical significance: ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

$2_{266-296}$ cross-reactivity prior to initial liposomal vaccination (figure 3.6 B and F) but negligible IgG1, IgG2a and IgG2b levels (figure 3.6 C-E). Upon first exposure to liposomal vaccine, both groups generated increased anti-ErbB- $2_{266-296}$ IgM and IgG isotype antibody levels. Anti-ErbB- $2_{266-296}$ IgM levels of both groups remained consistent throughout the duration of the experiment, with a slight boost in concentration at week 5, i.e. after the second liposomal vaccination (figure 3.6 B). The second liposomal vaccination can also be seen to increase IgG isotype concentrations. Pre-existing anti-OVA immunity led to consistently significantly ($p<0.05$) stronger anti-ErbB- $2_{266-296}$ IgG1 levels and initially stronger IgG2a and IgG2b levels (figure 3.6 C-E). Indeed the rapidity of the IgG1, IgG2a, and IgG2b generation in this group is one of the impressive hallmarks of the response achieved using this system. The PBS+E(O) group took (3-4 weeks) longer to class-switch and generate comparable IgG2a and IgG2b levels to the OVA+E(O) group (figure 3.6 D-E). Although able to class-switch to IgG1 antibodies, the PBS+E(O) group consistently produced significantly lower levels than the OVA+E(O) at every time point (figure 3.6 C). Both groups increased anti-ErbB- $2_{266-296}$ IgG3 production after liposomal vaccination, with some mice in both groups increasing production post-booster leading to increased within-group variation of IgG3 levels (figure 3.6 F).

Whilst demonstration of the signal by ELISA confirms target specificity for the rat peptide assurance that the TUBO cells could be targeted was needed.

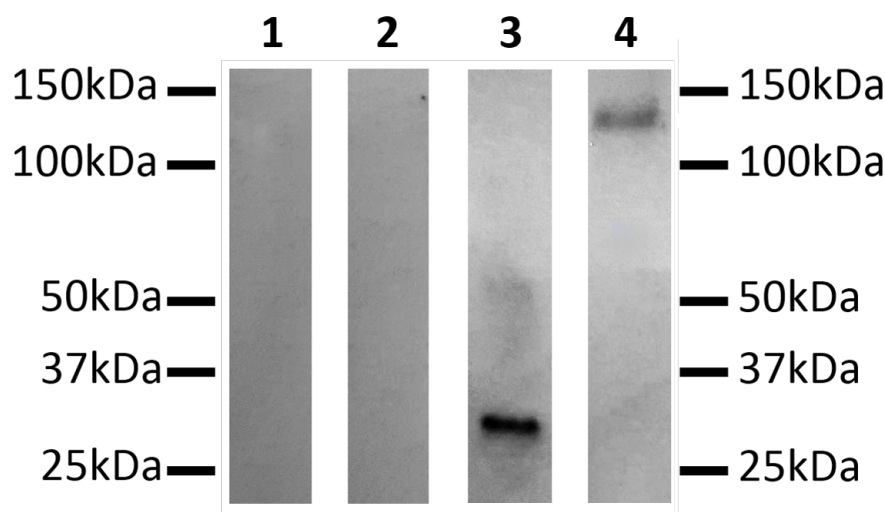


Figure 3.7: Western blots were used to test the ability of plasma from mice with and without anti-ErbB-2_{266–296} antibodies to bind to recombinant ErbB-2 protein and ErbB-2 in cell lysate from *in vitro* TUBO cells. Lanes 1 and 2 were incubated with a 1:1000 dilution of plasma from mice in the PBS+(O) group. While lanes 3 and 4 were incubated with a 1:1000 dilution of plasma from mice in the OVA+E(O) group. Lanes 1 and 3 were loaded with the extracellular domain of a recombinant rat ErbB2 protein while lanes 2 and 4 contain TUBO cell lysate. Recombinant ErbB-2 protein (Abcam, UK: ab191967) used consists of only the external domain of the protein and, therefore, has a molecular weight of 29 kDa while cellular ErbB-2 has an estimated molecular weight of 137 kDa. TUBO cells are a mouse mammary carcinoma cell line that overexpress rat ErbB-2.

Hence, antibodies generated against the ErbB-2 peptide were tested for their ability to bind to recombinant ErbB-2 protein and ErbB-2 in *in vitro* TUBO cell lysate by western blot. Lanes 1 and 2 in figure 3.7 were probed with plasma from naive mice in the PBS+(O) group with no ELISA detectable anti-ErbB-2_{266–296} antibodies, while lanes 3 and 4 were probed with plasma taken from mice in the OVA+E(O) group one week before tumour challenge. Lanes 1 and 3 contain the extracellular domain of a recombinant rat ErbB-2 protein while lanes 2 and 4 contain cell lysate from *in vitro* grown TUBO cells. The recombinant ErbB-2 protein used has a molecular weight of 29 kDa, while cellular ErbB-2 has an estimated molecular weight of 137 kDa. In lane 3, a band can be seen at approximately 30 kDa which corresponds to the extracellular domain of a recombinant rat ErbB-2 protein indicating

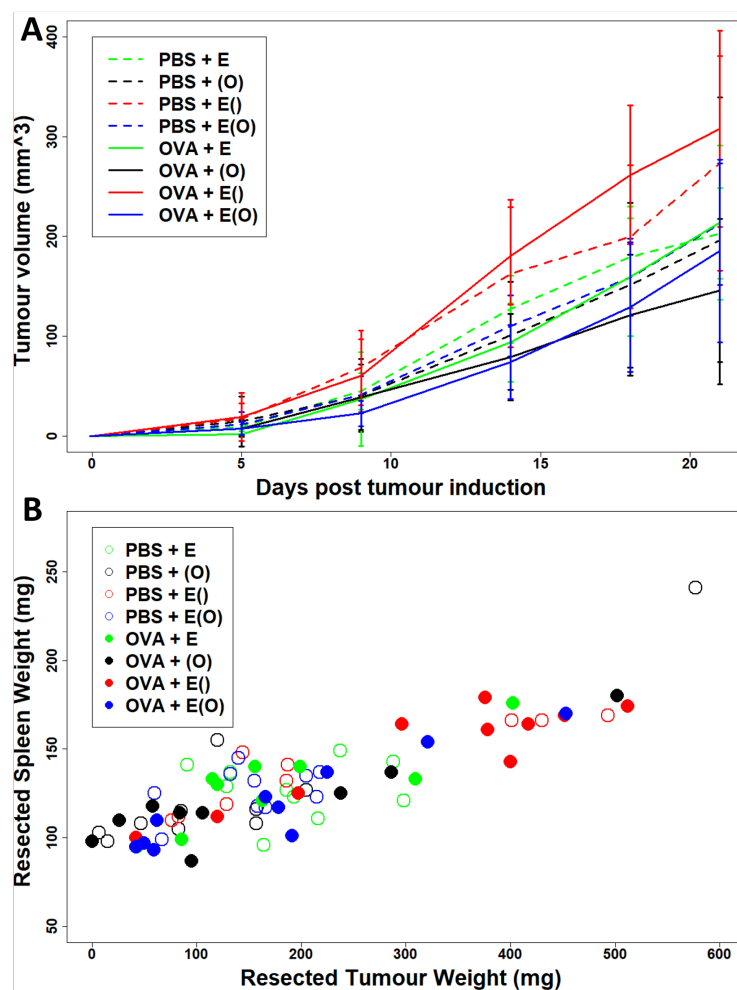


Figure 3.8: Testing resistance to challenge with ErbB-2 overexpressing cells. BALB/c mice were subcutaneously injected with 1×10^5 TUBO tumour cells and checked daily for the presence of tumours. Tumours were measured twice a week with callipers in three orthogonal directions (x, y, and z) with volume calculated as $xyz/2$. Panel A shows the average tumour growth curve, bars representing 95% confidence intervals. Panel B shows the comparison of the weight of tumour and spleen resected from mice at cull.

that the OVA+E(O) treatment produces antibodies that specifically target the desired ErbB-2_{266–296} peptide sequence. This band does not appear in lane 1. Similarly, a band can be seen at approximately 140 kDa in TUBO lysate probed with anti-ErbB-2_{266–296} antibodies (lane 4) but not when anti-ErbB-2_{266–296} antibodies are absent (lane 2), showing the vaccine-generated anti-ErbB-2 antibodies are able to bind to ErbB-2 protein on cells.

Having characterised the level and nature of the antibody response gener-

ated, it was deemed worthwhile to test if the response would provide protection against tumour challenge (figure 3.8). The take rate of the tumours was 100% in all groups, though the OVA+E(O) group was the last to reach this 100% appearance of palpable tumours. Whilst tumour growth profiles largely aligned with expectations, they were characterised by high variability and a slow overall growth rate. Hence no meaningful difference could be observed. It should be noted that while not group-dependent, there is a strong positive correlation between the weights of tumour and spleens at cull, indicating the presence of an immune response against the tumours. Indeed, Blamey and Evans found that rats bearing tumours had enlarged spleens compared to the non-tumour bearing rats and suggest this is likely due to the reaction to foreign antigen and phagocytic activity [269].

3.3.3 Optimisation of the dosing regime

While high levels of anti-ErbB-2_{266–296} antibodies were generated in the larger-cohort animal study (section 3.3.2), they had no significant impact on inhibiting tumour growth, despite their proven ability to bind to the target TUBO cells. In response, the dosing regime was subsequently optimised to increase the anti-ErbB-2 antibody titres in order to increase the chance of achieving inhibition of tumour progression. Three parameters of the dosing regime were optimised: the concentration of ErbB-2 administered; the number of booster doses given; and whether booster doses were liposomal vaccine particles or free ErbB-2 peptide (table 3.4). Changes in formulation method from method B to C (table 2.1) allowed for increased control over the amount of ErbB-2 peptide conjugated to the liposomal surface. This in turn allowed for easy testing of the effect of liposomally-conjugated ErbB-2 concentration administered has on the anti-ErbB-2_{266–296} antibody levels achieved. The effect of an

Group notation	Liposomal vaccination (week 0)	ErbB-2 peptide concentration	Booster dose type	Extra booster at week 1	Number of mice
LowE2	E(O)	Low	Peptide	No	3
LowE3	E(O)	Low	Peptide	Yes	3
LowE(O)2	E(O)	Low	Liposome	No	3
LowE(O)3	E(O)	Low	Liposome	Yes	3
HighE2	E(O)	High	Peptide	No	3
HighE3	E(O)	High	Peptide	Yes	3
HighE(O)2	E(O)	High	Liposome	No	3
HighE(O)3	E(O)	High	Liposome	Yes	3

Table 3.4: Number of mice in each group for optimisation of the dosing regime. Mice first received two identical pre-vaccinations of OVA peptide with immunoadjuvant, Addavax, on weeks -4 and -2. On week 0, mice were dosed with E(O) liposomes containing either a high or low concentration of ErbB-2 on their surface. Mice then received either one (on week 4) or two (on weeks 1 and 4) booster doses of either free ErbB-2 peptide or E(O) liposomes with the same concentration of ErbB-2 as the original vaccination dose on week 0. E(O) denotes liposomes encapsulating OVA peptide, and surface decorated with ErbB-2 peptide. An ErbB-2 peptide concentration of ‘Low’ represents 4 $\mu\text{g/mL}$, while one of ‘High’ represents 100 $\mu\text{g/mL}$. All liposomes were formulated by method C (table 2.1).

extra booster dose, one week after initial dosing was also tested. Furthermore, using liposomal vaccine particles as a booster may not be necessary due to the anti-ErbB-2_{266–296} responses already developed, and thus free ErbB-2 may be sufficient to boost the responses. This approach would reduce costs if it were to achieve translation.

All mice first received two identical pre-vaccinations of OVA peptide with immunoadjuvant, Addavax, on weeks -4 and -2 (figure 3.9A). On week 0, mice were dosed with E(O) liposomes containing either a high or low concentration

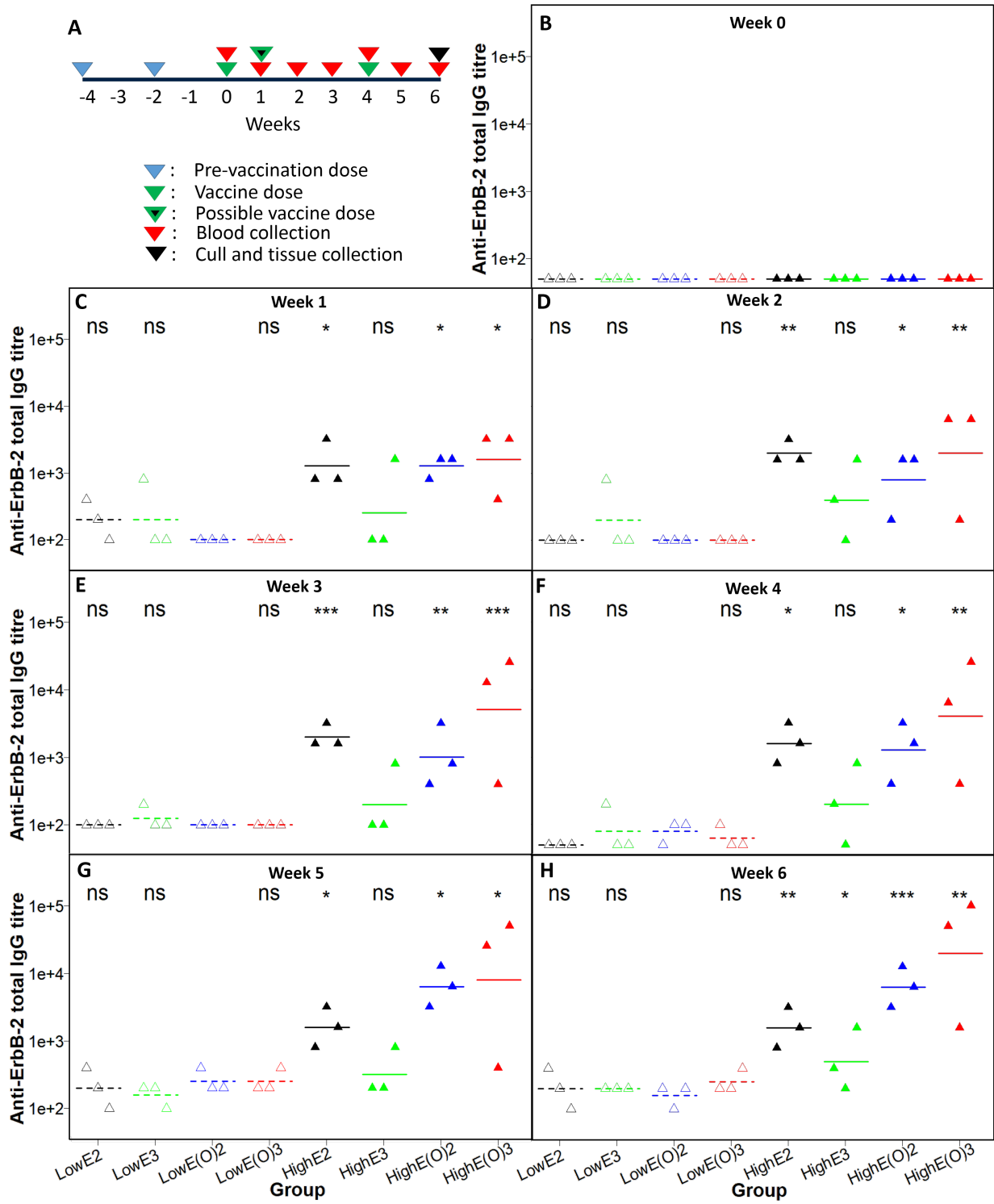


Figure 3.9: Anti-ErbB-2 IgG titres in BALB/c mice to find a more optimal dosing regime. Weekly plasma samples were taken from mice and tested by ELISA to detect an antibody response to the ErbB-2 peptide. Panel A shows a timeline of the experiment and panels B-H show weekly titres of individual mice from the day of first liposomal vaccination (Panel B) to cull (Panel H). Kruskal-Wallis statistical tests were first used to find if differences existed between all groups, followed by Conover-Iman tests to find pairwise differences between groups. Lines represent geometric means of $n=3$. Indicators of statistical significance are shown for comparisons between the LowE(O)2 group and all other groups: ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

of ErbB-2 on their surface (table 3.4). Mice then received either one (on week 4) or two (on weeks 1 and 4) booster doses (figure 3.9A) of either free ErbB-2 peptide or E(O) liposomes with the same concentration of ErbB-2 as the original vaccination dose given on week 0 (table 3.4).

Weekly blood samples were taken to monitor anti-ErbB-2_{266–296} antibody titres (figure 3.9). The LowE(O)2 group had an identical dosing regime and amount (4 μ g/mL) to that of the OVA+E(O) group in the previous larger-cohort study (section 3.3.2). However, it was observed that their anti-ErbB-2 titre levels were much lower than seen previously. Controls were run using sample from studies in section 3.3.2 to verify no change to ELISA sensitivity had occurred. This decrease in response may be due to the presence of contaminants from the extrusion kit used in liposomal formulation methods A and B acting as extra adjuvants in previous studies. The switch to disposable extrusion cassettes for the study may, therefore, provide enhancements to production speed, conjugation efficiency and sample purity but at a cost of lower immunogenicity (although such contamination does not lend itself to translation).

Of the 12 mice primed with the high level of ErbB-2_{266–296}, 10 showed a substantial response just 1 week after dosing (figure 3.9). Booster doses of free ErbB-2 peptide did not lead to increases in titre levels when compared to booster doses of E(O) liposomes (figure 3.9). The inclusion of a second booster dose did not lead to a significant increase in titre levels. However, the HighE(O)3 group, after receiving their first booster dose on week 1, consistently had two members that produced the highest titres, whilst one of the mice showed only a mild response to treatment.

Anti-ErbB-2_{266–296} antibody responses of the LowE(O)2 (white box, included as a control), HighE(O)2 (blue box) and HighE(O)3 (red box) groups

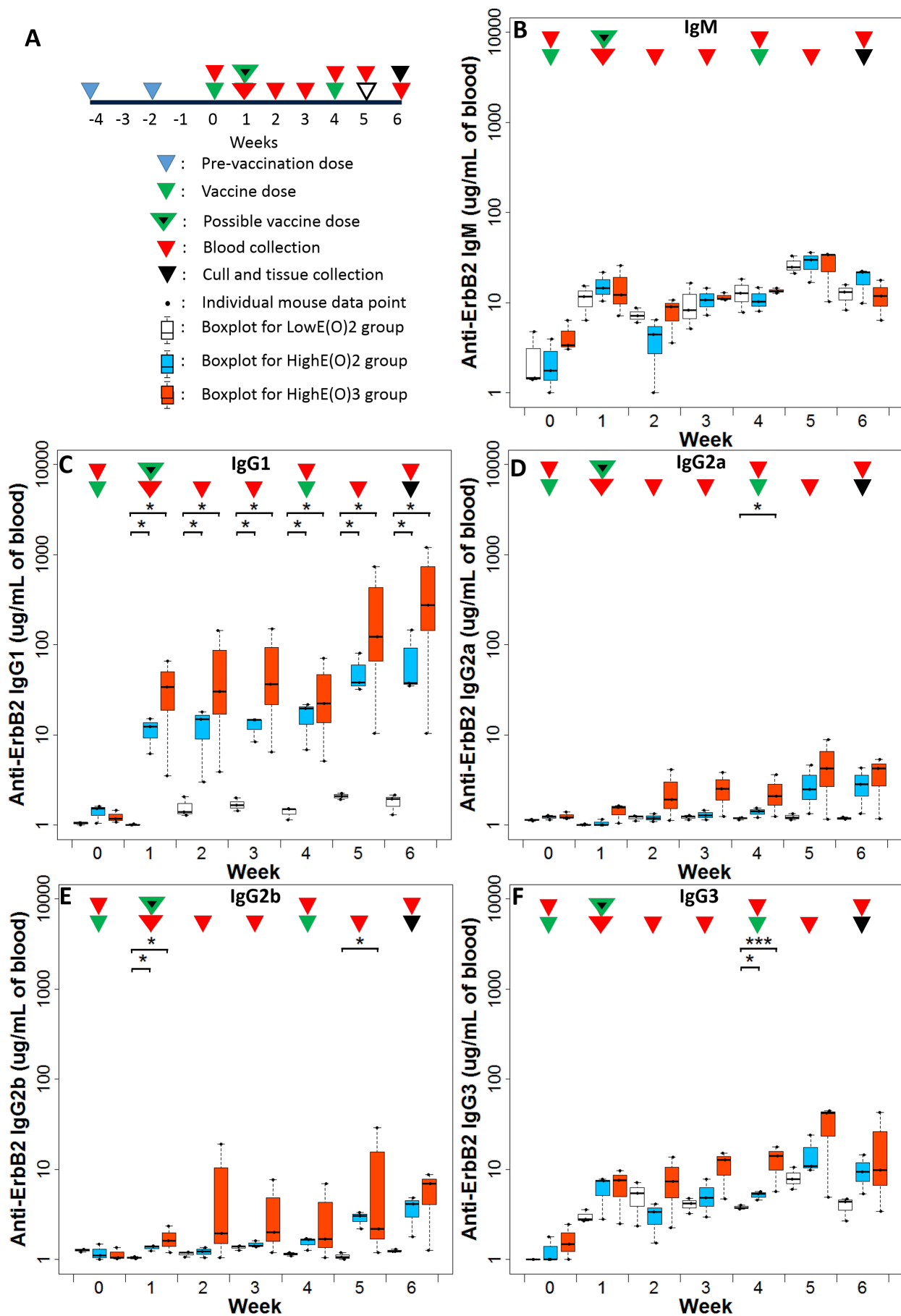


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Figure 3.10: IgM and IgG isotype concentrations in the blood of BALB/c mice in LowE(O)2, HighE(O)3, and HighE(O)2 groups were quantified by ELISA to determine an optimal dosing regime. Panel A shows experimental dosing regime and a key for the rest of the figure. Panels B, C, D, E, and F show the boxplots of whole blood concentrations of IgM, IgG1, IgG2a, IgG2b and IgG3, respectively. Each boxplot is made from $n=3$ data points. To avoid zero values for log-scaling on the y-axis, all data points had 1 added to them. Kruskal-Wallis and Conover-Iman tests statistical tests were used to find if differences existed between groups for each weeks blood samples. Indicators of statistical significance are shown for comparisons between the LowE(O)2 group and all other groups: *, $p<0.05$; ** $p<0.01$; ***, $p<0.001$.

were further characterised by measurement of IgM and IgG isotype concentrations in whole blood (figure 3.10). All three groups showed a low level of natural IgM antibodies with ErbB-2_{266–296} cross-reactivity prior to initial liposomal vaccination (figure 3.10 B) but negligible IgG1, IgG2a, IgG2b and IgG3 levels (figure 3.10 C-F). Upon first exposure to liposomal vaccine, all groups generated increased anti-ErbB-2_{266–296} IgM antibody levels (figure 3.10 B). While the week 1 booster received by the HighE(O)3 group appears to have had no effect on the IgM levels (figure 3.10 B), a further increase in IgM levels can be seen after the week 4 booster in all groups. High ErbB-2 concentration and extra dosing led to consistently stronger anti-ErbB-2_{266–296} IgG1 levels with levels in one mouse reaching 1.2 mg/mL in whole blood (figure 3.10 C). Similarly to the total IgG titres shown in figure 3.9, it can be seen that two of the mice in the HighE(O)3 group generated the strongest observed IgG1 responses, while one mouse generated only a mild response (figure 3.10 C). IgG2a and IgG2b class-switching was relatively low, occurring only in those dosed with high ErbB-2 concentrations, after receiving their respective first booster dose (figure 3.10 D-E). Anti-ErbB-2_{266–296} IgG3 levels increased after first exposure to the vaccine particles, with a further increase in all groups after the week 4 booster dose (figure 3.10 F).

It has, therefore, been observed that administration of doses containing

higher concentrations of ErbB-2 peptide led to increased antibody titres, and that boosting with liposomal vaccine particles is more effective than boosting with free peptide. However, with such small group sizes it is difficult to detect differences in similar responses, such as those in the HighE(O)2 and HighE(O)3 groups. While the average overall responses of the HighE(O)2 and HighE(O)3 groups were comparable throughout the duration of the experiment, two members of the HighE(O)3 group were consistently the highest responders. It remains unknown as to why the third member of this group did not respond similarly. As three vaccination doses were needed by Allen, et al., who first described the peptide ErbB-2_{266–296}, to demonstrate tumour growth inhibition in BALB/c mice [63] the HighE(O)3 group was chosen as the optimal dosing regime to take forward.

3.3.4 Larger-cohort tumour challenge study with optimised dosing regime

Once it had been established that higher and extra dosing using samples prepared by method C (table 2.1) could provide a titre advantage, this regime was tested with a larger sample size and tumour challenge. Hence, a repeat of the larger-cohort study in section 3.3.2 with the optimal dosing regime found in section 3.3.3 was next explored to see if anti-ErbB-2_{266–296} antibody levels could be increased, to subsequently inhibit tumour growth (table 3.5). Due to the comparable responses of the PBS+E and OVA+E groups in the previous larger-cohort study, only the OVA+E control group was used in this study, similarly for the PBS+E() and OVA+E() groups (table 3.5). As the PBS+(O) and OVA+(O) had never shown signs of anti-ErbB-2_{266–296} antibody responses prior to tumour challenge, they were replaced with a PBS+PBS control (table 3.5). The dosing regime used was HighE(O)3, as found in section 3.3.3. All

Pre-vaccination	Liposomal vaccination	Group notation	Number of mice
PBS + Addavax	PBS	PBS+PBS	8
	E(O)	PBS+E(O)	8
OVA + Addavax	E	OVA+E	8
	E()	OVA+E()	8
	E(O)	OVA+E(O)	8

Table 3.5: Number of mice in each group for larger-cohort vaccination study with an optimised dosing regime. Addavax is an immunoadjuvant similar to MF59; OVA is a peptide of Ovalbumin; E denotes free ErbB-2 peptide; E(), liposomes surface decorated with ErbB-2 peptide; E(O), OVA peptide encapsulating, ErbB-2 peptide surface decorated liposomes. Liposomes were formulated by method C (table 2.1) and mice received 10 μ g of ErbB-2 peptide.

mice first received two identical pre-vaccination doses of either PBS or 10 μ g OVA peptide with the immunoadjuvant Addavax, at weeks -4 and -2. Then on weeks 0, 1, and 4 mice were injected with identical doses of PBS, E, E(), or E(O). Weekly plasma samples were collected for the first 6 weeks, from week 0 onwards, and then one final time at cull. On week 5, mice were challenged with TUBO tumour cells. Tumours were allowed to grow for 25 days before sacrifice.

Figure 3.11 B-H shows the anti-ErbB-2_{266–296} total IgG titres of individual mice over the duration of the experiment. At week 0 (figure 3.11 B), the day of initial liposomal dose, there was no detectable antibody response, showing that the mice do not have a detectable pre-existing humoral IgG response towards the peptide target. After just 1 week (figure 3.11 C), all mice in the OVA+E(O) (solid blue circles) group generated high antibody levels (GMT of 2467), with the PBS+E(O) (empty blue circles) and OVA+E() (solid red circles) groups also demonstrating a detectable antibody response with a GMT 7.3-, and 9.5-

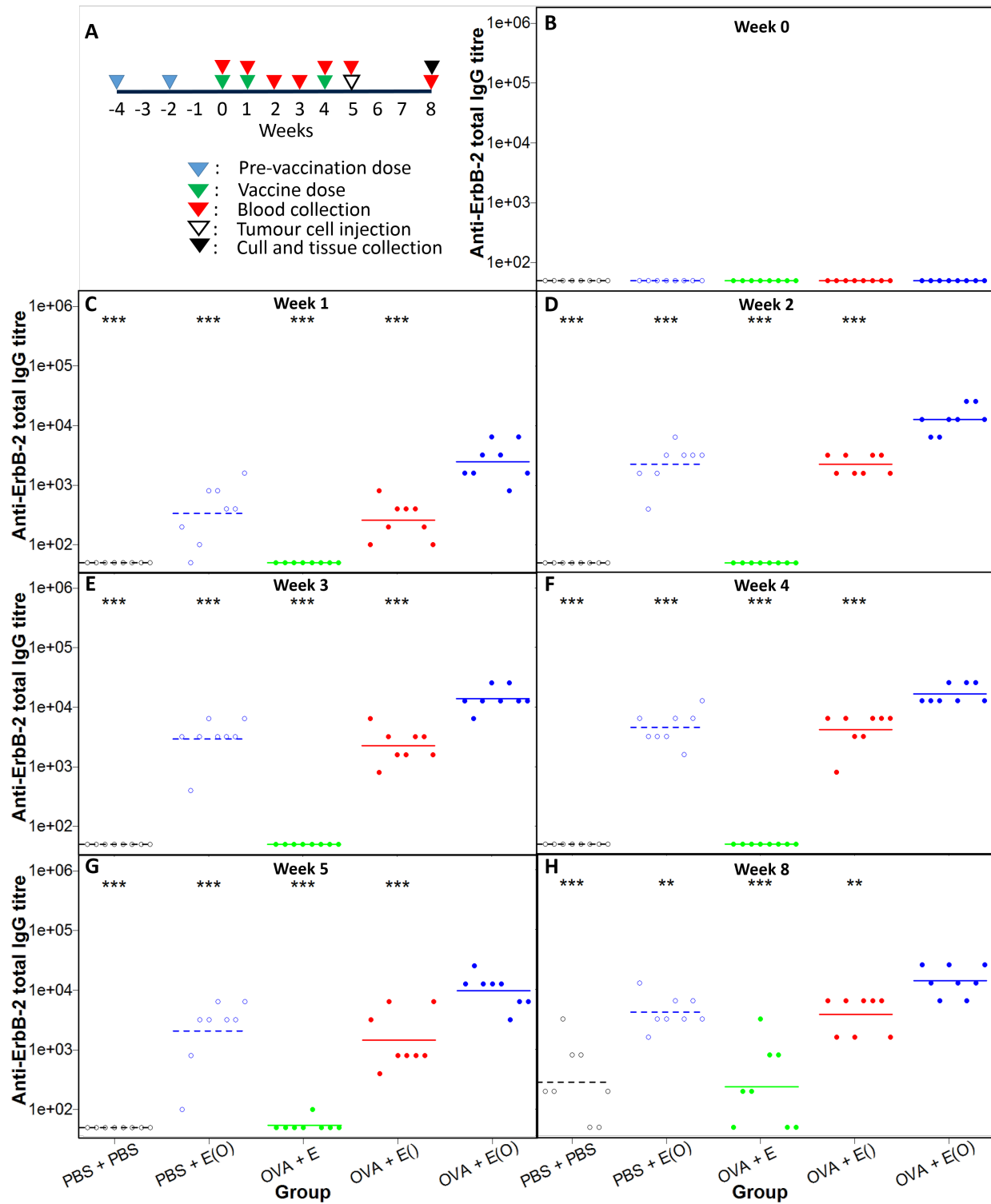


Figure 3.11: Anti-ErbB-2 IgG titres in BALB/c mice for a larger-cohort vaccination study with an optimised dosing regime. Weekly plasma samples were taken from mice and tested by ELISA to detect an antibody response to the ErbB-2 peptide. Panel A shows a timeline of the experiment and panels B-H show weekly titres of individual mice from the day of first liposomal vaccination (Panel B) to cull (Panel H). Dosing regime used was HighE(O)3, as found in section 3.3.3. Kruskal-Wallis statistical tests were used to find if differences existed between all groups, followed by Conover-Iman tests to find pairwise differences between groups. Lines represent geometric means of $n=8$. Indicators of statistical significance are shown for comparisons between the OVA+E(O) group and all other groups: ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

fold lower than those in the OVA+E(O) group, respectively. On week 1, mice also received their first booster dose. At week 2 (figure 3.11 D), one week post first boost, the IgG levels of all liposomally vaccinated mice increased, with both the PBS+E(O) and OVA+E() vaccinated groups having GMTs 5.7-fold lower than the OVA+E(O) vaccinated group (GMT of 12800). These titres were maintained through to week 4 (figure 3.11 E-F) when the third and final dose was given. At week 5, the third liposomal dose did not appear to have any effect in increasing the antibody levels further (figure 3.11 G). At week 8, the antibody levels of the liposomally vaccinated mice increased marginally, while those in the PBS+PBS group showed detectable anti-ErbB-2₂₆₆₋₂₉₆ antibody titres for the first time, once again demonstrating the immunogenicity of the peptide sequence on the native protein on the tumour cells (figure 3.11 H). To summarise, this data adds further evidence to the value of the approach as a maximal response is only achieved when a peptide with pre-existing immunity against it is encapsulated within the liposome.

Antibody responses in PBS+E(O), OVA+E(), and OVA+E(O) groups were further characterised by IgM and IgG isotype concentrations in whole blood (figure 3.12). All groups showed low levels of natural IgM and IgG3 antibodies with ErbB-2₂₆₆₋₂₉₆ cross-reactivity prior to initial liposomal vaccination (figure 3.12 B and F) but negligible IgG1, IgG2a and IgG2b levels (figure 3.12 C-E). Upon first exposure to the vaccine particles, all groups generated increased anti-ErbB-2₂₆₆₋₂₉₆ IgM and IgG isotype antibody levels. Anti-ErbB-2₂₆₆₋₂₉₆ IgM levels of all three groups remained relatively consistent throughout the duration of the experiment (figure 3.12 B). The IgG3 concentrations increased with each vaccine dose for each group. Mice dosed with E(O) liposomes generally had higher IgG3 levels than those which received E() liposomes, indicating that the presence of the OVA peptide, regardless of pre-existing immunity to-

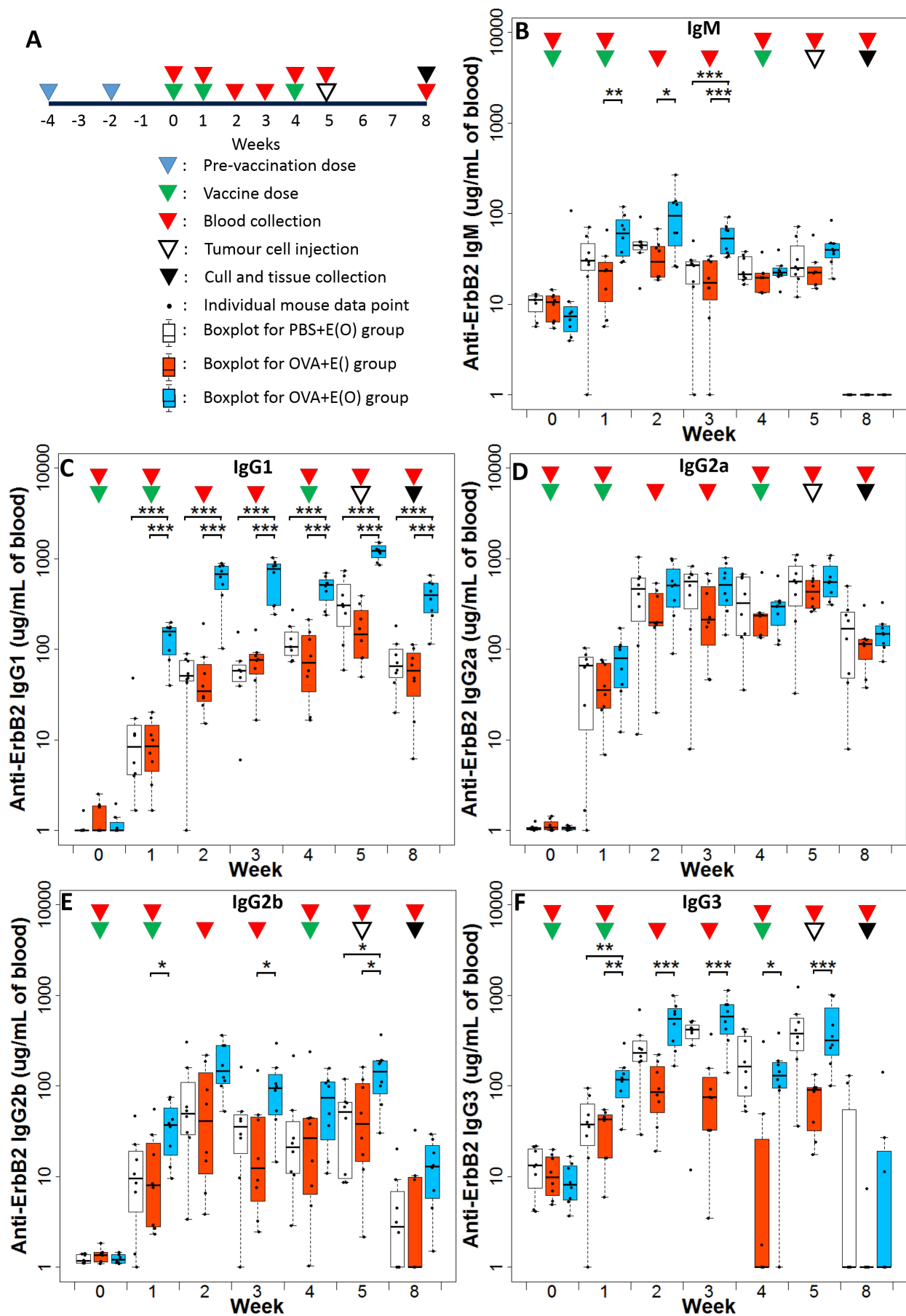


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Figure 3.12: IgM and IgG isotype concentrations in the blood of BALB/c mice in the PBS+E(O), OVA+E(), and OVA+E(O) groups were quantified by ELISA. Panel A shows experimental dosing regime and a key for the rest of the figure. Panels B, C, D, E, and F show the boxplots of whole blood concentrations of IgM, IgG1, IgG2a, IgG2b and IgG3, respectively. Each boxplot is made from $n=8$ data points. To avoid zero values for log-scaling on the y-axis, all data points had 1 added to them. Kruskal-Wallis and Conover-Iman tests statistical tests were used to find if differences existed between groups for each weeks blood samples. Indicators of statistical significance are shown for comparisons between the OVA+E(O) group and all other groups: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

wards it, aided in the production of anti-ErbB-2_{266–296} IgG3 (figure 3.12 F). The OVA+E(O) group showed high levels of IgG1 class-switching compared to other groups, leading to consistently stronger anti-ErbB-2_{266–296} IgG1 levels (figure 3.12 C). All groups showed strong IgG2a and IgG2b class-switching compared to those seen in the previous larger-cohort study in section 3.3.2 (figure 3.12 D-E). However, minimal difference between IgG2a and IgG2b levels from samples from different treatment groups was observed, indicating that this increase may simply be due to the increased concentration of ErbB-2 in this study.

Once the level and nature of the antibody response generated had been characterised again, the response was tested to see whether the raised antibody response was capable of providing cell kill of ErbB-2 overexpressing cells. Plasma from mice, both positive and negative for anti-ErbB-2_{266–296} antibodies, was tested *in vitro* for ability to induce cell death. Figure 3.13 shows that increasing the concentration of anti-ErbB-2_{266–296} antibody positive plasma increases the amount of cell death, whereas equal concentrations of antibody negative plasma or PBS does not. Flow cytometry of cells challenged only with media showed that $82.2\pm2.1\%$ of events were in the live cell population (by size and granularity), whereas cells challenged with PBS or anti-ErbB-2_{266–296} negative plasma had a marginal decrease in the proportion of live cells with $68.9\pm1.0\%$ and $65.8\pm0.7\%$ events, respectively, showing as non-debris (figure

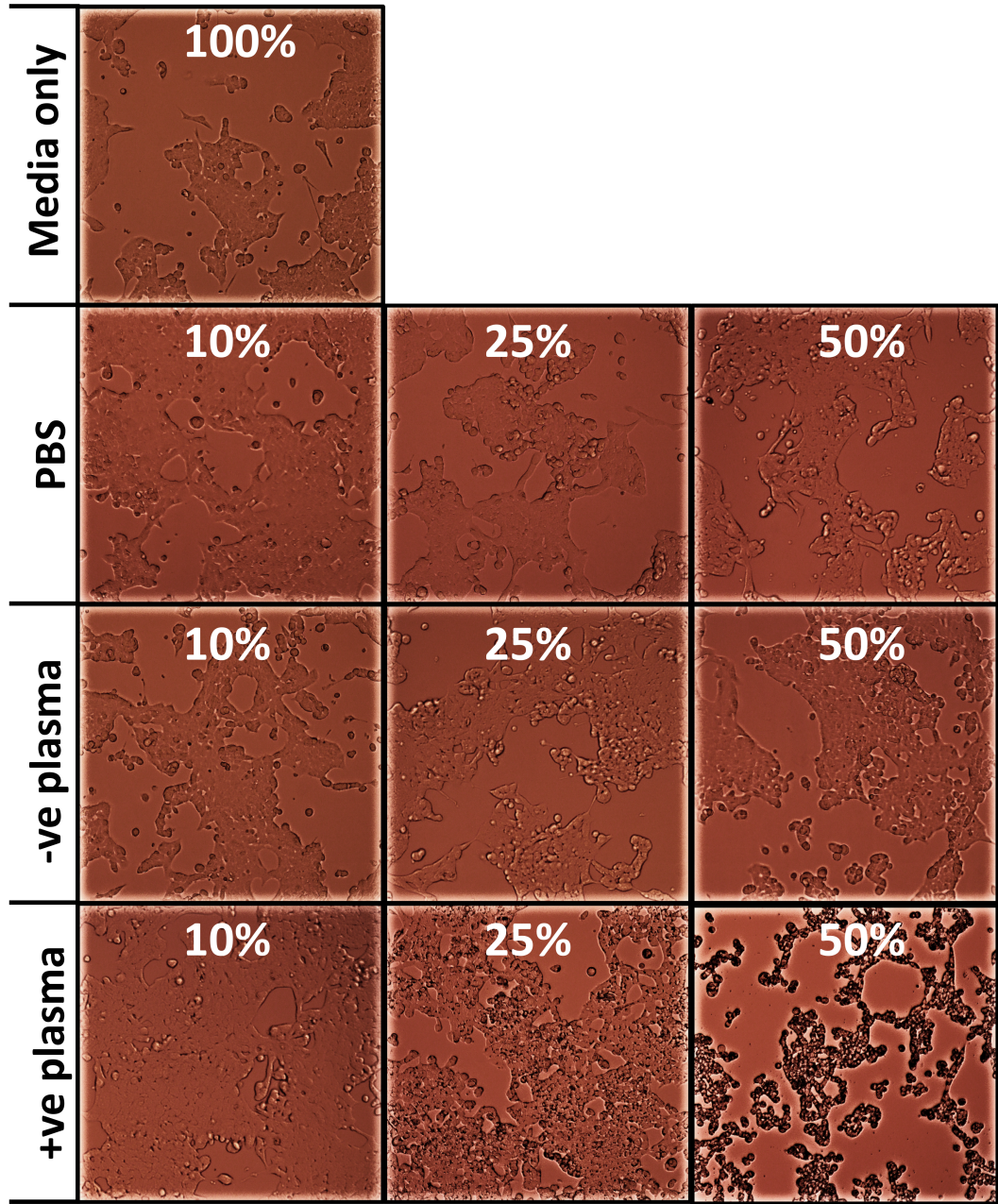


Figure 3.13: Cell death of TUBO cells incubated with varying dilutions of PBS or anti-ErbB-2_{266–296} positive or negative plasma. Negative plasma was pooled plasma from pre-vaccine blood samples, while positive plasma was pooled plasma from mice in the OVA+E(O) group prior to tumour challenge. Starting plasma samples were at a 1:10 dilution and complement-deactivated. Images shown are representative of 5 replicate samples. Cell death was assessed by visual inspection and non-debris gating by flow cytometry (figure 3.14).

3.14A-C). Cells challenged with anti-ErbB-2_{266–296} positive plasma from the OVA+E(O) group, however, showed that only $4.0 \pm 0.2\%$ of events were live cells (figure 3.14D). Notably the plasma was heat-treated and there were no

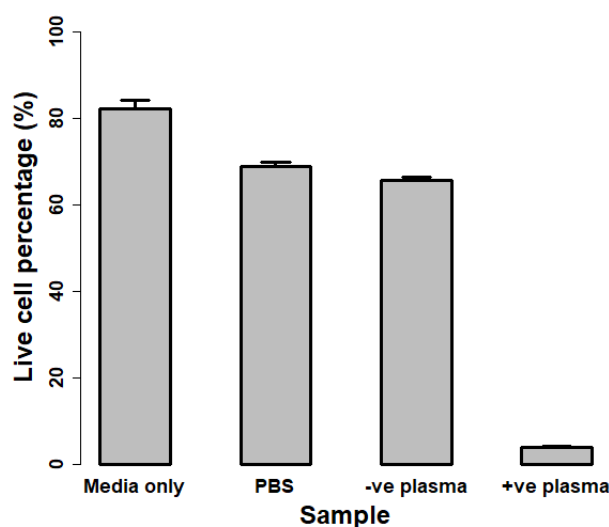


Figure 3.14: Histogram of live cells found by flow cytometry of TUBO cells incubated with media only, a 1:1 dilution of PBS in media, or anti-ErbB-2_{266–296} negative or positive plasma. Negative plasma was pooled plasma from pre-vaccine blood samples, while positive plasma was pooled plasma from mice in the OVA+E(O) group prior to tumour challenge. Plasma samples were complement-deactivated and diluted 1:1 with fresh media and thus had a final dilution of 1:20. Data represents the average (n=3) proportion of events contained within the forward scatter-side scatter debris gating, ± 1 standard deviation.

leukocytes present so this only accounts for the direct (non ADCC or CDC) mediated death that could be achieved [270].

After it had been demonstrated that the optimised vaccination regimen produced an antibody response which could directly kill ErbB-2 expressing cells *in vitro*, it was hypothesised that response would be effective in preventing tumour growth *in vivo*. This was because the benefit of ADCC and CDC could be added to the direct killing mechanism the anti-ErbB2 antibodies. This time a third of the tumour cell number was administered (3.4×10^4 vs 1×10^5) to reduce the proliferation of the tumours and give the immune system an increased chance at inhibiting tumour growth (figure 3.15). The take rate of the tumours was reduced, with 3 mice having no signs of palpable tumours at the time of cull. The reduction in take rate was unlikely to be due to treatment as 2 of the mice without tumours were in the PBS+PBS group, while the remaining mouse was in the OVA+E(O) group. With the exception of the PBS+PBS group, tumour

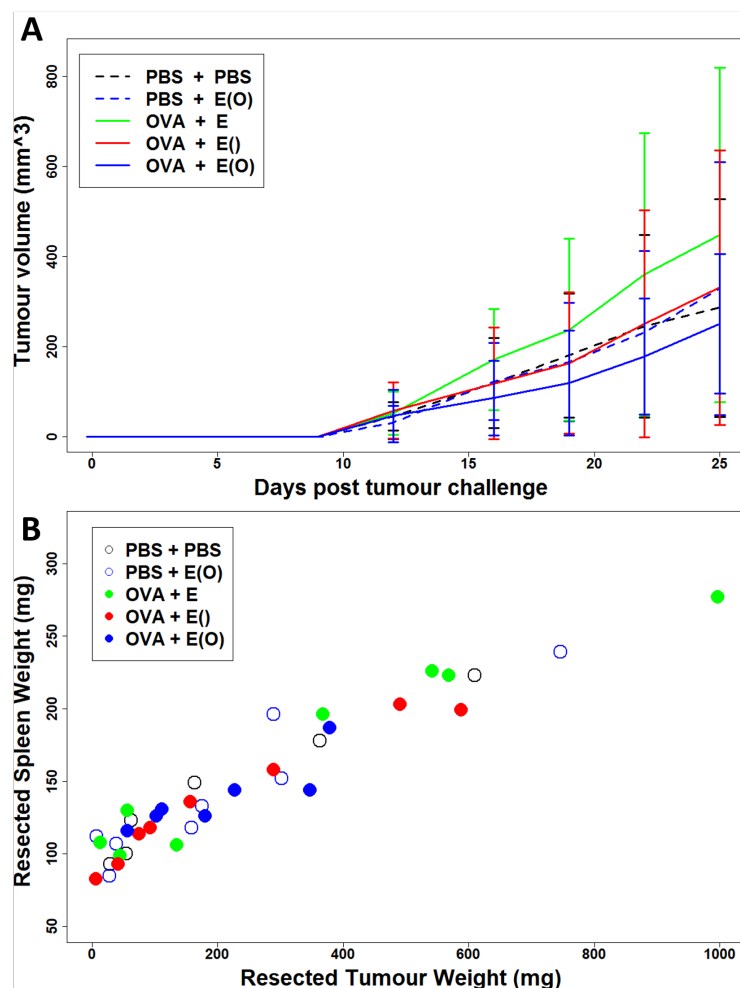


Figure 3.15: BALB/c mice were subcutaneously injected with 3.4×10^4 TUBO tumour cells and checked daily for the presence of tumours. Tumours were measured twice a week with callipers in three orthogonal directions (x, y, and z) with volume calculated as $xyz/2$. Panel A shows the average tumour growth curve, bars representing 95% confidence intervals. Panel B shows the comparison of the weight of tumour and spleen resected from mice at cull. The take rate of the tumours did not reach 100%, with 3 mice having no signs of palpable tumours at the time of cull. These mice are not included in the above figure as the lack of tumour was likely due to insufficient number of cells being used to challenge.

growth profiles largely aligned with expectations, however, there was high within-group variability (figure 3.15). Hence no significant difference could be observed. As seen previously (figure 3.8), while not group-dependent, there was again a strong positive correlation between the weights of tumour and spleens at cull, indicating the presence of an immune response against the tumours (figure 3.15).

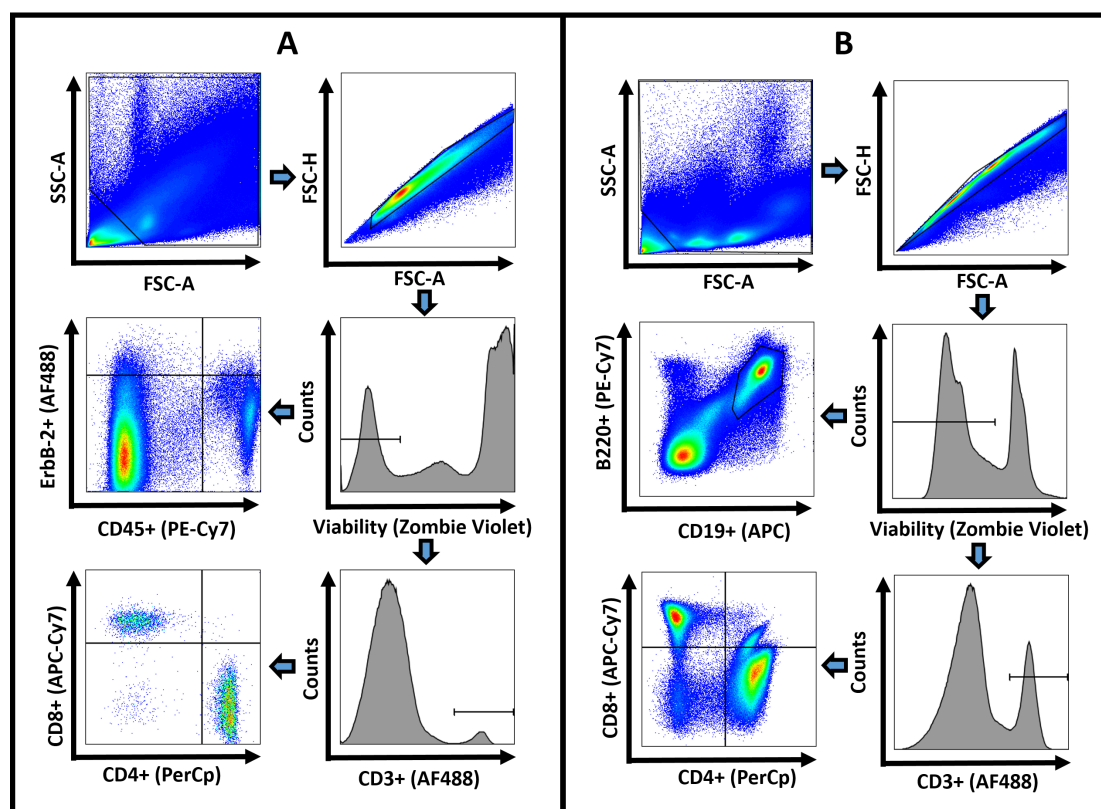


Figure 3.16: Gating strategy for flow cytometry of isolated single-cell suspensions from resected tumours (A) and spleens (B) of BALB/c mice. Tumours and spleens were resected from 4 mice in each group with the largest tumours and processed into single-cell suspension as described in section 3.2.3. Gating strategies were chosen based on comparing unstained, single-stained and full-minus-one (FMO) stained samples. Images shown here are the result of 20 samples pooled together for each respective tissue type.

At week 8, the 4 largest tumours in each group were resected, along with the corresponding mouse's spleen, for cellular analysis by flow cytometry. Resected tumours were processed into single-cell suspensions (see methods section 3.2.3) and examined for ErbB-2 expression on non-immune cells (i.e. CD45-ve) and for the presence of tumour-infiltrating lymphocytes. Meanwhile, isolated splenocytes were tested for B cell populations (B220+CD19+), helper T cell populations (CD3+CD4+), and cytotoxic T cell populations (CD3+CD8+). Figure 3.16 shows the gating strategy for single-cell suspensions of tumour and spleen samples. Images shown in figure 3.16 are the result of concatenating 20 samples. Notably, the majority of cells isolated from the tumours are ErbB-2-

ve CD45-ve and thus likely consist mostly of tumour cells that have lost their ErbB-2 overexpression since implantation or those that were selected for their resistance to anti-ErbB-2 antibody attack (figures 3.4 and 3.16A). In the concatenated samples of splenocytes, a distinct double positive CD4+CD8+ population can be seen (figure 3.16B). Double positive CD4+CD8+ T cells have been well-described in T cell developmental stages in the thymus before differentiating into the single-positive CD4+CD8- or CD4-CD8+ lineages. However, the function of double positive CD4+CD8+ T cells outside of the thymus is unknown, with conflicting reports describing cytotoxic or suppressive roles for these cells for different diseases or even types of cancer [271].

Figure 3.17A shows the average percentage populations of cell types isolated from resected tumours for each group. As seen in the pooled samples of figure 3.16A, a very small proportion of live cells were ErbB-2+CD45- (0.8-3.0%), indicating the vast majority of tumour cells recovered from these tumours have lost ErbB-2 overexpression since being implanted. The majority of isolated cells (67-98%) were ErbB-2-ve and CD45-ve and so were likely mainly consisting of fibroblasts and tumour cells that have lost their ErbB-2 expression. This may, therefore, be the reason for the lack of any significant difference between the tumour growth curves of any treatment group (figures 3.8 and 3.15). If more ErbB-2+CD45- tumour cells were present in the 4 large tumours analysed, it might be expected there would be a larger number of tumour-infiltrating lymphocytes (CD45+), specifically T cells (CD3+), in mice with high anti-ErbB-2 antibody titres due to ADCC and CDC. However, with low numbers of ErbB-2+CD45-, it is perhaps unsurprising to see no statistically significant differences between groups ErbB-2-CD45+ and CD3+ cell populations. The decision to assay the largest tumours from each group was necessitated by the amount of sample needed to account for processing losses

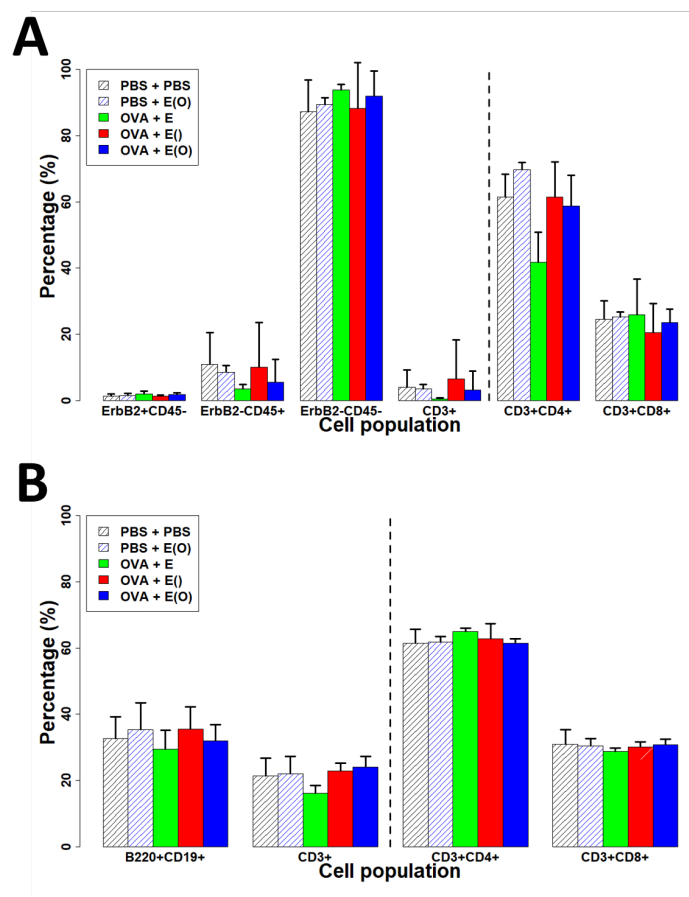


Figure 3.17: Bar chart showing the average percentage populations of cell types (± 1 standard deviation) isolated from resected tumours (panel A) and spleens (panel B). Gating strategy for flow cytometry of isolated single-cell suspensions from resected tumours and spleens of mice is given in figure 3.16. Data represents the percentage of all non-debris, singlet, live events gated for. Data for CD3+CD4+ and CD3+CD8+ are further subsetting to be percentages of CD3+ gated events. Tumours and spleens were resected from 4 mice in each group with the largest tumours and processed into single-cell suspension as described in section 3.2.3.

but ultimately may have selected the mice that were the least responsive to therapy.

The cellular immunological profile was explored by looking at splenocytes to see if the treatment had an effect on the number of circulating B and T cell populations (figure 3.17B). No differences could be seen in the proportion of circulating B (B220+CD19+) or T cells (CD3+) between groups, nor for the proportion of helper (CD4+CD8-) and cytotoxic (CD4-CD8+) T cells (figure

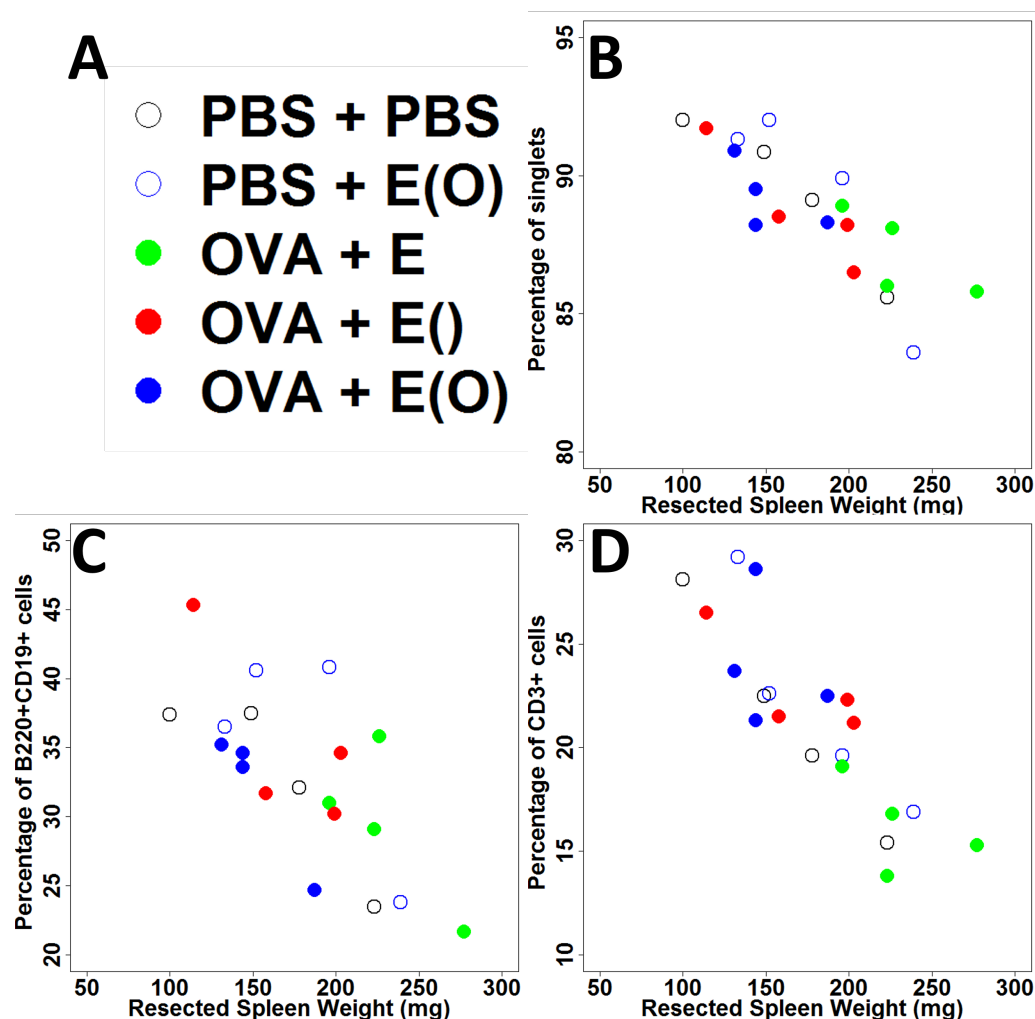


Figure 3.18: Linear trends between resected spleen weight and isolated cellular populations. Panel A shows a legend for the plots, panels B-D compare the spleen weight to the percentage of isolated singlets (panel B), B220+CD19+ (panel C), and CD3+ (panel D).

3.17B). This is, again, likely due to the fact that tumours which were analysed had lost ErbB-2 overexpression and thus none of the mice from any of the groups had an immune system primed to target the tumour cells.

While there were no group dependencies on the cellular populations analysed, a negative linear trend between the spleen weight and some of the cell populations was seen (figure 3.18). Sample cell numbers were first adjusted by the weight of the resected tissue prior to staining. Figure 3.18 shows that the increase in size of the spleen resulted in a lower proportion of singlets being

isolated from the samples. The relationships in figures 3.18 C and D indicate that the increase in spleen weight in response to the increase in tumour size is not due to an increase in the number of B cells or T cells. Therefore, this increase in size may be due to either an increase in other leukocyte populations or possible liver metastasis which is known to increase spleen size [272]. Although the latter of these explanations is extremely unlikely in the context of a subcutaneously implanted tumour.

3.4 Summary

This chapter describes a novel development of the liposomal vaccine platform described by Hills, et al. [57] to target the cancer antigen ErbB-2 which is overexpressed in 15 - 30% of all breast cancers, as well as in many other cancers such as ovarian, renal, colon and lung [258]. Many patients with ErbB-2 overexpressing tumours have previously shown an ability to mount a weak immune response towards this antigen, showing that it is possible to overcome the self-tolerance associated with it [239, 240]. Trastuzumab, a monoclonal humanised antibody that targets ErbB-2, licensed in 1998, was one of the first target-specific drugs to be used in the clinic [273]. A large proportion of treatment regimes for ErbB-2 overexpressing tumours now include Trastuzumab due to its favourable therapeutic index [274–276]. Pertuzumab, licensed in 2012, is a more recent addition to the options for treatment of ErbB-2 overexpressing cancers. The phase III trial which led to its licensure compared the response of patients with ErbB-2+ metastatic breast cancer treated with either Trastuzumab and docetaxel alone or in combination with Pertuzumab [260]. It was found that the combination including Pertuzumab had an increased median progression-free survival of 18.5 months compared to 12.4 with no change in the treatment safety profile [260]. A follow up study revealed that patients

treated with the combination of Pertuzumab, Docetaxel and Trastuzumab had a median overall survival of 56.5 months rather than 40.8 [261]. Like Trastuzumab, it is a humanised monoclonal antibody, but in contrast it binds to a region of ErbB-2 that inhibits ErbB-2 dimerising with ErbB-3, rather than ErbB-2 homodimerisation [262, 277] (figure 3.1). The peptide used here, originally described by Allen, et al. [63], mimics the Pertuzumab binding region, and hence appeared to be a good target. The antibodies generated in response to the vaccine described here could potentially provide the same therapy as Pertuzumab, by inhibiting ErbB-2 dimerisation with ErbB-3, without the need to redose.

The proposed mechanism of action for the vaccination platform was first validated by comparing the anti-target antibody responses generated by FVB/n and BALB/c mice (section 3.3.1). FVB/n mice, which are unable to present the helper OVA peptide on the surface of their B cells [246, 247], generated similar anti-ErbB-2_{266–296} total IgG responses with or without the help of non-cognate CD4+ T cells (figure 3.3 A). BALB/c mice, which are able to present the OVA peptide on their B cell surface for T cell recognition, showed an increase in anti-ErbB-2_{266–296} total IgG levels only when mice had pre-existing anti-OVA immunity and were vaccinated with the full vaccine construct, E(O) (figure 3.3 B). This provided further exquisite proof that the mechanism proposed by Hills, et al. [57] and developed here is capable of generating increased antibody levels by non-cognate T-helper cell stimulation of B cells, activated by a liposomal vector with precise spatial separation of B cell receptor and T helper cell peptides.

The mechanism of action and necessity for each component of the vaccine was then explored for its influence on anti-ErbB-2_{266–296} antibody responses in a tumour challenge study with BALB/c mice (section 3.3.2). It was found that

mice with pre-existing anti-OVA immunity, achieved by OVA dosing, which were then vaccinated with E(O) liposomes, generated rapid (within 1 week) and substantially (10.5-fold) and significantly ($p < 0.001$) raised antibody titres (figure 3.5). The rapidity of the response demonstrates one of the advantages of the mechanism of action. In comparison, DNA-based vaccines tend to take a long time (often >2 weeks) and require multiple administrations before antibody responses can be detected [278, 279]. Whilst even conjugate vaccines and live attenuated vaccines can require multiple doses before detectable IgG responses can be observed [280–283]. Mice lacking the pre-existing anti-OVA immunity and vaccinated with E(O) generated a lower and slower antibody response (figure 3.5). This response is probably due to the presence of a second peptide (OVA) presented by B cells, doubling the chance of low-level circulating CD4+ T cell, with TCR specific for a presented peptide, coming into contact with a B cell that engulfed and processed vaccine particles. The isotype profiles of the PBS+E(O) and OVA+E(O) groups were then characterised. Each isotype interacts with the immune system differently [265] and in a different way to their human counterparts [266, 267]. It was evident that there was higher class-switching in the mice with pre-existing anti-OVA immunity (figure 3.6), which led to increased IgG1, IgG2a and IgG2b isotype levels from just one week after initial exposure to ErbB-2 peptide. This rapid class-switched antibody response provides a dynamic immune response against the target antigen soon after vaccination. Notably, the liposomal booster vaccine increased these responses further (figure 3.6 C-E). IgM is the first antibody isotype produced after initial antigen exposure [268], the IgM responses of all mice in both groups was very tightly distributed with very little difference between mice (figure 3.6B). While IgG3 levels were again similar between groups, there was a large within-group variation in whole blood concentration after booster

dosing (figure 3.6 F). IgG3 is the earliest produced IgG isotype and is able to fix complement effectively which leads to a cascade of inflammatory responses [266]. IgG1 was the main anti-ErbB-2_{266–296} antibody isotype generated by vaccination, this isotype is the same isotype as the pre-clinical studies' version of Pertuzumab, prior to humanisation [284]. Therefore, this particular anti-ErbB-2_{266–296} antibody isotype should not only bind to the same region of the protein as Pertuzumab, but also interact with the immune system identically to its pre-clinical precursor. In moving to the clinic, Pertuzumab was humanised and was made with the human IgG1 isotype, which, in contrast to its murine counterpart, is a strong activator of complement and effector cells [267]. The production of murine IgG1 is increased in the presence of T cell-dependent B cell activation, such is the case for the proposed mechanism of action [266]. Its contribution to protective immunity is largely unclear, as it is unable to fix complement and only engages with the inhibitory Fc γ RIIb receptor of effector cells [266]. Murine IgG1's main role is, therefore, believed to be similar to that of the human IgG4 isotype, in limiting inflammatory responses and neutralising target molecules from binding their receptor [266]. The IgG1 levels produced here are approximately a dramatic 10,000-fold increase compared to those seen by Hills, et al., while the IgG2a (equivalent isotype to IgG2c produced by C57Bl/6 mice) and IgG3 levels were comparable and the IgG2b levels were approximately 2-3 fold lower [57]. These differences may be due to the different strains of mice used or simply the different peptide being targeted. Indeed, several studies have found that C57Bl/6 mice produce lower levels of the IgG1 isotype compared to BALB/c mice [285–288].

After challenge with rat ErbB-2 overexpressing TUBO tumour cells, anti-ErbB-2_{266–296} IgG levels could be detected by ELISA in mice not previously exposed to the ErbB-2 peptide, confirming the peptide is representative of the

epitope exposed on the tumour cells (figure 3.5 G-H). A western blot was used to further confirm that the peptide is representative of the epitope on TUBO cells, by probing with plasma samples from a high-responding mouse in the OVA+E(O) group and comparing to a pre-vaccination plasma sample from the PBS+(O) group (figure 3.7). Despite high-titre IgG antibody levels and confirmation of the target epitope's presence on the tumour cells at the point of implantation, no statistically significant differences between the tumour growth curves were observed (figure 3.8).

In response, the dosing regime and liposome manufacture was then optimised to find a regime that could maximise the anti-ErbB-2₂₆₆₋₂₉₆ titre levels generated (section 3.3.3). The effect of administered ErbB-2₂₆₆₋₂₉₆ concentration was explored and it was found that a higher dosing level induced increased total IgG titres (figure 3.9). It was also observed that the same dosing regime as the previous larger-cohort study was unable to induce such high titres as before. This was likely due to changes in the formulation method from method B to the cleaner method C (table 2.1), with less risk of contamination. The contamination occurring in method B is thought to have acted in an adjuvanting capacity in the first larger-cohort vaccination study. The free ErbB-2 peptide was shown to be ineffective at boosting an existing anti-ErbB-2₂₆₆₋₂₉₆ response compared to using the liposomal vaccine as a booster, indicating that even after the generation of an anti-ErbB-2₂₆₆₋₂₉₆ immune response, the vaccine particle was still better able to boost the response. This may be due to the same mechanism of action being repeated, or it may simply be due to the adjuvanting effects of the liposome. Lastly, the effect of a second booster dose was unclear due to the small sample size and because one mouse, treated with two booster doses, only responded mildly to treatment. However, 2/3 of mice in the HighE(O)3 group consistently had the highest anti-ErbB-2₂₆₆₋₂₉₆ IgG

titres after being administered the first boost (figure 3.9). The dosing regime of the first tumour challenge, LowE(O)2 (see table 3.4), and the two groups with the highest GMTs, HighE(O)3 and HighE(O)2, were further compared by isotyping the antibody responses (figure 3.10). Those which were dosed with high ErbB-2 concentrations generated high anti-ErbB-2_{266–296} IgG1 levels, particularly the two high responders in the HighE(O)3 group. Slight IgG2a and IgG2b class-switching could only be seen in mice administered high ErbB-2 concentrations after their respective first dose. It was difficult to reveal differences between the HighE(O)3 and HighE(O)2 treatment regimes because of small sample sizes and such similar responses. The HighE(O)3 group was chosen as the optimal dosing regime to take forward as three vaccination doses were needed by Allen, et al., who first described the utility of delivering the ErbB-2_{266–296} peptide [63].

A repeat larger-cohort study was consequently performed with the optimal dosing regime found in section 3.3.3 with the aim of further increasing antibody responses and testing for an inhibition of tumour growth (section 3.3.4). It was again shown that the OVA+E(O) group induced the highest antibody titres compared to all other groups with 7.3-fold higher responses just 1 week post-vaccination (figure 3.11). Of particular note was the similarity between the PBS+E(O) group and the OVA+E() group as neither of these could recruit non-cognate CD4+ T cells to help activate B cells and in turn stimulate increased antibody production (figure 3.11). Isotyping of the PBS+E(O), OVA+E(), and OVA+E(O) groups' antibody responses showed stronger IgG2a and IgG2b class-switching than seen in previous studies (figure 3.12 D-E). This is likely due to the increased ErbB-2 concentrations administered as there was minimal difference between any of the three groups examined. IgG2a and IgG2b both have similar functions in mice, with IgG2a

levels linked to an increase in cytotoxic T cell levels and IgG2b levels linked to T cell independent B cell activation [266]. Both isotypes are able to fix complement and have strong interactions with $\text{Fc}\gamma\text{R}$ effector cells [266]. The OVA+E(O) group again showed the highest levels of IgG1 class-switching compared to the PBS+E(O) and OVA+E() groups (figure 3.12 C). Mice dosed with E(O) liposomes generally had higher IgG3 levels than those which received E() liposomes, indicating that the presence of the OVA peptide, regardless of pre-existing immunity towards it, aided in the production of anti-ErbB-2_{266–296} IgG3 (figure 3.12 F). The ability of the raised antibodies to induce direct cell killing was demonstrated by addition of complement inactivated plasma to TUBO cells *in vitro* (figures 3.13 and 3.14). This is likely due to the blockade of ErbB-2/ErbB-3 dimer formation, and thus a reduction of ErbB-2 activation [259, 262]. Cuello, et al. previously demonstrated that anti-ErbB-2 therapy could induce apoptosis in ErbB-2 overexpressing cell lines, but not in cell lines with low levels of ErbB-2 expression [289]. This may explain why plasma positive for anti-rat-ErbB-2 could induce death in TUBO cells *in vitro* despite its co-expression of mouse ErbB-2. A primary function of ErbB-2 is suppression of apoptosis to enhance cell survival, and thus inhibition of its activation will increase cellular apoptosis [290, 291]. This, therefore, demonstrates the ability of the vaccine-induced immune response to inhibit the growth of ErbB-2 overexpressing tumour cell lines. The fact that the OVA+E(O) plasma could induce highly efficient cell kill within 24 hours, without CDC or ADCC mechanisms, gave confidence that the growth of TUBO tumours in these mice would be retarded. However, again, despite the high anti-ErbB-2 antibody titres, no statistically significant differences in the tumour growth curves could be seen between any group (figure 3.15). The reduced take rate in this study compared to those reported in figure 3.8 was likely due to fewer cells being administered

to challenge the mice (figure 3.15) [263].

To probe if successful mechanism of action was being concealed by tumour growth and structure heterogeneity, mice with the 4 largest tumours from each group, at cull, had their tumours and spleens resected and processed into single-cell suspensions to analyse the cellular populations contained within them. The tumour cells were found to have lost their overexpression of ErbB-2 in all of the tumours from all of the treatment groups, giving reason as to why the anti-ErbB-2_{266–296} humoral responses had no effect in inhibiting the tumour take rate or growth rate (figures 3.16 and 3.17). TUBO cells are a transgenic cell line spontaneously developed by a BALB/c mouse transformed to express the rat ErbB-2 oncogene [263]. The loss of rat ErbB-2 expression may, therefore, be due to *in vivo* selective pressures increasing the burden on cells expressing this transgene, resulting in the loss of its expression in favour of the already existent mouse ErbB-2 protein [263]. The discrepancy between *in vitro* and *in vivo* inhibition may be due to differing amounts of anti-ErbB-2_{266–296} present in the vicinity of the cells, allowing the tumour cells *in vivo* more time to adapt to the selective pressures present. More studies are required to develop this approach for *in vivo* success (described in detail in chapter 5), such as the inclusion of either whole protein or multiple peptides into the vaccination particles to increase the diversity of the vaccine targets. A more refined approach to profiling the existing formulation may have involved assaying the speed with which implanted tumours were cleared of cells expressing rat ErbB-2, or implanting cells at a dilution series and comparing between groups the number at which take rate dropped to 0.

Allen, et al. conjugated the ErbB-2_{266–296} peptide to a measles virus fusion protein, in the presence of ISA720 adjuvant. Notably the titre level reported by Allen, et al. was evident one week after the second vaccination dose and

reached a peak GMT of approximately 100,000 one week after the third vaccination dose [63]. One week after the peak GMT was achieved, BALB/c mice were challenged with 1×10^5 TUBO cells and demonstrated tumour growth inhibition [63]. The reason for the disparity between the *in vivo* efficacy of the ErbB-2_{266–296} peptide in inhibiting tumour growth demonstrated here and by Allen, et al. may simply be due to higher titre levels achieved prior to implantation. The higher titre levels achieved by Allen, et al. may be a result of increased adjuvantation in each dose, or differences in the dosing regime.

In the next chapter, the vaccine platform is repurposed to target a well-conserved epitope of H3 influenza A viruses, namely the HA_{91–108} peptide first used by Müller, et al. in 1982 [248]. The peptide has since been incorporated into Biondavax's Multimeric-001 influenza vaccine which is currently in a phase III trial (NCT03450915: clinicaltrials.gov). The ability of the vaccination platform to again generate a high-titre, isotype-switched antibody response to a different peptide is an important demonstration of its broad applicability.

Chapter 4

Influenza vaccination

4.1 Introduction

In this chapter, the liposomal vaccination platform developed and tested in chapters 2 and 3 is repurposed to target a well-conserved epitope of H3 influenza A viruses. Haemagglutinin (HA) is a surface receptor of the influenza virus which is highly immunogenic, however it also is subject to a high rate of mutation. The protein is essential for viral entry into cells and works by binding to sialic acid on glycosylated cell surface proteins and initiating endocytosis of the virus [13]. The virus is then trafficked to an endosome where the low pH induces an irreversible conformational change in HA structure exposing a section of the protein known as the fusion peptide [13]. The fusion peptide is able to insert itself into the endosomal membrane, fusing the viral envelope and the endosomal membrane together, which leads to the release of viral RNA into the cytosol of the cell [13]. As HA plays such a key role in cellular infection, it is no wonder that it is the most popular target for influenza vaccination [292]. Due to the high mutation rate of HA, many studies are now targeted at inducing an immune response against well-conserved regions of the protein with a lower rate of mutation [292]. However, these regions are generally less immunogenic, making the production of a vaccine targeting these regions more difficult [293].

A well-conserved B cell epitope from a H3 influenza virus, designated HA_{91–108}, consisting of residues 91-108 of the H3 protein, was used here. This

peptide was first used by Müller, et al. in 1982 when they demonstrated a level of protective effect against viral challenge in mice vaccinated with the peptide conjugated onto a tetanus toxoid molecule emulsified in complete Freund's adjuvant [248]. They then incorporated the peptide into a recombinant salmonella flagellin and showed that they could induce significant levels of HA_{91–108}-specific serum IgG and mucosal IgA [294]. It was also demonstrated that a retro-inverso version of HA_{91–108} (whereby the chirality and sequence of the HA was inverted) had a 700-fold increase in half-life when in the presence of lung homogenate proteases [295]. This retro-inverso HA_{91–108} slightly increased the anti-virus serum IgG and lung IgA titres when compared to the unmodified HA peptide [295]. Both peptides were still able to provide 100% protection to mice challenged with a 50% lethal dose of virus, and also reduce the severity of infection by minimising weight loss [295]. Their vaccination platform was extended to include a helper T cell epitope and a cytotoxic T cell epitope of the influenza Nucleoprotein (NP) protein, both well-conserved between influenza strains [296–300]. This peptide combination vaccine was demonstrated to induce anti-influenza antibody responses [296, 298–300] and reduce viral loads in the lungs of mice, days after challenge in both young and aged mice [296–300]. It was also able to increase survival and minimise weight loss after challenge with several different H3 influenza strains [297, 300]. Six additional well-conserved peptide sequences have since been included in the vaccine, prior to its entry into clinical trials (table 1.1). This vaccine, designated M-001, is a single polypeptide consisting of three repeats of the 9 linear epitopes (table 1.1) expressed in *E. coli* [72]. In a phase II trial, M-001 was used to prime elderly participants (≥ 65) before being given a commercial trivalent influenza vaccine to see if responses to the trivalent vaccine could be improved [73]. Priming with M-001 led to increased seroconversion towards all

three strains used in the trivalent influenza vaccine compared to priming with a placebo [73]. Furthermore, serum taken from participants when this study was conducted in 2011 was later tested against the A/Switzerland/9715293/2013 (H3N2-like) strain that caused the 2014/15 influenza epidemic [74]. Serum from participants primed with M-001 gave improved seroprotection and seroconversion against this strain, which was not known to circulate in 2011/12 [74]. The vaccine is currently in a phase III trial (NCT03450915: clinicaltrials.gov).

Here, our vaccination platform is repurposed by using liposomal vaccine particles, surface decorated with HA_{91–108} instead of ErbB-2_{266–296}. The necessity of each component of the vaccination platform to generate high-titre isotype-switched antibody responses is again demonstrated for this peptide. The antibody responses generated are subsequently examined for their ability to neutralise two H3 influenza viruses from infecting cells *in vitro*. Lastly, the ability of the generated antibodies to bind to the whole protein in its native, denatured, and low pH forms is examined.

4.2 Material & Methods

4.2.1 Mice

Female BALB/c mice were purchased from Charles Rivers at 4 - 6 weeks old and housed in specific pathogen-free conditions at the University of Oxford animal facility and used between 5 and 15 weeks of age. Animal weights were recorded twice a week from the start of treatment until cull. Animal experimentation was performed in accordance with institutional and UK Home Office guidelines. All mice showed consistent weight gain throughout the experiment and there was no evidence of toxicity (piloerection, lack of movement/activity,

lack of socialisation) in any mouse.

4.2.2 Liposome formulation

Liposomes were formulated by method C (table 2.1) as described in chapter 2. Briefly, lipid cakes were formulated by lyophilisation before hydration with citrate buffer with/without OVA_{323–339}. Resulting liposomes were extruded using the t&t Scientific extruders (product codes: TT-030-0004 and TT-003-0010). Free OVA peptide was purified away by size-exclusion columns before addition of HA_{91–108} peptide. Liposomes were then left to react overnight at 4°C before purification by dialysis to remove unconjugated HA peptide. Physico-chemical characterisations were comparable to those of other method C formulations for ErbB-2_{266–296} incorporating liposomes (figure 2.9).

4.2.3 Vaccination

All injections were performed subcutaneously under anaesthetic. Mice first received a pre-vaccination dose of either PBS or 10µg OVA_{323–339} peptide mixed with Addavax (Invivogen, France: vac-adx-10) in a 1:1 dilution. An identical booster pre-vaccination was given two weeks later. After a further two weeks (week 0) mice were vaccinated with either PBS; free HA_{91–108} peptide, denoted ‘H’; HA_{91–108} presenting, empty liposomes, denoted ‘H()’; or OVA_{323–339} encapsulating, HA_{91–108} presenting liposomes, denoted ‘H(O)’ (figure 4.1). Dosing regime used was identical to that found in section 3.3.3. The administered concentration of HA_{91–108} and OVA_{323–339} in vaccine doses, where present, was 100 µg/mL and 5 µg/mL, respectively, at a 100µL dose per mouse. Two booster doses, identical to the vaccination dose given on week 0, were administered at weeks 1 and 4.

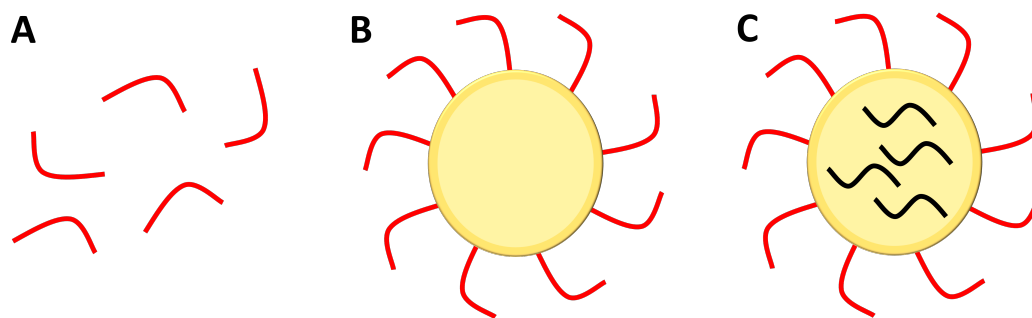


Figure 4.1: Diagrammatic representation of liposomes used in influenza vaccinations. Panel A represents free HA₉₁₋₁₀₈, denoted ‘H’; panel B represents HA₉₁₋₁₀₈ presenting, empty liposomes, denoted ‘H()’; panel C represents OVA₃₂₃₋₃₃₉ encapsulating, HA₉₁₋₁₀₈ presenting liposomes, denoted ‘H(O)’.

4.2.4 Plasma collection

As described in section 3.2.6, weekly blood samples were collected from the tail vein of mice after dosing with liposomal vaccines. Blood samples were immediately diluted 1:10 in PBS before being centrifuged at 1000 xg for 5 minutes. Supernatants were collected and frozen at -20°C until required.

4.2.5 ELISA for total anti-HA₉₁₋₁₀₈ IgG titre

ELISAs were performed as described in section 2.2.5 to quantify the anti-HA₉₁₋₁₀₈ total IgG titre in plasma samples using HA₉₁₋₁₀₈ as the initial antigen at a concentration of 10µg/mL. Plasma from mice was used at varying dilutions as the primary antibody and a HRP conjugated anti-mouse IgG antibody as the secondary (Sigma Aldrich, UK: A4416). The titre of plasma was defined to be the last dilution that gave an absorbance reading above 0.05 after subtraction of the average absorbance and 2 standard deviations of 16 blank wells treated with PBS instead of plasma as the primary.

4.2.6 Isotyping antibody responses

ELISAs were performed as described in section 2.2.5 to quantify anti-HA_{91–108} IgM, IgG1, IgG2a, IgG2b, IgG3 concentrations in mouse blood using HA_{91–108} as the initial antigen at a concentration of 10 μ g/mL. Plasma used from mice was at varying dilutions as the primary antibody and a HRP conjugated anti-mouse IgM, IgG1, IgG2a, IgG2b or IgG3 antibody as the secondary (Abcam, UK: ab97240, ab97245, ab97250, ab97260, ab97230). The concentration of plasma samples was calculated as in section 3.2.8 by using a standard curve of isotype controls as the initial antigen on the same ELISA plate as samples, with PBS replacing the primary antibody. Isotype plasma concentrations were then back calculated to find concentrations in whole blood. Calculated concentrations represent lower bounds of the actual blood concentration due to competitive binding between the different isotypes within samples.

4.2.7 Antibody avidity assay

Antibody avidity assays were performed using the chaotropic agent, sodium thiocyanate (NaSCN), to disrupt antibody-antigen interactions [301]. This assay was performed similarly to an ELISA (see section 2.2.5). After plates were coated with HA_{91–108}, blocked with 10% FBS in PBS, incubated with mouse plasma (individually diluted to a level calculated to give an OD₄₅₀ absorbance reading of 0.5), and washed 6 times with 0.05% Tween in PBS, wells were exposed to ascending concentrations (0 - 5M) of NaSCN at room temperature for 10 minutes. Plates were again washed 6 times and the presence of anti-HA_{91–108} antibodies was detected as in section 4.2.5. Resistance to thiocyanate elution was used as an indicator of avidity and the concentration of NaSCN required to reduce the ELISA signal by 50% was used as a measure

of avidity.

4.2.8 Microneutralisation assay

Neutralisation studies were performed in collaboration with Dr Pramila Rijal as described by Xiao, et al. and Powell, et al. [302, 303]. Briefly, pooled serum samples from each group were heat-inactivated by heating to 56°C for 30 minutes before being titrated by doubling dilutions and incubated with virus for 2 hours at 37°C. Two H3 viruses were used: A/Victoria/361/2011 and X-31. The HA_{91–108} peptide sequence is conserved on the A/Victoria/361/2011, while the X-31 virus has one different amino acid, with a Tyrosine being replaced by a Phenylalanine at residue 94 of the H3 protein. Viruses were pseudotype influenza viruses (S-FLU) [303], which are modified by replacement of their HA gene with an enhanced green fluorescent protein (eGFP) gene. S-FLU viruses are, therefore, able to infect cells and express eGFP, which acts as an assay reporter, but cannot replicate [303]. After incubation of S-FLU with plasma samples, 3x10⁴ MDCK-SIAT1 cells were added and left to incubate in a 37°C, 5% CO₂ incubator for 18 hours. eGFP fluorescence was then measured using a fluorescence plate reader. Percentage of infection was calculated by comparing the fluorescence of sample wells and the fluorescence of wells treated with virus growth medium instead of plasma.

4.2.9 Dot blot

Recombinant whole HA protein (Abcam, UK: ab217661) or HA_{91–108} peptide was diluted in a 1:3 dilution with either pH 4.0 acetate buffer to irreversibly change the conformation of the HA protein by simulating the acidic endosomal conditions; Laemmli sample buffer (Bio-Rad, USA: 1610747) with 5% β -mercaptoethanol before heating to 95°C for 5 minutes for denaturation; or

PBS to accordingly adjust concentration/target sequence copy number of native HA peptide and protein. Starting concentrations for protein and peptide were calculated to have approximately equal number of HA_{91–108} epitope copies: 1mg/mL whole HA protein (molecular weight 58kDa, containing 3 epitope copies per molecule) and 130 μ g/mL HA_{91–108} peptide (molecular weight 2.514 kDa, containing 1 epitope copy per molecule). Samples were dotted onto a 0.2 μ m pore size nitrocellulose membrane (Bio-Rad, USA: 1620095) and left to dry for at least 30 minutes. Membranes were blocked with 5% skimmed milk in PBS for 1 hour before being washed with 0.05% Tween-20 in PBS and incubated with the primary antibody sample at varying dilutions in PBS 0.5% skimmed milk for 1 hour. Primary antibody sample was either plasma from mice with/without anti-HA_{91–108} antibodies or MEDI8852 as a positive control. MEDI8852 is an anti-HA monoclonal antibody currently in phase II clinical trials (NCT03903718: clinicaltrials.gov) [304] that binds to a different region of the protein to the HA_{91–108} region and was gifted by Dr Pramila Rijal. Next, membranes were washed with 0.05% Tween-20 in PBS and incubated with either a HRP conjugated anti-mouse IgG antibody raised in goat (R&D Systems, USA: HAF007) or a HRP conjugated anti-human raised in goat (Sigma Aldrich, UK: AP112P) at a dilution of 1:1000 in PBS 0.5% skimmed milk for a further hour. Immobilon Western HRP Substrate (Merck Millipore, USA: WBKLS0500) was used for chemiluminescent detection of protein. Membranes were washed with 0.05% Tween-20 in PBS before addition of the substrate for 3 minutes. Excess substrate was then drained before exposing the membranes to X-ray films (ThermoFisher, UK: 10137683) before being developed using an Agfa CP 1000 film processor.

Pre-vaccination	Liposomal vaccination	Group notation	Number of mice
PBS + Addavax	PBS	PBS+PBS	8
	H(O)	PBS+H(O)	8
OVA + Addavax	H	OVA+H	8
	H()	OVA+H()	8
	H(O)	OVA+H(O)	8

Table 4.1: Number of mice in each group for influenza vaccinations. Addavax is an immunoadjuvant similar to MF59; OVA is a peptide of Ovalbumin; H denotes free HA peptide; H(), liposomes surface decorated with HA peptide; H(O), OVA peptide encapsulating, HA peptide surface-decorated liposomes.

4.2.10 Statistics

Statistical analysis was performed using R. ELISA results were first tested for normality by Shapiro-Wilks test with a p-value less than 0.05 and thus were analysed using non-parametric tests. Kruskal-Wallis and Conover-Iman tests were used to find differences between antibody responses of different groups. Differences were considered to be significant when the p-value was less than 0.05. Indicators of statistical significance: ns, $p > 0.05$, *, $p < 0.05$; ** $p < 0.01$; ***, $p < 0.001$.

4.3 Results

The liposomal vaccine was tested for its ability to raise an antibody response towards an influenza target after vaccination (table 4.1). Mice were treated with the same dosing regime as found in section 3.3.3. First, mice received two identical pre-vaccinations of either PBS or OVA peptide with immunoadjuvant, Addavax, on weeks -4 and -2 (figure 4.2 A). Then on weeks 0, 1 and

4, mice were injected with identical doses of free HA peptide, denoted H; HA_{91–108} presenting, empty liposomes, denoted H(); or OVA_{323–339} encapsulating, HA_{91–108} presenting liposomes, denoted H(O), formulated by method C (table 2.1). Weekly plasma samples were collected from the day of the first liposomal vaccination, which were subsequently tested for anti-HA_{91–108} antibody responses. Samples were characterised for their titre levels, isotype concentrations, avidity, binding ability to peptide or whole HA protein, and *in vitro* virus neutralisation ability.

Figure 4.2 B-H shows the anti-HA_{91–108} total IgG titres of individual mice for the duration of the experiment. At week 0 (figure 4.2 B), the day of initial liposomal dose, there was no detectable antibody response, showing that the mice do not have detectable levels of pre-existing IgG towards the peptide target. After just 1 week (figure 4.2 C), all mice in the OVA+H(O) group generated detectable antibody levels, with a GMT of 12800 at least 98-fold higher than any other group. This titre is the highest seen at one week after first vaccination compared to all the cancer studies described in chapter 3. It also compares favourably with the work of Ben-Yedidia, et al. who showed that using recombinant flagellin, containing the HA_{91–108} peptide sequence, a titre of 2187 was only evident one week after the third vaccination dose [299]. The level of antibody in this group was at least 98.7-fold higher than all other groups. This differential was approximately maintained throughout the study with control groups rarely exceeding a titre of 200. The fact that any titre and immunogenicity was detected for the free peptide is likely due to the inherent immunogenicity of an influenza peptide in comparison to the cancer peptide which has more resemblance to a ‘self’-antigen, as it only differed in 1 residue to the mouse ErbB-2. At week 2 (figure 4.2 D), one week post-booster, the GMT of the OVA+H(O) group dropped to 6979, while the GMTs

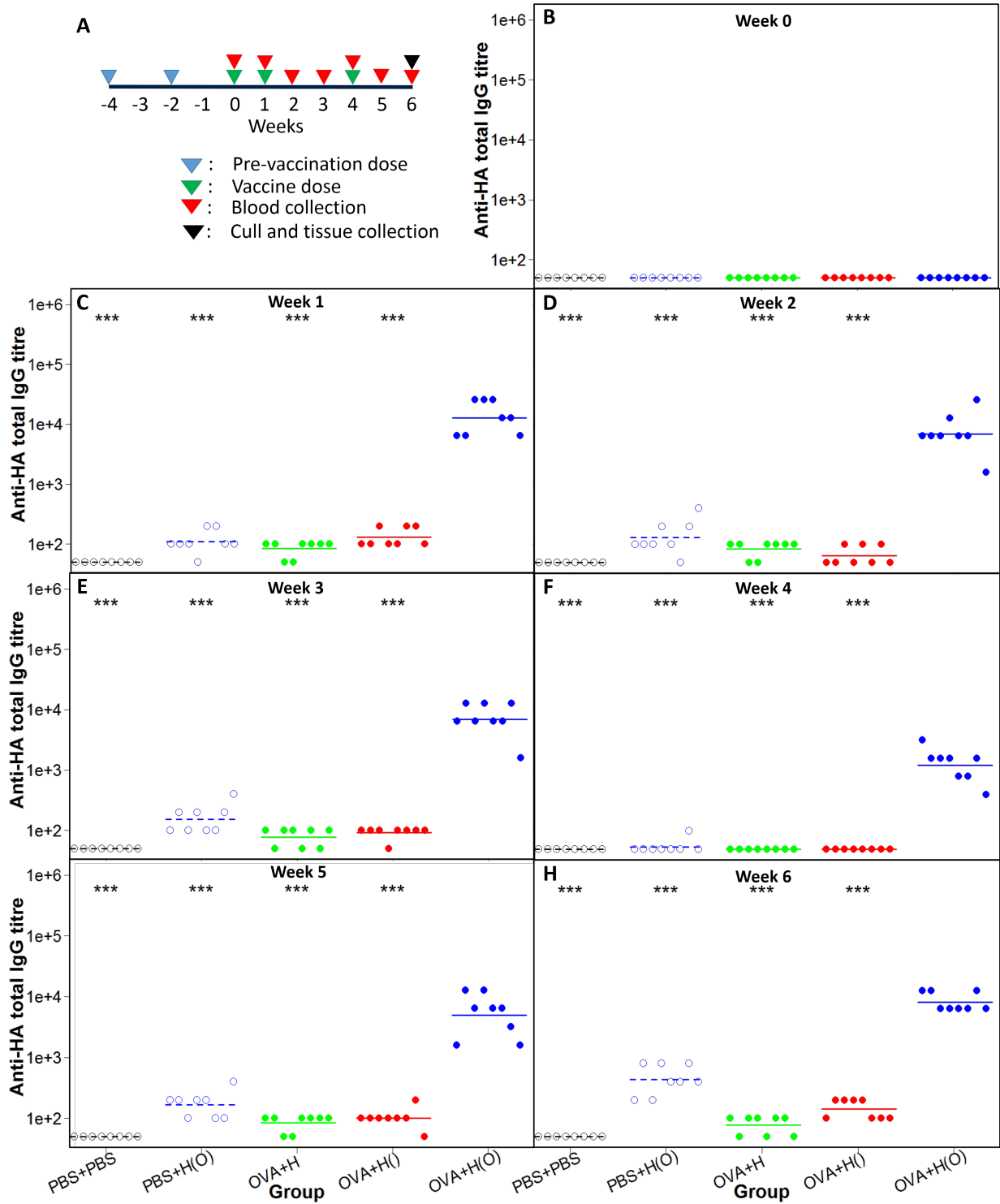


Figure 4.2: Anti-HA IgG titres in BALB/c mice in influenza vaccination study. Weekly plasma samples were taken from mice and tested by ELISA to detect an antibody response to the HA peptide. Panel A shows a timeline of the experiment and panels B-H show weekly titres of individual mice from the day of first liposomal vaccination (Panel B) to cull (Panel H). Kruskal-Wallis statistical tests were used to find if differences existed between all groups, followed by Conover-Iman tests to find pairwise differences between groups. Lines represent geometric means of $n=8$. Indicators of statistical significance are shown for comparisons between the OVA+H(O) group and all other groups: ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

of all other groups did not significantly change. By week 3 (figure 4.2 E), the anti-HA_{91–108} antibodies in all groups had remained constant. At week 4, the antibody levels of all groups declined, with the OVA+H(O) group's GMT dropping to 1233 (figure 4.2 F). At this timepoint, mice also received a second booster vaccination (figure 4.2 A), the effects of which were clearly seen on week 5, with increased GMTs for all groups exposed to HA_{91–108} (figure 4.2 G). At the time of cull (week 6), the OVA+H(O) groups GMT had increased to 8300 (figure 4.2 H). It can be seen that the mechanism of action is essential in order to generate a significant anti-HA antibody titre as no group in which the mechanism is hindered (by absence of pre-existing OVA immunity or absence of OVA within the liposomes) had a GMT above 500 at any point during the experiment (figure 4.2).

Antibody responses of PBS+H(O), OVA+H(), and OVA+H(O) groups were further characterised by IgM and IgG isotype concentrations in whole blood (figure 4.3). Prior to initial liposomal vaccination all groups showed low level of natural IgM and IgG3 antibodies with HA_{91–108} cross-reactivity (figure 4.3 B and F) but negligible IgG1, IgG2a and IgG2b levels (figure 4.3 C-E). Upon first exposure to the vaccine particles, the OVA+H(O) group generated increased anti-HA_{91–108} IgM levels, peaking one week after first vaccination before exponentially declining back to a base level identical to that of the PBS+H(O) and PBS+H() groups (figure 4.3 B). The IgG3 concentrations in the OVA+H(O) group increased after vaccination with significantly higher levels (4.2-fold, $p < 0.01$) than all other groups (figure 4.3 F). As seen consistently in chapter 3, IgG1 is the dominant antibody isotype produced by this mechanism of action in BALB/c mice. This is to be expected as production of murine IgG1 is increased in the presence of T cell-dependent B cell activation, which the mechanism of action used here relies heavily upon [57, 266]. The

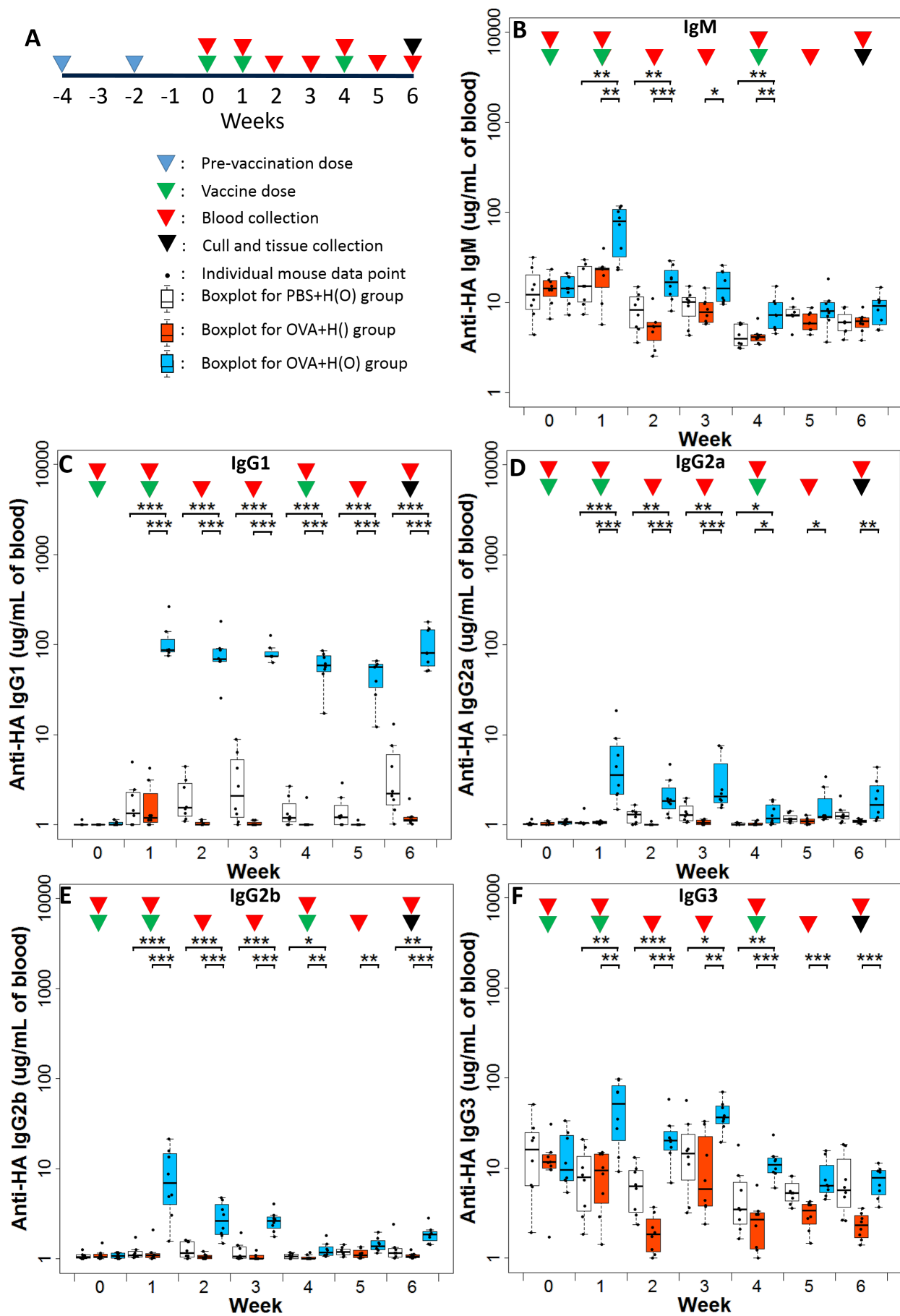


Figure 4.3: Caption located on following page.

Figure 4.3: IgM and IgG isotype concentrations in the blood of BALB/c mice in the PBS+H(O), OVA+H(), and OVA+H(O) groups were quantified by ELISA. Panel A shows experimental dosing regime and a key for the rest of the figure. Panels B, C, D, E, and F show the boxplots of whole blood concentrations of IgM, IgG1, IgG2a, IgG2b and IgG3, respectively. Each boxplot is made from n=8 data points. To avoid zero values for log-scaling on the y-axis, all data points had 1 added to them. Kruskal-Wallis statistical tests were used to find if differences existed between all groups, followed by Conover-Iman tests to find pairwise differences between groups. Indicators of statistical significance are shown for comparisons between the OVA+H(O) group and all other groups: ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

OVA+H(O) group consistently had significantly higher levels of IgG1 class-switching compared to other groups after initial vaccination (figure 4.3 C). The IgG2a and IgG2b levels of the OVA+H(O) group were also significantly ($p<0.001$) increased compared to the PBS+H(O) and OVA+H() groups (figure 4.3 D-E). However, despite similar target peptide concentrations to those used in the cancer studies being administered, described in section 3.3.4, the IgG2a and IgG2b antibody levels were not as high for this influenza peptide study, this discrepancy may simply be due to the fact that a different peptide is being targeted. Similarly to the cancer studies discussed in chapter 3, the IgG1 levels produced here are approximately increased 10,000-fold compared to those seen by Hills, et al., while the IgG2a (equivalent isotype to IgG2c produced by C57Bl/6 mice) and IgG3 levels were comparable, and the IgG2b levels were approximately 2-3 fold lower [57]. These differences may be due to the different strains of mice used or different inherent properties of the peptide being targeted. Several studies have demonstrated that C57Bl/6 mice produce lower levels of the IgG1 isotype [285–288], but higher levels of IgG2b [285, 288, 305] compared to BALB/c mice.

The avidity of the anti-HA_{91–108} antibodies generated by the PBS+H(O), OVA+H(), and OVA+H(O) groups was also characterised by calculation of the concentration of NaSCN required to reduce the signal given by ELISA

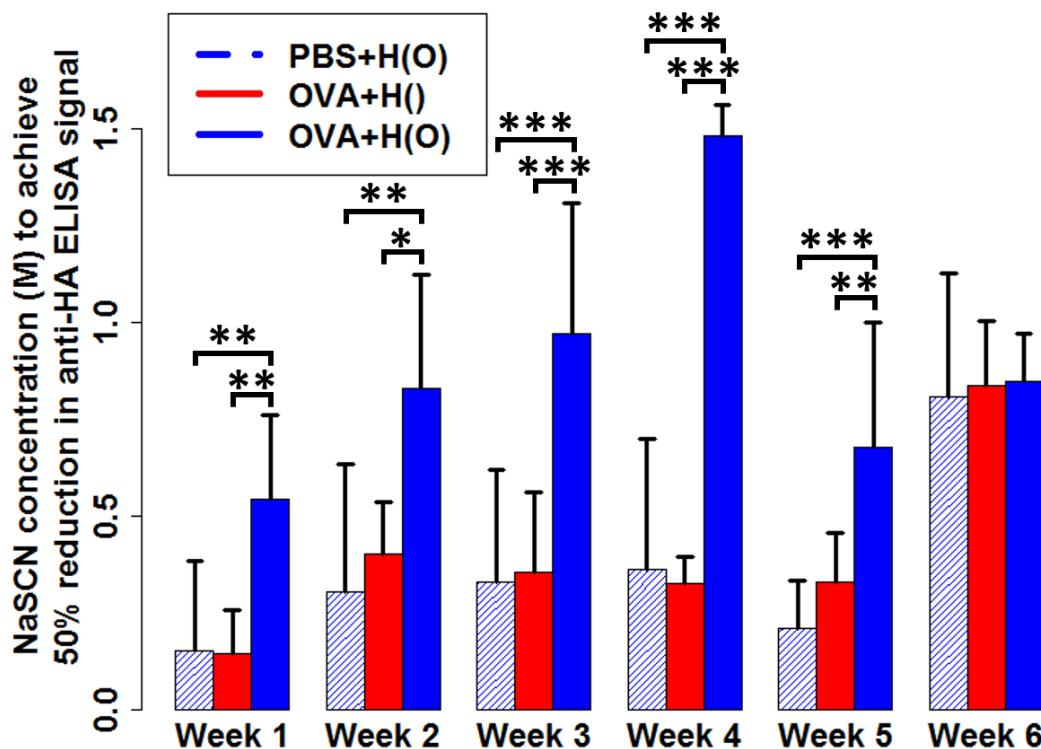


Figure 4.4: Antibody avidity of anti-HA₉₁₋₁₀₈ antibodies generated by BALB/c mice in the PBS+H(O), OVA+H(), and OVA+H(O) groups. The concentration of the NaSCN required to decrease the anti-HA₉₁₋₁₀₈ ELISA signal by 50% was used as a measure of antibody avidity. Data represents average of n=8 samples, while error bars represent 1 standard deviation. Kruskal-Wallis statistical tests were used to find if differences existed between all groups, followed by Conover-Iman tests to find pairwise differences between groups. Indicators of statistical significance are shown for comparisons between the OVA+H(O) group and all other groups: ns, p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001.

by 50%. It can be seen that the avidity of the anti-HA₉₁₋₁₀₈ antibodies in the OVA+H(O) group is significantly (p<0.05, 2.2-fold) higher than those of the PBS+H(O) or OVA+H() groups for weeks 1-5 (figure 4.4). After the administration of the week 4 booster dose there was a stark decrease in antibody avidity in the OVA+H(O) group, while the PBS+H(O) and OVA+H() groups' avidity began to increase, resulting in no statistical difference between groups' antibody avidity by week 6. This decrease in the average avidity of the OVA+H(O) group may be due to memory B cells with low anti-HA₉₁₋₁₀₈ an-

tibody avidity increasing their antibody production after booster vaccination, diluting the pool of higher avidity antibodies. Indeed, between weeks 4 and 5 the anti-HA₉₁₋₁₀₈ titre increased 4-fold after administration of the booster vaccine (figure 4.2 F-G).

Plasma from each group, taken at week 1, was pooled separately, heat-inactivated, and examined for its ability to neutralise two H3 influenza viruses (figure 4.5). The HA₉₁₋₁₀₈ peptide is 100% conserved on the A/Victoria/361/2011 virus, while the X-31 virus has one different amino acid, replacing a Tyrosine for a Phenylalanine at residue 94 of the H3 protein. All groups showed similar natural very low neutralisation ability (1:40 plasma dilution providing 50% protection) to the A/Victoria/361/2011 virus indicating that the therapy has had no differential effect on improving the neutralisation of the virus (figure 4.5 A). Similarly, there was no difference between the neutralising ability of any group against the X-31 virus (figure 4.5 B). In fact, high plasma concentrations appear to marginally aid the cellular infection of the virus compared to virus growth media alone (figure 4.5 B).

Having not detected any neutralising ability of the anti-HA₉₁₋₁₀₈ antibodies, it was necessary to determine if the antibodies are able to bind to the whole HA protein present on the virus and not just the HA₉₁₋₁₀₈ peptide. Figure 4.6 A shows that OVA+H(O) treated mouse sample plasma, positive for anti-HA₉₁₋₁₀₈ antibodies, can bind to the HA₉₁₋₁₀₈ peptide and the whole protein. Meanwhile, plasma lacking anti-HA₉₁₋₁₀₈ antibodies (i.e. from a PBS+PBS treated mouse) does not show any binding ability to the peptide or whole protein in native or denatured form (figure 4.6 A). It can be seen that once the peptide has been exposed to denaturing conditions, the antibodies are no longer able to bind, demonstrating that the peptide has some form of conformational structure and is not just a linear epitope (figure 4.6 A). This

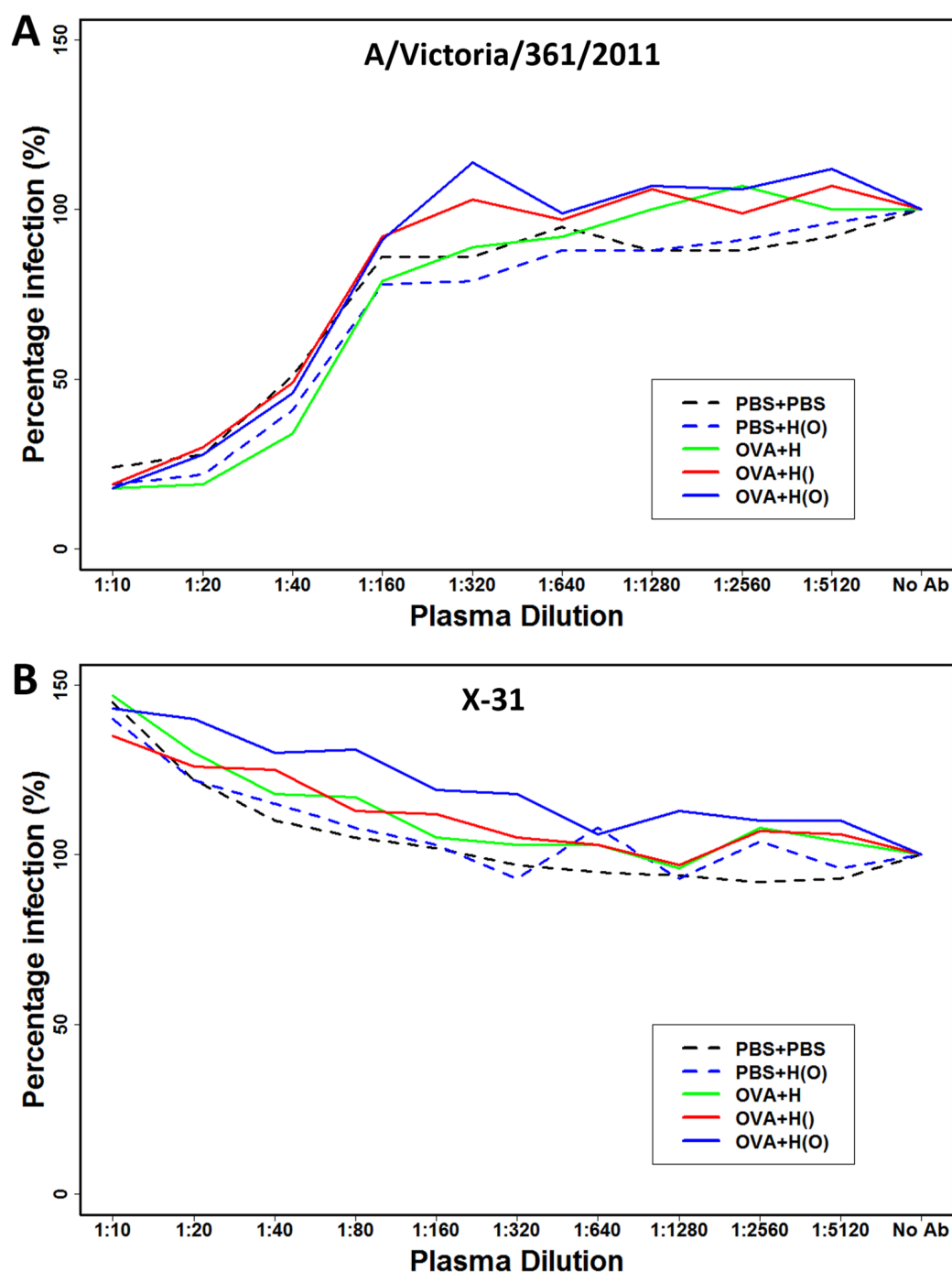


Figure 4.5: Virus neutralisation ability of pooled plasma from vaccinated BALB/c mice. Plasma from each group, taken at week 3, was pooled separately and examined for its ability to neutralise two H3 influenza viruses. The HA_{91–108} peptide is 100% conserved on the A/Victoria/361/2011 virus (panel A), while the X-31 virus (panel B) has one different amino acid, replacing a Tyrosine for a Phenylalanine at residue 94 of the H3 protein. Data represents average of n=2 wells.

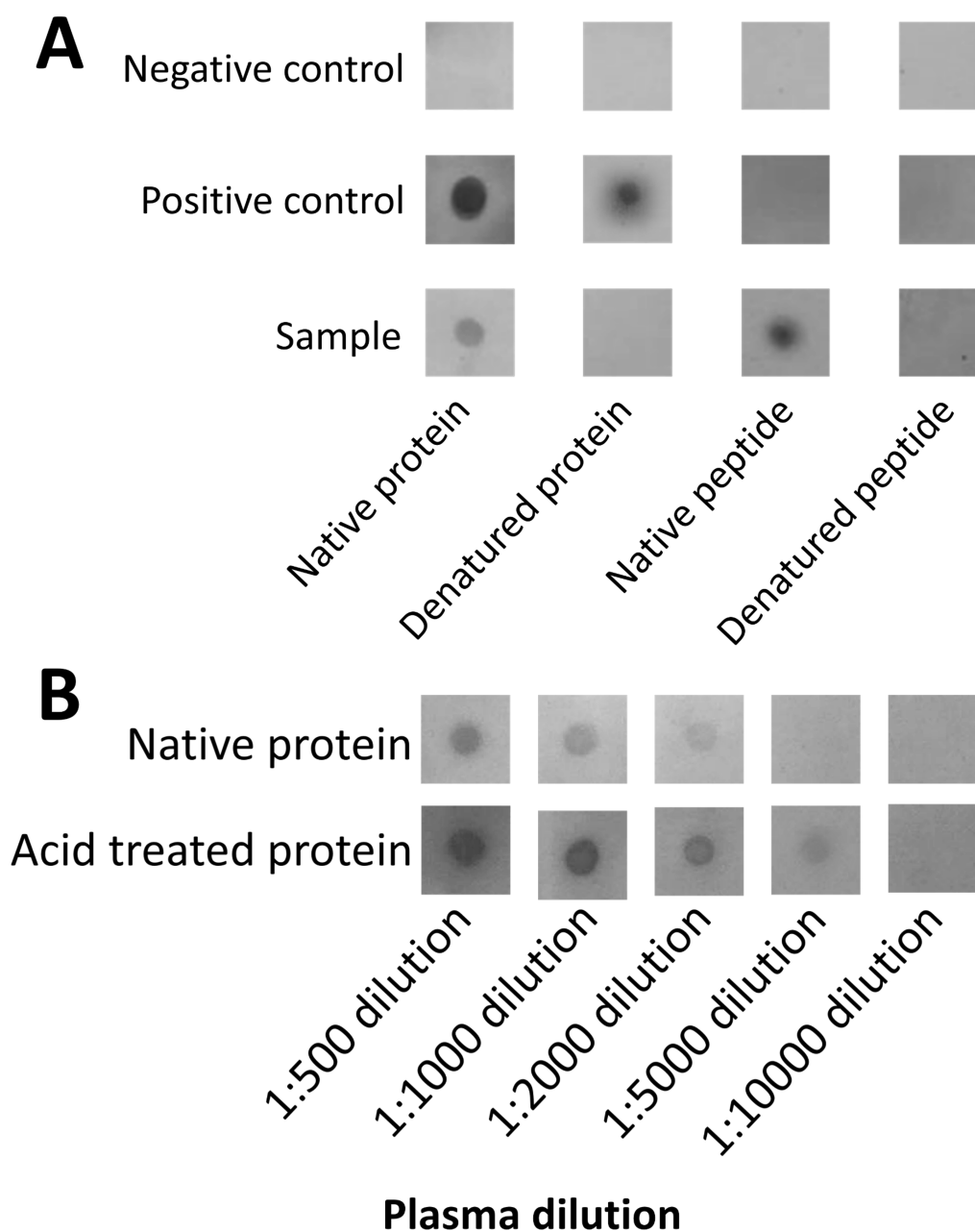


Figure 4.6: Dot blot of antibody binding to recombinant whole HA protein and HA₉₁₋₁₀₈ peptide. Panel A shows the binding ability of pre-vaccine plasma (negative control), MEDI8852 (positive control) and high anti-HA₉₁₋₁₀₈ antibody titre plasma (from OVA+H(O) treated BALB/c mouse) to whole HA protein and HA₉₁₋₁₀₈ peptide in either native or denatured form. Panel B shows the binding ability of the high anti-HA₉₁₋₁₀₈ antibody titre plasma at different dilutions to bind to the recombinant whole HA protein in its native form or after being exposed to acidic conditions (pH 4.0).

structure clearly mimics the epitope of the whole HA protein, though maybe not perfectly since the signal given by the anti-HA₉₁₋₁₀₈ antibodies, binding

to the native protein, is lower than that of an equal number of copies of the peptide for which they are raised against (figure 4.6 A). It can also be seen that the anti-HA_{91–108} antibodies are unable to bind to the whole protein that has been exposed to denaturing conditions, again indicating that the target epitope is not available when quaternary and tertiary protein structure is broken (figure 4.6 A). The MEDI8852 antibody can be seen to strongly bind to the native whole protein and, to a reduced extent, the denatured form of the whole protein (figure 4.6 A).

HA is a pH sensitive protein which irreversibly changes conformation in the presence of acidic conditions [306, 307], such as those of cellular endosomes, to expose its fusion peptide and facilitate endosomal escape [13]. Because of this conformational change, it was worth exploring if it impacts the binding efficiency of the anti-HA_{91–108} antibodies to the whole protein. Figure 4.6 B shows the binding efficiency is improved when binding to the acid-treated protein compared to the native protein. The detection of binding to the native protein is lost at 1:5000 plasma dilution, while for the acid-treated protein the binding detection is lost at 1:10000. This demonstrates that while the HA_{91–108} peptide does mimic the epitope region of the native whole protein, it more closely resembles the epitope conformation on the low pH whole protein conformation (figure 4.6). This may explain how the ability of the antibodies raised by this approach to bind the target epitope presented by the HA peptide is not translated into an ability to effectively bind the whole protein or inhibit infection. It is worth noting that virus neutralisation studies were performed by first incubating virus with dilutions of plasma then adding to cells.

4.4 Summary

This chapter demonstrates the adaptability of the liposomal vaccine platform, explored in chapter 3 and by Hills, et al. [57], to be easily retargeted to the influenza HA protein. Indeed, the same liposome platform and chemistry that was used to link ErbB-2 was used to create H(O) liposomes with very similar physiochemical properties. The HA protein is a highly immunogenic surface protein of the influenza virus which has 18 variants (H1-18) [14]. The two main influenza A viruses that infect humans have the H1 and H3 subtypes, and thus they are the main focus of influenza vaccination [14]. However, HA is subject to a high rate of mutation and so yearly vaccination against the circulating virus strains is required to mitigate infection. Systems are in place to predict forthcoming strains of seasonal influenza, however, owing to extended production and distribution timelines, there are often mismatches between the predicted and actual circulating strains. In 2014/15, due to the emergence of drifted H3 strains, seasonal influenza vaccines were only 19% effective in the USA [16, 17]. While in 2010/11, when circulating influenza strains closely matched predicted strains, seasonal vaccines were found to be 60% effective [17, 18]. In response to the high mutation rate of HA, several groups have discovered well-conserved epitopes on HA that provide possibilities of developing a ‘universal’ long-term vaccine which would not require annual administration due to changes in circulating strains [242]. These well-conserved epitopes, however, tend to have lower immunogenicity than their non-well-conserved counterparts [308]. They, therefore, require the inclusion of an adjuvanting component such as a conjugated helper molecule [296, 299] or an emulsion [72, 309]. Thus, a well-conserved epitope of the HA protein is an ideal candidate for the vaccination platform explored in this thesis.

The well-conserved epitope explored here was the HA epitope first described by Müller, et al. [248], which consists of residues 91-108, designated HA_{91–108}. This group demonstrated that vaccination of mice and rabbits with this peptide, conjugated to helper proteins, led to *in vitro* virus neutralisation and improved survival after viral challenge *in vivo* [248, 294, 295]. Specifically, titres of 12,000 were detected in n=10 mice, one week after the third dose of 50µg. Notably, this serum was not subject to *in vitro* neutralisation analysis but in n=10 mice, exposure to a 50% lethal dose of A/Texas/1/77 influenza virus showed a 100% survival rate compared to a 50% survival rate in non-vaccinated mice [295]. The HA_{91–108} peptide has since been incorporated into a multi-epitope ‘universal’ influenza vaccine candidate, M-001, which is currently in a phase III trial (NCT03450915: clinicaltrials.gov). M-001 is a single polypeptide consisting of three repetitions of nine linear epitopes (table 1.1) expressed in *E. coli* [72]. In a phase II trial, M-001 was used to prime elderly participants (≥ 65) before being given a commercial trivalent influenza vaccine to see if responses to the trivalent vaccine could be improved [73]. Priming with M-001 led to increased seroconversion towards all three strains used in the trivalent influenza vaccine compared to priming with a placebo [73]. Furthermore, serum taken from participants when this study was conducted in 2011 was later tested against the A/Switzerland/9715293/2013 (H3N2-like) strain that caused the 2014/15 influenza epidemic [74]. Serum from participants primed with M-001 had improved seroprotection and seroconversion against this strain which was not known to circulate in 2011/12 [74].

With the change in target peptide from the cancer epitope ErbB-2_{266–296} (explored in chapter 3) to HA_{91–108}, the mechanism of action and the necessity for each vaccine component was again explored for its ability to raise an anti-target antibody response in BALB/c mice. With the HA_{91–108} peptide, the

necessity of each vaccine component can clearly be seen by the great disparity when comparing the total anti-HA₉₁₋₁₀₈ IgG titres of the OVA+H(O) group, which has all the components necessary for the mechanism of action, to all other groups in which the mechanism of action is hindered. The benefit of OVA+H(O) was evident at all time points, with a 98.7-fold higher titre being observed just one week after initial dosing (figure 4.2). These titres reached just 1 week after vaccination are comparable to those reached by Ben-Yedidia, et al. after 3 vaccination doses of 50 μ g of the same HA₉₁₋₁₀₈ peptide conjugated to an ovalbumin protein in the presence of adjuvant [295]. This shows that this liposomal approach provides a remarkable benefit. It can be seen that the HA₉₁₋₁₀₈ peptide is naturally more immunogenic than the ErbB-2₂₆₆₋₂₉₆ cancer epitope used in chapter 3 by observing that the OVA+H group had detectable anti-HA₉₁₋₁₀₈ antibodies, albeit a low amount, one week after initial exposure. On the other hand, mice vaccinated with free ErbB-2₂₆₆₋₂₉₆ never showed a detectable anti-ErbB-2₂₆₆₋₂₉₆ antibody response prior to challenge (figures 3.5 and 3.11). The HA₉₁₋₁₀₈ peptide also demonstrated the highest anti-target antibody titre seen one week after vaccination in mice that received the full treatment (OVA+E(O) or OVA+H(O) groups). After the first vaccine dose, the anti-HA₉₁₋₁₀₈ antibody titres were seen to consistently decline until week 4, when the second booster was given, after which point the titres began to climb again (figure 4.2). The first booster dose (at week 1) did not have an effect in raising the anti-HA₉₁₋₁₀₈ antibody titres. This may be because it was too close to initial dosing to have an effect or vaccine particles may have been rapidly cleared by the high titre immune response present at the time of administration. Determination of the anti-HA₉₁₋₁₀₈ antibody avidity revealed that while the titres of the OVA+H(O) group declined between weeks 1-4, their avidity was increasing (figure 4.4). However, after the week 4 booster dose was

administered the average avidity decreased. This may be due to low-avidity memory B cells increasing their production of low avidity anti-HA_{91–108}, which in turn dilutes the pool of high avidity antibodies. Indeed, the antibody titre levels of the OVA+H(O) group increase 4-fold between weeks 4 and 5. The highest avidity seen by the OVA+H(O) group required 1.48 M of NaSCN to reduce the ELISA signal by 50%. Similarly, Hills, et al. found a concentration of approximately 1.4 M of NaSCN was needed [57]. In contrast, for the control groups just 0.36 M NaSCN was required. Analysis of anti-HA_{91–108} antibody binding to native and modified (denatured or acid-treated) whole protein and peptide revealed that the peptide has some form of structure that is essential for mimicking the conformation of the epitope on the native protein (figure 4.6 A). Similarly, the antibodies could bind to the whole recombinant HA protein but not in its denatured form (figure 4.6 A). However, anti-HA_{91–108} antibody binding was seen to improve when the native protein was exposed to acidic conditions (figure 4.6 B), emulating the conditions of cellular endosomes, which irreversibly changes the protein’s conformation [306, 307].

Isotyping of the PBS+H(O), OVA+H(), and OVA+H(O) groups’ antibody responses showed that prior to vaccination all groups had low levels of natural IgM and IgG3 antibodies with HA_{91–108} cross-reactivity but negligible IgG1, IgG2a and IgG2b levels (figure 4.3). After the first vaccination, the OVA+H(O) group generated increased anti-HA_{91–108} IgM levels, peaking one week after first vaccination before exponentially declining back to a base level identical to that of the PBS+H(O) and PBS+H() groups (figure 4.3 B). This once again demonstrates the need for pre-existing anti-OVA immunity prior to vaccination with the full liposomal construct, H(O), to induce a rapid immune response. Similarly, the OVA+H(O) group showed significantly ($p < 0.01$) higher (7.2-fold) anti-HA_{91–108} IgG3 concentrations after vaccination compared

to other groups (figure 4.3 F). The IgG2a and IgG2b levels of the OVA+H(O) group were also significantly ($p < 0.001$) increased compared to the PBS+H(O) and OVA+H() groups (figure 4.3 D-E). However, despite the fact that the target peptide concentrations administered were similar to the cancer studies described in section 3.3.4, the IgG2a and IgG2b antibody levels were not as high for the influenza peptide, this discrepancy may simply be due to the different peptide being targeted. As seen consistently in chapter 3, IgG1 is the dominant antibody isotype produced by this mechanism of action, with anti-HA₉₁₋₁₀₈ IgG1 having far superior levels compared to the PBS+H(O) and OVA+H() groups where the mechanism is hindered (figure 4.3 C). In mice the IgG1 isotype demonstrates low activation of ADCC and CDC. The significant levels of IgG1 generated by the mechanism are to be expected as the production of IgG1 is increased in the presence of T cell-dependent B cell activation [266]. This may, however, hinder use of the HA₉₁₋₁₀₈ peptide as the target epitope: it was observed that while able to bind to the whole HA protein (figure 4.6), the plasma was unable to neutralise the virus *in vitro* (figure 4.5). This indicates that this epitope may not be a neutralising target to viral infection and may only act as a location to mark the virus for immune clearance by other mechanisms such as CDC or ADCC, mechanisms which are not provided in *in vitro* studies and may not also be optimal for a strategy which raises high levels of murine IgG1 antibodies. Indeed, the mouse IgG1 isotype could potentially inhibit ADCC and CDC by competitive binding preventing other isotypes from binding [266, 310]. There are several possible reasons for the discrepancy between the ability of the raised antibodies to bind to target peptide and protein and the absence of neutralising antibody. One such reason may be that the epitope targeted requires low pH activation to be fully available for antibody binding. Such binding is non-neutralising due to minimal chance

of co-encapsulation of antibody and virus into endosomes. Hence, while the peptide was demonstrated by Müller, et al. to produce *in vitro* neutralising antibodies, they may have predominantly been of a different isotype, capable of fixing complement, which they did not claim to have deactivated in their studies [248]. Similarly, this may be the reason why several studies afterwards demonstrated *in vivo* protection [294, 295]. However, there have been conflicting reports. One study stated that vaccination with this peptide, while able to produce an anti-HA_{91–108} antibody response, did not confer protection from viral challenge [311]. It would, therefore, be prudent for future studies that any target epitope, which is to be used with the mechanism of action, be one that is not only essential to pathogen activity, i.e. either a protein binding site (e.g. ErbB-2_{266–296}) but is also fully available for binding during the time the virus is present in the extracellular environment. This said, it was only in the case of the complete mechanism of action, in the OVA+H(O) group, that a diverse class-switched, high-titre antibody response was generated.

Chapter 5

Conclusions & future work

The overall aim of the DPhil thesis presented here was to further develop the vaccination platform described by Hills, et al. [57]. The main objective of the work was to demonstrate the ability of the vaccination platform to generate a high-titre, class-switched antibody response that was able to inhibit the growth of tumour cells or infection of a pathogen (flu). The liposomal formulation and characterisation methods were also further developed, and to demonstrate the ease of adaptability of the vaccination platform, the vaccine was repurposed to target an epitope of an influenza virus. Notably, for both cancer and flu epitopes, dramatic mechanism-dependent increases in antibody titre (10.5-fold and 98.7-fold, respectively) were detected within 1 week. In this final chapter, an overview of the research is presented, along with suggested avenues for future work.

5.1 General discussion and conclusions

As outlined in chapter 1, many novel vaccination technologies are being employed to develop an arsenal of next-generation vaccines that are able to provide protection against many different types of cancer, and to provide long-lasting immunity against both seasonal and emerging pandemic influenza strains. However, the low immunogenicity of cancer antigens and the high mutation rate of influenza antigens mean that these methods still face challenges. In terms of cancer, there have been some tremendous successes in the field of cancer vaccination with two prophylactic VLP vaccines (Recombivax HB and

Gardasil) and one therapeutic dendritic cell vaccine (Provenge) being licensed to date. While these successes are remarkable, they do not pave a clear way for the production of other cancer vaccines. Recombivax HB and Gardasil are prophylactic cancer vaccines which prevent hepatitis B and HPV viruses from inducing liver and cervical cancers, respectively. There are, however, only a select number of cancers that have been linked to viruses to provide simple viral epitopes as targets. Provenge is the only licensed therapeutic cancer vaccine to date, however, given its high-cost, high-complexity, and modest clinical efficacy [119, 120], it does not obviously contribute to the journey away from live and attenuated vaccines, into an era of vaccines with lower complexity and cost-of-production that are more suited to the treatment of large populations within the confines of reducing healthcare resources. In terms of influenza, one of the main focuses has been on the development of a ‘universal’ vaccine that contains well-conserved regions of the virus strains so that yearly vaccinations are not required. However, these well-conserved regions of the virus tend to be less immunogenic and, therefore, require the inclusion of an adjuvanting component such as a conjugated helper molecule [296, 299] or an emulsion [72, 309]. There is yet to be a licensed ‘universal’ influenza vaccine. Therefore, current flu vaccine are heavily dependent on prediction of strain prevalence, a situation that can leave the vaccine ineffective.

The first peptide explored as a target in this thesis was an epitope of the ErbB-2 cancer-associated antigen. ErbB-2 is overexpressed on 15-30% of all breast cancers, as well as in many other types of cancer [258]. Many patients diagnosed with ErbB-2 overexpressing tumours have shown an ability to mount a weak immune response towards this antigen, showing that it is possible to overcome the self-tolerance associated with it [239, 240]. The epitope of the ErbB-2 protein used here (ErbB-2_{266–296}) was first described by

Allen, et al. and is part of an ongoing phase I clinical trial (NCT01376505: clinicaltrials.gov) [63, 65, 66]. This peptide mimics the ErbB-2 binding region of Pertuzumab [63], which is a FDA-approved, monoclonal antibody used in the clinic to treat patients with ErbB-2 overexpressing breast cancers. A phase III trial comparing the response of patients with ErbB-2+ metastatic breast cancer treated with either Trastuzumab and Docetaxel alone or in combination with Pertuzumab found that, when combined with Pertuzumab, the median progression-free survival was increased from 12.4 months to 18.5 months without affecting the treatment safety profile [260]. A follow up to this study showed that the median overall survival of patients had increased from 40.8 months to 56.5 months in those with Pertuzumab included in their treatment [261]. Pertuzumab works by inhibiting ErbB-2/ErbB-3 dimerisation, which is the most potent dimerisation in the ErbB family [262]. The ErbB-2_{266–296} peptide used here should, therefore, mimic Pertuzumab's inhibition of ErbB-2/ErbB-3 dimerisation and downregulate the level of ErbB-2 signalling.

The second peptide explored as a target was an epitope of the HA influenza antigen. The HA protein is a highly immunogenic surface protein of the influenza virus which is subject to a high rate of mutation [14]. Due to this high rate of mutation, yearly vaccination against the circulating viral strains is required to mitigate infection. Systems are in place to predict forthcoming strains of seasonal influenza, however, owing to extended production and distribution timelines, there are often mismatches between the predicted and actual circulating strains. In 2014/15, due to the emergence of drifted H3 strains, seasonal influenza vaccines were only 19% effective in the USA [16, 17]. While in 2010/11, when circulating influenza strains closely matched predicted strains, seasonal vaccines were found to be 60% effective [17, 18]. The HA epitope used here (HA_{91–108}) was first described by Müller, et al. and is currently

incorporated into an ongoing phase III clinical trial (NCT03450915: clinical-trials.gov) [72–74, 248]. This epitope is well conserved on the majority of H3 influenza strains and, therefore, presents an appealing target for long lasting anti-influenza immunity.

The vaccination platform proposed in this thesis has the potential to generate a rapid, dynamically class-switched, high-titre humoral immune response. The plug-and-play nature of this platform allows this response to be re-targeted against any desired antigenic target, regardless of its inherent immunogenicity, whether it has ‘self’ resemblance like a cancer epitope or low immunogenicity like a well-conserved epitope of an influenza virus. The objectives of this thesis can be outlined as follows:

1. To improve liposomal formulation and characterisation methods, specifically:
 - (i) Improve scalability of the formulation method.
 - (ii) Improve the peptide incorporation efficiency.
 - (iii) Confirm the internal helper peptide is exclusively inside to the liposome.
2. Confirm the mechanism of action by demonstrating the requirement of helper peptide presentation for increased anti-target specific antibody production.
3. Determine the efficacy of the proposed mechanism of action targeted against a cancer epitope in inhibiting tumour growth.
4. Demonstrate the adaptability of the mechanism of action by repurposing the vaccination platform to target an epitope of viral origin.

The first research objective was addressed in chapter 2. The original formulation method described by Hills, et al. [57] made use of the thin-film hydration method first described by Bangham, et al. [249]. While this method is useful for small scale laboratory testing, it would be impractical for large scale production as, realistically, it can only produce one small (<5mL) batch of one type of liposome per day. The first alteration made to this formulation method, therefore, was a transition to lyophilisation, first described by Szoka and Papahadjopoulos [250]. Though this method takes longer to produce a batch of liposomes (2-3 days vs 1 day), it allows for an increased laboratory production (<100mL) of liposomes that are not necessarily identical formulations. Another benefit in choosing lyophilisation over thin-film hydration is that, when employing thin-film hydration, only one liposome batch can be produced each day resulting in high batch to batch variability. In contrast, in the case of lyophilisation, the ability to produce multiple batches concurrently reduces this batch to batch variability and, therefore, makes the process more reliable and reproducible.

Switching from the Avanti extrusion kit to the t&t Scientific kit resulted in an astonishing increase in the concentration of external peptide conjugated to the liposomal surface (table 2.2). This may be due to either a component of the Avanti kit or an impurity, introduced by insufficient cleaning before use, catalysing hydrolysis of the reactive maleimide linker and resulting in fewer maleimide linkers available for target peptide conjugation post-extrusion. Impurities introduced by the Avanti extrusion kits were also the likely cause of higher anti-ErbB-2_{266–296} titre levels seen in the first tumour challenge model compared to during the dosing regime optimisation study (sections 3.3.2 and 3.3.3).

Free OVA_{323–339} or OVA_{323–339} on the surface of the liposome surface could

potentially diminish B cell receptor binding of the external ErbB-2 or HA peptide. It was, therefore, imperative to confirm that the OVA peptide was exclusively located inside of the liposome. This was demonstrated by protein A purification columns and ELISA, which showed only a slight decrease in sample OVA concentration after purification (figure 2.2). This mild decrease ($\approx 15\%$) in concentration is likely due to dilution factors during the purification process. Alternatively, this decrease may have been caused by small amounts of OVA peptide conjugated to the outer surface of the liposomes. However, the majority of the OVA peptide can clearly be seen to be exclusively located inside of the liposomes. After storage for 15 months at 4°C , no leakage of internally encapsulated peptide or shedding of surface conjugated peptide was detected (table 2.5). Therefore, the majority of OVA peptide has been shown to be internal to the liposome with minimal (if any) leakage over time.

Liposomes were designed to have an approximate diameter of 200 nm, which falls within the expected range for a virus particle (20-400 nm). This should enable increased *in vivo* half life compared to larger liposomes (>500 nm) [255]. Their small size also enables them to be freely trafficked to draining lymph nodes where they can come in direct contact with B cells [256, 257]. Liposomes were also demonstrated to be stable after 15 months' storage at 4°C , with minimal change in size (figure 2.10) over this time period. The mechanism of action explored in this thesis requires vaccine particles to be degraded once engulfed by B cells in order to facilitate peptide fragment presentation on their MHC II proteins for T cell recognition. Therefore, vaccine particles were designed to be anionic to prevent them from escaping this endosomal degradation like some cationic particle [125]. Anionic liposomes are also less cytotoxic than their cationic counterparts [312]. Indeed, the anionic liposomes, formulated here, were shown to be non-cytotoxic *in vitro* (figure 2.7). The

ease of adaptability of the liposomal formulation was also demonstrated by comparing the physico-chemical characterisations of liposomes with different target peptides.

Chapter 3 began by addressing the second research objective outlined above by making use of the FVB/n mouse strain, which is unable to complete the mechanism of action explored here with OVA_{323–339} as the helper peptide (section 3.3.1). This is because they lack the MHC class II haplotype (I-A(d)) capable of presenting the OVA peptide for helper T cell recognition [246, 247]. Indeed, all FVB/n mice exposed to ErbB-2_{266–296} presenting liposomes generated comparable anti-ErbB-2_{266–296} IgG responses, regardless of the existence of pre-existing anti-OVA immunity or the inclusion of internal OVA peptide in the liposomes (figure 3.3 A). BALB/c mice, however, do possess the I-A(d) MHC II haplotype capable of presenting the OVA peptide [313]. In contrast to the antibody responses seen in the FVB/n mice, BALB/c mice demonstrated that individuals with pre-existing anti-OVA immunity, vaccinated with E(O) liposomes, generated significantly increased anti-ErbB-2_{266–296} IgG levels compared to groups which lacked necessary components of the mechanism of action (figure 3.3 B). This, therefore, provided further exquisite proof that the mechanism of action, proposed by Hills, et al. and developed here, is capable of generating increased antibody levels by non-cognate helper T cell activation of B cells, activated by a liposomal vector with precise spatial separation of B cell receptor and helper T cell peptides.

The third research objective was then first explored by an initial large cohort tumour challenge study (section 3.3.2). This demonstrated that mice which could conduct the full mechanism of action (OVA+E(O) vaccinated mice) were able to generate a rapid, significantly raised IgG response (figure 3.5). However, mice in the PBS+E(O) group which lacked pre-existing anti-

OVA immunity, but were still vaccinated with E(O) liposomes, also generated an anti-ErbB-2_{266–296} antibody response (figure 3.5). This was likely due to the presence of a second peptide presented by B cells, doubling the chance of low-level circulating CD4⁺ T cell, with TCR specific for a presented peptide, coming into contact with a B cell that engulfed and processed vaccine particles. The rapidity of the response demonstrates one of the advantages of the mechanism of action. In comparison, DNA-based vaccines tend to take a long time (often >2 weeks) and require multiple administrations before antibody responses can be detected [278, 279]. Meanwhile, even conjugate vaccines and live attenuated vaccines can require multiple doses before detectable IgG responses can be observed [280–283]. The isotype profiles of the two highest responding groups, PBS+E(O) and OVA+E(O), were then characterised. Higher levels of class-switching were seen in the OVA+E(O) group just one week after first vaccination, leading to significantly higher IgG1, IgG2a and IgG2b levels (figure 3.6). These responses were seen to increase even further after administration of the booster vaccination (figure 3.6 C-E). IgM is the first antibody isotype produced after initial antigen exposure [268]. Both groups showed comparable, tightly distributed levels throughout the duration of the experiment with increases after each vaccination (figure 3.6B). While IgG3 levels were again similar between groups, there was a large within-group variation after booster dosing (figure 3.6 F). The mean IgG1 levels produced saw a staggering 10,000-fold increase compared to those seen by Hills, et al., while the IgG2a (equivalent isotype to IgG2c produced by C57Bl/6 mice) and IgG3 levels were comparable and the IgG2b levels were approximately 2-3 fold lower [57]. These differences are likely due to the different strains of mice or simply the different peptide being targeted. Indeed, several studies have shown that C57Bl/6 mice produce lower levels of the IgG1 isotype compared to BALB/c

mice [285–288]. Mice were challenged with ErbB-2 overexpressing tumour cells which were allowed to grow for 3 weeks. Anti-ErbB-2_{266–296} IgG titres could subsequently be detected in those mice that had not previously been exposed to the ErbB-2_{266–296} peptide (PBS+(O) and OVA+(O) groups) (figure 3.5). This indicates that the peptide is representative of the epitope located on the TUBO tumour cells. Indeed, the antibodies generated by the OVA+E(O) group against the peptide were then demonstrated to be able to bind to the whole ErbB-2 protein either in recombinant form or from TUBO cell lysate (figure 3.7). However, despite high-titre IgG antibody levels and confirmation of the target epitope’s presence on the tumour cells at the point of challenge, no statistically significant differences between the tumour growth curves were seen for any group (figure 3.8). This is in contrast to the findings of Allen, et al. who showed a GMT of approximately 100,000 could be generated by a 3 dose delivery regime of ErbB-2_{266–296} peptide conjugated to a measles virus fusion protein, in the presence of ISA720 adjuvant, which would lead to protection from TUBO tumour cell implantation [63]. These differences may be explained by the higher titre levels achieved prior to implantation by Allen, et al., which in turn may be the result of increased adjuvantation in each dose, or differences in the dosing regime. There was, however, a strong correlation between the weights of the resected tumours and spleens, indicating there was an ongoing anti-tumour immune response in the mice, although it was not group-specific (figure 3.8).

In response to this, the dosing regime was then explored to find an improved regime that induces increased anti-ErbB-2_{266–296} titre levels (section 3.3.3). The liposomal manufacture method also was improved by switching from method B, using the Avanti extruder, to method C, using the t&t Scientific extruders (table 2.1). This allowed for improved control over the concen-

tration of ErbB-2 peptide conjugated to liposomes. It was demonstrated that administration of a higher concentration (10 μg) of ErbB-2_{266–296} on liposomes led to higher anti-ErbB-2_{266–296} IgG titres (figure 3.9). Administration of the same concentrations of ErbB-2 peptide used in the initial tumour challenge model (0.4 μg) led to lower IgG titres than seen previously. This was likely due to the change from a self-assembled extruder kit to a pre-assembled, sterile, disposable extruder kit with less risk of contamination. The contamination occurring in method B is thought to have acted in an adjuvanting capacity in the first large-cohort vaccination study. Boosting the response with free ErbB-2 peptide was shown to be insufficient to increase the anti-ErbB-2_{266–296} IgG titres compared to boosting with E(O) liposomes. This may be due to the same mechanism of action as explored here, or it may simply be due to the adjuvanting effects of the liposome. Lastly, it was examined whether an extra boosting dose, one week post initial vaccination, would improve the IgG titres. However, due to small sample sizes and one mouse, treated with two booster doses, only responding mildly to treatment compared to the other members of the group, no difference could be discerned. Despite this, the remaining two members of the HighE(O)3 group consistently had the highest anti-ErbB-2_{266–296} IgG titres after being administered the first boost at week 1, one week after the initial vaccination (figure 3.9). The dosing regime of the first tumour challenge, LowE(O)2 (see table 3.4), and the two groups with the highest GMTs, HighE(O)3 and HighE(O)2, were further explored by isotyping of the antibody responses (figure 3.10). All those which received the high concentration of ErbB-2_{266–296} generated high levels of ErbB-2_{266–296}-specific IgG1, particularly the two highest responders of the HighE(O)3 group. Mild levels of IgG2a and IgG2b were also seen in mice administered with the high concentration of ErbB-2_{266–296}. Because HighE(O)3 and HighE(O)2 had simi-

lar responses, and because of the small sample sizes, it was difficult to perceive differences between these treatment regimes. The HighE(O)3 group was chosen as the optimal dosing regime to take forward as three vaccination doses were needed by Allen, et al., who first described the peptide ErbB-2_{266–296}, to demonstrate tumour growth inhibition in BALB/c mice [63].

This improved dosing regime was then implemented in a second tumour challenge study with the aim of inducing higher anti-ErbB-2_{266–296} antibody levels and inhibition of tumour growth (section 3.3.4). It was again demonstrated that the only group that could execute the mechanism of action (the OVA+E(O) group) had the highest anti-ErbB-2_{266–296} IgG titres (figure 3.11). Both of the liposomally-vaccinated groups that could not recruit non-cognate CD4+ T cells to help activate B cells (the PBS+E(O) and OVA+E() groups) were seen to have comparable IgG responses (figure 3.11). Isotyping of the PBS+E(O), OVA+E(), and OVA+E(O) groups' antibody responses revealed stronger IgG2a and IgG2b class-switching than seen in previous studies (figure 3.12 D-E). This is likely due to the increased ErbB-2_{266–296} concentrations administered compared to the previous tumour challenge study, as there was minimal difference between any of the three groups examined. The OVA+E(O) group, again, showed the highest levels of IgG1 class-switching compared to the PBS+E(O) and OVA+E() groups (figure 3.12 C). Mice dosed with E(O) liposomes generally had higher IgG3 levels than those which received E() liposomes, indicating that the presence of the OVA peptide, regardless of pre-existing immunity towards it, aided in the production of anti-ErbB-2_{266–296} IgG3 (figure 3.12 F). Plasma samples positive for anti-ErbB-2_{266–296} antibodies were then shown to be capable of inducing *in vitro* cell death of TUBO cells (figures 3.13 and 3.14). This is likely due to the blockade of ErbB-2/ErbB-3 dimer formation, and thus a reduction of ErbB-2 activation. It has previously

been demonstrated that cell lines that overexpress ErbB-2 are susceptible to apoptosis following anti-ErbB-2 therapy, whereas cell lines that do not overexpress the receptor were not [289]. This may explain why plasma positive for anti-rat-ErbB-2 could induce apoptosis in TUBO cells *in vitro* despite co-expression of mouse ErbB-2 in these cells. A primary function of ErbB-2 is suppression of apoptosis to enhance cell survival, thus inhibition of its activation will increase cellular apoptosis [290, 291]. However, again, despite the high anti-ErbB-2 antibody titres, no statistically significant differences in the tumour growth curves *in vivo* could be seen between any group (figure 3.15). The reduced take-rate in this study was likely due to fewer cells being administered to challenge the mice (3e4 vs 1e5 cells) (figure 3.15A) [263]. To probe if successful mechanism of action was being concealed by tumour growth and structure heterogeneity, the mice with the 4 largest tumours from each group, at cull, had their tumours and spleens resected and processed into single-cell suspensions to analyse the cellular populations contained within them. Tumour cells were found to have lost their ErbB-2 overexpression since being implanted, giving a reason as to why the anti-ErbB-2_{266–296} humoral response had no impact in the tumour take or growth rates (figures 3.16 and 3.17). TUBO cells are a transgenic cell line spontaneously developed by a BALB/c mouse that was transformed to express the rat ErbB-2 oncogene [263]. The loss of rat ErbB-2 expression is then likely due to *in vivo* selective pressures increasing the burden on cells expressing this transgene, resulting in the loss of its expression in favour of its already existing mouse ErbB-2 protein.

These studies, therefore, demonstrate the ability for the mechanism of action to rapidly induce a high-titre, isotype-switched antibody response against a peptide that mimics an epitope of the cancer antigen, ErbB-2. IgG1 was consistently the main anti-ErbB-2_{266–296} antibody isotype generated by vac-

cination. This isotype is the same as the pre-clinical studies' version of Pertuzumab, prior to humanisation [284]. Therefore, this particular anti-ErbB-2_{266–296} antibody isotype should not only bind to the same region of ErbB-2 as Pertuzumab, but also interact with the immune system in the same way as to its clinical precursor. However, in moving to the clinic, Pertuzumab was humanised and was made with the human IgG1 isotype, which, in contrast to its murine counterpart, is a strong activator of complement and effector cells [267]. Murine IgG1 production is increased in the presence of T cell-dependent B cell activation, such is the case for the proposed mechanism of action [266]. Its contribution to protective immunity is largely unclear, as it is unable to fix complement and only engages with the inhibitory Fc γ RIIb receptor of effector cells [266]. Murine IgG1's main role is, therefore, believed to be similar to that of the human IgG4 isotype, in limiting inflammatory responses and neutralising target molecules from binding their receptor [266]. The antibody responses were subsequently demonstrated to be capable of growth inhibition and cell death induction of an ErbB-2 overexpressing tumour cell line *in vitro*. The likely mechanism for this is a blockade of ErbB-2/ErbB-3 dimer formation, inhibiting ErbB-2 from suppressing apoptosis. The discrepancy between *in vitro* and *in vivo* tumour growth inhibition is likely due to differing amounts of anti-ErbB-2_{266–296} present in the vicinity of the cells, allowing the tumour cells *in vivo* more time to adapt to the selective pressures present. Profiling of the rat ErbB-2 expression during the development of the tumours would be an important step in validating this model if it were to be used again.

The fourth research objective was addressed in chapter 4 by repurposing the liposomal vaccine platform to target the well-conserved HA_{91–108} epitope of H3 influenza viruses. Using the improved dosing regime found in section 3.3.3, mice with/without pre-existing anti-OVA immunity were vacci-

nated with either PBS, free HA peptide, H() liposomes, or H(O) liposomes. It was clearly seen that the full mechanism of action generated significantly higher anti-HA₉₁₋₁₀₈ IgG titres compared to all other groups (figure 4.2). The OVA+H(O) group demonstrated the highest anti-target IgG titre one week post-initial vaccination seen thus far (figure 4.2). This is indicative of the natural immunogenicity of a viral-based epitope compared to a cancer epitope which has a high level of 'self'-resemblance [314]. The natural immunogenicity of the peptide could also be seen by the detection of an anti-HA₉₁₋₁₀₈ IgG titre in mice just one week post-vaccination with free HA₉₁₋₁₀₈ peptide (figure 4.2). No such detectable humoral response was seen previously in mice vaccinated with free ErbB-2₂₆₆₋₂₉₆ peptide (figures 3.5, 3.9, and 3.11). The OVA+H(O) groups IgG titres peaked at week 1, one week after initial vaccination. The titre levels then began to wane continuously until week 4, when the second booster vaccine was administered, after which the responses began to climb again (figure 4.2). The first booster dose, administered on week 1, did not raise the anti-HA₉₁₋₁₀₈ antibody titres. This may be because it was too close to initial dosing to have an effect or because vaccine particles may have been rapidly cleared by the high titre immune response present at the time of administration. Determination of the anti-HA₉₁₋₁₀₈ antibody avidity revealed that while the titres of the OVA+H(O) group declined between weeks 1-4, their avidity was increasing (figure 4.4) and demonstrated substantially improved avidity compared to control groups. However, after the week 4 booster dose was administered the average avidity decreased. This may be due to low-avidity memory B cells increasing their production of low avidity anti-HA₉₁₋₁₀₈, which in turn dilutes the pool of high avidity antibodies. Indeed the antibody, titre levels of the OVA+H(O) group increase 4-fold between weeks 4 and 5.

Isotyping of the PBS+H(O), OVA+H(), and OVA+H(O) groups' antibody responses showed that all groups had low levels of natural IgM and IgG3 antibodies with HA₉₁₋₁₀₈ cross-reactivity but negligible IgG1, IgG2a and IgG2b levels prior to vaccination, similar to that seen for the cancer studies in chapter 3 (figure 4.3). One week after initial vaccination, the OVA+H(O) group generated increased anti-HA₉₁₋₁₀₈ IgM levels, which was seen to exponentially decline over the following weeks back to a base level identical to that of the PBS+H(O) and PBS+H() groups (figure 4.3 B). Similarly, the OVA+H(O) group showed significantly higher anti-HA₉₁₋₁₀₈ IgG2a, IgG2b, and IgG3 concentrations after initial vaccination compared to the PBS+H(O) and OVA+H() groups (figure 4.3 D-F). However, despite the fact that the target peptide concentrations administered were similar to the cancer studies described in section 3.3.4, the IgG2a and IgG2b antibody levels were not as high for the influenza peptide. This discrepancy may simply be due to the different peptide being targeted. It was again observed that mice vaccinated with liposomes encapsulating the OVA peptide and surface-decorated with the target peptide (E(O) or H(O) liposomes) generated higher IgG3 levels compared to mice vaccinated with similar liposomes without the internal OVA peptide (E() and H() liposomes) (figure 4.3 F). This indicates that the encapsulated OVA peptide was a big determining factor in IgG3 levels, regardless of the existence of pre-existing immunity towards it. As seen consistently in chapter 3, IgG1 was the dominant antibody isotype produced by the mechanism of action, with anti-HA₉₁₋₁₀₈ IgG1 having far superior levels compared to the PBS+H(O) and OVA+H() groups where the mechanism is hindered (figure 4.3 C). The significant levels of IgG1 generated by the mechanism are to be expected as the production of IgG1 is increased in the presence of T cell-dependent B cell activation [266]. This may, however, hinder use of the HA₉₁₋₁₀₈ peptide as the

target epitope. Analysis of anti-HA_{91–108} antibody binding to native and modified (denatured or acid-treated) whole protein and peptide revealed that the peptide has some form of structure that is essential for mimicking the conformation of the epitope on the native protein (figure 4.6 A). Similarly, the plasma containing anti-HA_{91–108} antibodies could bind to the recombinant whole HA protein but not in its denatured form (figure 4.6 A). However, anti-HA_{91–108} antibody binding was seen to improve when the native protein was exposed to acidic conditions (figure 4.6 B), emulating the conditions of cellular endosomes, which irreversibly change the protein's conformation [306, 307]. Despite this, while able to bind to the whole HA protein (figure 4.6), the plasma was unable to neutralise the virus *in vitro* (figure 4.5). This indicates that this epitope may not be a neutralising target for viral infection and may only act as a location to mark the virus for immune clearance by other mechanisms such as CDC or ADCC. If so, the murine IgG1 isotype inhibits these by competitive binding, preventing other isotypes from binding [266, 310]. Indeed, while the peptide was demonstrated by Müller, et al. to produce *in vitro* neutralising antibodies, they may have predominantly been of a different isotype, capable of fixing complement, which they did not claim to have deactivated in their studies [248]. Similarly, this may be the reason why several studies afterwards demonstrated *in vivo* protection [294, 295]. However, there have been conflicting reports. One study stated that vaccination with this peptide, while able to produce an anti-HA_{91–108} antibody response, did not confer protection from viral challenge [311]. It would, therefore, be prudent for future studies that any target epitope, which is to be used with the mechanism of action, be one that is not only essential to pathogen activity, i.e. either a protein binding site (e.g. ErbB-2_{266–296}), but is also fully available for binding during the time the virus is present in the extracellular environment. This said, it was only

in the case of the complete mechanism of action, in the OVA+H(O) group, that a diverse class-switched, high-titre, high-avidity antibody response was generated.

5.2 Future work

In order to improve the efficacy of the proposed mechanism of action to produce an increased antibody response capable of providing a protective response, a number of suggestions are made for potential future work.

The primary focus, when attempting to increase the immune response generated by the mechanism of action, should be improving the poor loading efficiency of the helper peptide within liposomes. Currently, the majority of OVA peptide added to dried lipids during formulation is not encapsulated, but is instead purified away and discarded. Not only is this wasteful, but it also means that there is less peptide within the liposomes to be presented for T cell activation. Logically, an increase in internal helper peptide concentration would lead to an increase in anti-helper CD4+ T cell activation. It is, therefore, imperative that a controllable method for effective loading of the helper peptide into the liposomes is explored. Several studies have been carried out in the attempt to improve the poor loading efficiency of liposomes. One such study found that hydration of lipids with buffer containing the peptide to be loaded, in the presence of 30% ethanol, increased the loading efficiency 5-fold compared to hydration with peptide containing buffer alone [315]. Other groups have utilised several rounds of freeze-thaw cycles to increase the loading efficiency [316], however, with the risk of maleimide hydrolysis, this method may not be well suited to the current liposome formulation. The concentration of cholesterol incorporated into the liposome structure also plays a role, as while cholesterol increases the structural stability of liposomes, it also

has been shown to inhibit liposomal loading [317]. One class of methods for increasing the liposomal loading efficiency is called active-loading, by which pre-formed liposomes are filled with drug molecules that are able to diffuse into the liposome but not diffuse back out [318]. These methods have been used for a wide variety of hydrophobic and ionisable molecules. However, proteins and peptides are generally more hydrophilic with minimal charge, and, therefore, are not able to freely diffuse through the lipid bilayer [318]. Therefore, active loading methods may be of little use in increasing the concentration of helper peptide encapsulated within the liposomes. Assessment of B cell interactions with liposomal particles would also provide further proof of the mechanism of action. One method of doing this could be to encapsulate fluorescently-tagged peptides within the liposomes and use immunofluorescence microscopy to observe the uptake of the liposomes by B cells.

As described by Hills, et al. the use of OVA_{323–339} as the helper peptide is simply for proof of concept purposes [57]. In terms of clinical translatability, the use of an epitope with high seroprevalence in a population as the helper peptide would negate the need to pre-vaccinate against the helper peptide first to establish anti-helper CD4+ T cells. Hills, et al. recommended a cytomegalovirus which has a high degree of seroprevalence worldwide. Cytomegaloviruses are ubiquitous, yet they are very species specific [319, 320], and are a common cause of infections in humans and many other animal species. Human cytomegalovirus (HCMV) infects the majority of the human population with seroprevalence generally increasing with age [321]. In the USA, seroprevalence ranges from 36.3% in those ages 6 - 11 up to 90.8% in those aged over 80 [322]. In Italy, it was shown that the anti-cytomegalovirus antibody prevalence increased from 28% in two year-olds to 95.7% in 45 - 54 year-olds [323]. A study in Finland followed a cohort of children for 8 years

and found that the prevalence of anti-cytomegalovirus antibodies increased from 27% when they were 7 months of age to 41% at 8 years of age [324]. While blood donors from populations in Asia and Africa have demonstrated seropositivity rates of 90 - 100% [325–329]. Sylwester, et al. found that a sample of 33 healthy seropositive individuals had a median of 4% of the CD4+ T cells pool directed against HCMV epitopes, with the maximum value being approximately 40% [330]. Meanwhile, Sester, et al. found that a sample of 161 seropositive patients had a median HCMV-specific CD4+ T cell population size of 1.87%, with a maximum of 43.8% [331]. For translation of the mechanism of action, HCMV, therefore, makes an attractive source for the liposomally-encapsulated helper antigen.

The degree of helper CD4+ T cell activation may also be boosted by the inclusion of multiple helper epitopes encapsulated within the liposomes. This would lead to a larger pool of CD4+ helper T cells capable of activating B cells that have engulfed the vaccine particles. Pre-existing immunity would, therefore, be required for each of the included helper epitopes. The simplest way to scale this would be to encapsulate a whole protein rather than an array of peptides. However, this presents challenges of its own: the protein would need to be immunogenic and have no exposed cysteines on its surface in order to mitigate the risk of whole protein binding to the maleimide linker. Otherwise, this may inhibit the exclusive spatial segregation of helper and target peptides/proteins. Another factor is the expense of recombinant whole proteins compared to that of custom-synthesised peptides. The formulation method may also need refining as currently the extrusion step is executed at 50°C, which may well denature the protein (though this may not present an issue as the protein will still undergo endosomal degradation).

Further investigation is also required as to why the surface conjugation ef-

efficiency increased dramatically after switching to the single-use t&t Scientific extruder kits as this remains unexplained. One method for investigating the differences in the extruder kits' effects on the number of available maleimides for conjugating the target peptide is described by Martínez-Jothar, et al. which uses UV-Vis spectroscopy to measure the number of hydrolysed maleimide regions [332]. This would then be a method of demonstrating if the Avanti extruders are hydrolysing the maleimide linkers before the external target antigen can conjugate to them.

In order to produce a diversified immune response, multiple target epitopes may be combined onto a single liposome, either in the form of multiple synthetic peptides or a whole protein. The addition of extra target peptides would allow for antibody production against multiple different regions of a single protein or multiple different target proteins. For the cancer studies explored in chapter 3, antibodies were produced against a peptide that mimics the binding region of Pertuzumab [63], and should, therefore, inhibit ErbB-2/ErbB-3 dimerisation. ErbB-2 can be activated by the formation of a dimer with any of the four members of the ErbB family [259]. Thus, the inclusion of multiple epitopes onto the vaccine, that would prevent ErbB-2 dimerisation with other members of the ErbB family, should inhibit the activation of ErbB-2 further. One candidate for this would be the peptide described by Garrett, et al. which mimics the binding region of Trastuzumab, and should inhibit ErbB-2/ErbB-2 dimerisation [64]. These two peptides have already been combined in an ongoing phase I clinical trial [65, 66]. Pertuzumab and Trastuzumab are already used clinically in combination to treat ErbB-2 overexpressing cancers [62]. Therefore, it is of interest to combine the two peptides mimicking their respective binding regions into the vaccine to provide active immunity against these cancers by inhibition of ErbB-2 signalling. The inclusion of mul-

multiple target peptides would be extremely easy to achieve in comparison to the inclusion of a whole protein. While the inclusion of whole protein would provide a multitude of antigenic regions to produce a dynamic immune response against, there are many challenges to its incorporation. Firstly, the current formulation method requires maleimide-thiol conjugation; therefore, the target protein would be required to have a free cysteine available for conjugation. While disulfide bonds could be broken to produce this free cysteine, it risks denaturing the protein beyond recognition from the original native protein, leading to antigenic regions no longer being mimics for the desired target. Alternatively, the formulation method could be modified to use NHS-amine conjugation. The main difficulty with this approach is that amines are far more common than thiols in proteins, and there are likely to be multiple regions for liposomal conjugation on the surface of the target protein [333]. This is an issue for two reasons. Firstly, it provides little control of protein orientation, which may be necessary to effectively display antigenic regions for B cell receptor recognition [334]. Secondly, with multiple amines available on the surface of a protein, there is a high risk of multiple liposomes binding to the same protein, leading to aggregation unless sufficient amounts of protein are available. Perhaps the most appealing method is, therefore, the SpyTag/SpyCatcher coupling to conjugate a protein to the liposomal surface [60]. This would provide a simple method for specifically conjugating a target protein in a designated orientation. However, assurances would be needed that the addition of the SpyTag/SpyCatcher sequence to the target protein would not modify the protein structure or immunogenicity, which is essential for antibody production. The inclusion of whole protein as the target for the mechanism of action may also only be desirable for select target proteins e.g. ErbB-2. However, it may be less suited to highly mutative proteins, e.g. HA,

as the immune response will likely focus on the highly immunogenic, highly mutative regions of the protein rather than the well-conserved regions.

In order to demonstrate the *in vivo* protective ability of the mechanism of action, further determination of an improved dosing regime is required. It was shown that increasing the concentration of ErbB-2_{266–296} peptide increased the antibody titre, and that free ErbB-2 peptide was insufficient to boost the immune response compared to boosting with E(O) liposomes. However, the effect of the number of booster doses administered was unclear due to large within-group variation and small sample sizes. Therefore, further testing is required. Similarly, optimisation will be required for each individual target, as the cancer studies found that of the three E(O) doses, the third had, at best, a minimal effect (figure 3.11), while for the influenza studies, of the three H(O) doses, the second dose had minimal effect (if any) (figure 4.2).

The mechanism of action was demonstrated here to mainly produce an IgG1 response in BALB/c mice. IgG1's production is increased in the presence of T cell-dependent B cell activation, such is the case for the proposed mechanism of action [266]. However, Hills, et al. demonstrated that the mechanism of action led to an IgG response predominantly consisting of the IgG2b isotype in C57Bl/6 mice [57]. Several studies have demonstrated that BALB/c mice produce higher levels of the IgG1 isotype than C57Bl/6 mice [285–288], but lower levels of IgG2b [285, 288, 305]. This information, combined with the fact that human IgG isotypes interact with the immune system differently to those of murine origin, creates the necessity for a representative animal model to enable future successful translation into the clinic of this method [266, 267]. Indeed, a report by D'Alessio, et al. highlighted that in order for improved development of a universal influenza vaccine much consideration must be given to the choice of the type and strain of animal used pre-clinically

[335]. Ferrets and mice are the most common laboratory animals used for influenza research due to their susceptibility to infection without modification of the virus, however, both species have overly strong T-cell responses compared to humans [335]. The use of humanised mice provides an interesting avenue for evaluation of vaccine efficacy. Humanised mice are immunodeficient mice, engrafted with human cells or tissues [336, 337]. They, therefore, have the potential to be a relatively good model for mimicking how a vaccine or drug would perform in the clinic. However, humoral immunity, which is the main aim of the vaccination platform described in this thesis, in humanised mice has many limitations, such as incomplete B cell development and impaired class-switching, as seen in many models such as the BLT and Hu-HSC mouse models [336, 337]. Therefore, more consideration is required in the decision of which animal model to use.

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