

Imperial College London

Heterologous Prime-Boost Vaccine Regimens

Against *Chlamydia trachomatis*

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“The world could show nothing to me,
So what good would living do me?”

Brian Wilson & Tony Asher, The Beach Boys

Abstract

Chlamydia trachomatis is the most common bacterial sexually transmitted disease in man and despite decades of effort, there is still no protective vaccine. Left untreated, genital infection can lead to pelvic inflammatory disease, ectopic pregnancy, and infertility. However, infection-induced immunity in both animal models and humans indicates a strong role for CD4⁺ Th1-biased immune responses. Using a multi-component vaccine approach we assessed the immunogenicity and protective efficacy of novel plasmid DNA, Adenovirus 5 (HuAd5) and modified vaccinia Ankara (MVA) vectors each containing a major outer membrane protein (MOMP) transgene and recombinant MOMP protein in various heterologous prime-boost regimens in BALB/c and B6C3F1 mice.

During the course of the prime-boost regimens, serum and vaginal MOMP-specific antibody titres, subtypes, avidities and neutralisation abilities were assessed, alongside IFN- γ ELISpot and CD4⁺ and CD8⁺ T cell polyfunctionality (IFN- γ , TNF- α , and IL-2). Regimens were grouped on the distinct MOMP-specific immune environments they elicited, with these regimens taken through into *C. trachomatis* vaginal challenge studies in two mouse models to shed light on the relative contribution of each environment to protective immunity.

The DNA-HuAd5-MVA-Protein (D.A.M.P.) vaccine regimen resulted in a significant reduction in *C. trachomatis* vaginal shedding at day 3 post-infection in both BALB/c and B6C3F1 mouse strains. This significant reduction was lost when D.A.M.P. vaccinated mice were depleted of their CD4⁺ T cells prior to challenge, indicating the protection is CD4⁺ T cell mediated. *C. trachomatis* EB serum neutralisation profiles were similar between protective and non-protective vaccine regimens and combined with passive transfer experiments into naïve C57BL/6 mice and IFN- γ knock-out C57BL/6 mice we concluded that the antibody response did not play a significant role in this vaccine-induced protection.

As well as infecting the genital tract, *Chlamydia trachomatis* is also the causal agent of trachoma, the leading cause of infectious blindness in the world. Recently, Kari *et al.* revealed serum anti-MOMP antibodies correlated with the reduction in chlamydial ocular burden in non-human primates, while anti-PmpD and anti-Pgp3 serum antibodies correlated with chlamydial eradication. We therefore investigated if we could induce such anti-chlamydial antibodies on the murine eye through heterologous prime-boost vaccinations. We uncovered a vaccination regimen that induced significantly greater anti-MOMP ocular antibodies, and employed this regimen for the additional chlamydial antigens, of which all induced ocular antigen-specific IgG antibodies. This is the first investigation into such vaccination regimens to induce chlamydial specific ocular antibodies and provides a new model for the screening of future potential trachoma vaccines.

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Declaration of Originality

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Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cells
ASC	Antibody secreting cell
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
CD	Cluster of differentiation
CMI	Cell-mediated immunity
DC	Dendritic cells
ddPCR	Droplet digital polymerase chain reaction
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EB	Elementary body
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunoSpot assay
FcR	Fc Receptor
g	The acceleration due to gravity
GC	Germinal centre
GST	Glutathione S-transferase
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
hr	Hour
HuAd5	Human adenovirus serotype 5
i.d.	Intradermal
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
i.vag.	Intravaginal
ICS	Intracellular cytokine staining
IFA	Immunofluorescence assay
IFN	Interferon
IFU	Inclusion forming units
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilodalton

KO	Knock-out
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
min	Minutes
MPLA	Monophosphoryl lipid A
MVA	Modified vaccinia virus Ankara
NLR	NOD-like receptors
OD	Optical density
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	0.05% Tween-20 in phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
RB	Reticulate body
RLR	Rig-like receptor
RNA	Ribonucleic acid
RT	Room temperature
s.c.	Subcutaneous
SFU	Spot forming unit
TCR	T-cell receptor
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
WT	Wild type
α	Amino acid

Chapter 1

Introduction

1 Introduction

1.1 The importance of vaccines

The use of vaccines has been instrumental in the prevention and control of infectious disease and remains one of the most cost-effective public health interventions. In the 1790s, Edward Jenner was the first to demonstrate that inoculation with cowpox virus could confer protection against smallpox in man [1]. To date smallpox is the only human infectious disease to be eradicated through vaccination, closely followed by poliomyelitis which has had its incidence reduced by >99% [2]. Though not eradicated, vaccination still controls and reduces the global morbidity and mortality associated with several other infectious diseases such as diphtheria, measles and rubella [3].

1.2 Vaccine immunology

Vaccine-mediated protection requires activation of the human immune system, with the establishment of an effective immune response requiring both the innate and adaptive arms of the immune system. Most current licensed vaccines (Hepatitis B, measles, pertussis, rabies) mediate protection through the induction of immunoglobulin (Ig) G serum antibodies [4]. However the partially effective Bacillus Calmette-Guerin (BCG) vaccine appears to use T cells producing interferon (IFN)- γ as the main effector mechanism [5]. It is becoming apparent that for diseases including malaria and HIV, it is likely that vaccine induced protection may require effective

engagement of both the humoral and cellular arms of the immune system. In addition to the humoral and cellular arms of immune responses the system has traditionally been further sub-divided into the innate (inherited) and the adaptive (learned) immune system.

1.2.1 Innate Immune System

Once barrier immunity is breached, the innate immune system is the first point of contact of vaccines with the human immune system. The innate immune system acts to clear or control pathogens as well as activate the adaptive immune system, however it does not confer immunological memory.

1.2.1.1 Innate Immune Cells

Natural killer (NK) cells, basophils, eosinophils, $\gamma\delta$ T cells and mast cells are the non-phagocytic leukocytes of the innate immune system. NK cells are cytotoxic, releasing perforin and granzyme upon activation following their encounter with damaged cells or cells downregulating their major histocompatibility complex (MHC) class I molecule expression due to viral infection [6]. Basophils and eosinophils release histamine upon pathogen encounter, contributing to defence against parasites but pushing the immune response towards allergic and inflammatory responses [7]. $\gamma\delta$ T cells are innate-like cells, with characteristics similar to lymphocytes of the adaptive immune system, such as gene arrangement of their T cell receptor (TCR). Though

they do not recognise MHC presented antigens, they do release cytokines such as IFN- γ [8].

Dendritic cells, macrophages and neutrophils are the phagocytic cells of the innate immune system. Upon interaction with various cell surface receptors (CD205, macrophage mannose receptor, scavenger receptors) they internalise pathogens, forming a phagolysosome which results in the destruction of the phagocytosed pathogen via antimicrobial peptides, proteases and reactive oxygen species [9].

1.2.1.2 Innate responsive molecules

Pathogen-associated molecular patterns (PAMPs) and common structural motifs of pathogens are recognised by pathogen recognition receptors (PRRs), which lead to the activation of the innate immune system through distinct signaling pathways. Several PRRs are expressed by innate immune cells such as the Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-like receptors (RLRs) [10]. Nine functional TLRs have been discovered in humans (and 11 in mice) and are broadly classified according to their ligands [11]. TLRs 1, 2, 4 & 6 recognise lipids and are expressed on the cell surface of many cells of the immune system, whereas TLRs 3, 7, 8 & 9 recognise nucleic acid and are expressed in endosomal compartments. TLR agonists have been extensively used in adjuvant development [12] and are discussed in 1.3.4.1.

NLRs are a recently identified family of intracellular PRRs, with 22 identified in humans to date. The ligands and functions of many of these receptors are not known,

but their primary role of those that have been more fully described is to recognise cytoplasmic PAMPs and endogenous danger signals [13]. RLRs are cytoplasmic RNA helicases critical for host antiviral responses, and when activated lead to the transcription of interferons and other pro-inflammatory cytokines [14].

1.2.1.3 Dendritic Cells

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that act as messengers between the innate and the adaptive immune systems. Therefore the activation of these cells is essential in generating a protective immune response. DCs circulate in peripheral tissues including the dermis and epidermis where they endocytose antigen, process it for display on MHC molecules before migrating to secondary lymphoid organs where they display antigen to T cells [15]. DCs can also present antigen to B cells in high endothelial venules (HEVs) [16]. Once activated by PAMPs, DCs mature and secrete cytokines such as IL-12 and IL-23, further modulating the immune response induced [17]. Pattern recognition receptors (PRRs) expressed on DCs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-like receptors (RLRs) [10].

Plasmacytoid dendritic cells (pDC) are a subset of dendritic cells, constituting 0.2-0.8% of the peripheral cell blood population in humans [18]. These cells express TLR7 and TLR9 and play a major role in antiviral immunity by rapidly producing large amounts of type-1 interferon (mainly IFN- α and IFN- β) after viral stimulation through the induction of the TLR pathway [19].

1.2.2 Adaptive Immune System

If the innate immune system fails to clear or neutralise a pathogen then the adaptive arm of the immune system is the next line of defence and if activated appropriately, can create a pathogen-specific and potential effective response and establish a long-lasting memory immune response. This adaptive arm of the immune system is the target of vaccination.

1.2.2.1 T cells

T lymphocytes play a central role in cell-mediated immunity, and recognise antigen presented by APCs (B cells and DCs) on MHC molecules in the presence of co-stimulatory signals. They are distinguished from other lymphocytes by the presence of a T-cell receptor (TCR), and are named as such due to their development and maturation in the thymus. CD4⁺ T cells recognise exogenous antigen displayed on MHC class II molecules of the APC, while CD8⁺ T cells recognise endogenous antigen degraded in the cells cytoplasm which is then displayed on MHC class I molecules. However, there is also the phenomenon of cross-presentation in which exogenous antigen is loaded onto MHC class I molecules resulting in the activation of CD8⁺ T cells by this alternative mechanism, a function generally carried out by specialised DC populations.

1.2.2.2 CD4+ T cells

CD4+ T cells act through the recruitment and activation of target cells mainly through the secretion of cytokines and chemokines. Naïve conventional CD4+ T cells have at least 4 distinct fates depending on the pattern of signals they receive during their initial interaction with antigen: Th1, Th2, Th17, and T regulatory cells. Within the scope of this thesis and relevancy to chlamydia vaccination, only Th1 and Th2 induction has been measured and investigated.

Under the stimulation of IFN- γ and IL-2, naïve CD4+ differentiate into Th1 T cells through the expression of the transcription factor T-bet [20]. Th1 cells mediate immune responses against intracellular pathogens. They principally secrete IFN- γ and tumor necrosis factor (TNF)- α , with IFN- γ being important in activating macrophages to increase their microbicidal activity, recruiting NK cells, as well as promoting opsonising antibody protection and isotype class switching of B cells [21].

Th2-type cells are differentiated in the presence of IL-4 through the expression of the transcription factor GATA-3 [22]. Th2 cells mediate the activation and maintenance of the humoral immune response by secreting IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25, which are responsible for strong antibody production, eosinophil activation, and the inhibition of several macrophage functions [23]. These cytokines counteract the Th1 responses, with IL-4 being a positive feedback cytokine and the major mediator of IgE class switching in B cells [24].

1.2.2.3 CD8+ T cells

CD8+ T cell's major role is to directly kill infected cells. Upon recognition of antigen displayed on MHC class I of an infected cell, CD8+ T cells secrete the pro-inflammatory cytokines IFN- γ and TNF- α , recruiting phagocytes to the site of infection. Furthermore, CD8+ T cells induce the death of their target cells through the direct release of perforin and granzymes. Perforin mediated membrane pore formation allows granzymes to enter the target cell's cytoplasm where they trigger the caspase cascade eventually leading to apoptosis. Another way apoptosis is induced is through the activation of FasL on CD8+ T cells that binds to the death receptor Fas on the target cells [25].

1.2.2.4 B cells and Antibodies

B cells are lymphocytes, distinguishable by the presence of the B cell receptor (BCR) on their outer surface. This specialised receptor, unique to each B cell, allows a B cell to bind a specific antigen. The principle function of B cells is to make antibodies through VDJ recombination, which constitute the secreted form of the BCR and can provide systemic humoral immunity throughout the body. There are three types of B cells: B1 B cells, marginal zone (MZ) B cells and follicular B cells. B1 and MZ B cells have innate-like properties, not circulating through lymph nodes, and can be activated without T cell help. Follicular B cells require T cell help from Tfh cells for activation, resulting in clonal expansion and differentiation of these cells. All B cells make IgM and IgD, but through signals received from the T cell help and the resulting

cytokine environment, the B cells can undergo class switching to produce isotypes IgG, IgA and IgE.

B cells can further undergo somatic hypermutation and clonal selection in an organised germinal centre to produce high affinity antibodies. Following contact with antigen in secondary lymphoid organs, B cells migrate to the interface of the T cell zone within the B cell follicle. Here antigen bound to the BCR is taken up and processed before being presented to T cells on MHC class II [26]. T cells recognising this peptide-MHC complex secrete IL-6 and IL-21 and synthesise CD40L, which interacts with CD40 on the B cell to initiate B cell proliferation and affinity maturation, and finally to effect maturation of the activated B cell into an antibody secreting cell. Activated B cells can produce either short-lived plasma cells or can enter the germinal centre reaction to produce long-lived plasma cells and memory B cells (mBCs). These mBCs can redifferentiate back into antibody secreting plasma cells after re-encountering antigen and thus make up humoral immunological memory that provides long-term immunity.

The circulation of B cells and their entry and retention in secondary lymphoid organs is dependent upon adhesion molecules and chemokine receptors [27] [28]. The expression of LFA-1 and VLA-4 permit entry into the lymphoid tissue, with the chemokine receptors CXCR5 and CCR7 directing localisation within the tissue. CXCR5 is expressed on all mature B cells and mediates the B cell migration to follicles in response to CXCL13. If the B cell does not encounter a cognate antigen it exits the

lymphoid organ in response to the sphingosine-1-phosphate (S1P) molecule, but on antigenic encounter the B cells are retained in the lymphoid organ due to the up regulation of CCR7 [29]. The ligands for CCR7 (CCL19 and CCL21) attract antigen-activated B cells to the T cell zone where cognate T cell-B cell interactions occur, and later the chemokine CXCL12 promotes the migration of plasmablasts to the bone marrow where they develop into long-lived plasma cells [30].

Antibodies have a variety of effector functions. These include neutralisation, complement activation, as well as initiating phagocytosis in macrophages and neutrophils and inducing NK cell antibody dependent cellular cytotoxicity through interactions with Fc receptors (FcRs) [26].

1.2.3 Mucosal Immunology

Mucosal surfaces are a major site of entry for pathogens, with mucosal surfaces having the largest area of contact of the immune system with the environment. The mucosal compartment of the immune system displays functional independence from the systemic compartment, with lymphoid tissues in the mucosae as opposed to the spleen, lymph nodes and bone marrow of the systemic system [31]. Mucosal epithelial cells provide both a physical barrier and a site for innate immunity. Physical barriers include the presence of mucus secreted from goblets cells and the expulsion of particles via beating ciliated epithelial cells [32]. Intraepithelial lymphocytes constitute a major component of the mucosal epithelium, consisting of

various T cell subsets with CD8+ T cells predominating over CD4+ T cells [33].

The mucosal immune system can be separated into inductive and effector sites. The cellular basis of immune responses in the gastrointestinal and female reproductive tracts involves the migration of lymphocytes from these mucosal inductive sites to the effector tissues via the lymphatic system. The mucosa-associated lymphoid tissue (MALT) network including the gut-associated lymphoid tissues (GALT) and nasopharyngeal-associated lymphoid tissues (NALT) provides the source of memory T and B cells which then move onto effector sites [34]. The MALT also contains APCs, including DCs, which can carry antigen to mesenteric lymph nodes to initiate mucosal T and B cell responses, while mucosal homing receptors on activated T cells direct these migrating cells to the mucosal lamina propria [35].

The lamina propria regions of female reproductive tract contain antigen-specific effector cells including IgA-producing plasma cells, and alongside CD4+ T cell help, these support the production of IgA antibodies [36]. Polymeric IgA (pIgA) from the lamina propria binds the polymeric Ig receptor (pIgR) on epithelial cells, upon which pIgA is internalized and transported across the epithelia. The released pIgA results in secretory IgA (sIgA) antibodies [37]. An overwhelming majority of mucosal antibodies in humans are produced in the mucosal tissues, with only a fraction of the antibodies deriving from circulation [38]. Accordingly, protection against respiratory and intestinal diseases including influenza and cholera correlate more strongly with external secretion antibodies than serum antibodies [39].

1.3 Vaccine Delivery Platforms

Issues with the safety profile of live vaccines, the weak immunogenicity of subunit vaccines and immunisation failure due to poor patient compliance to booster doses are a few of the reasons which necessitated the development of a new generation of vaccines to promote effective immunisation. Attempts have been made to deliver vaccines through carriers such as liposomes or nanoparticles or ISCOMs, as they spatially and temporally control antigen presentation to the immune system. This allows reduced doses of weak immunogens to still stimulate immune responses [40].

In some instances the immunologically protective immunity may be complex and necessitate the induction of strong cellular immunity rather than a threshold titre of neutralising antibodies. To that end, replication-deficient viral vectored vaccines, such as adenoviruses and poxviruses, and DNA-based vaccines have emerged as promising delivery platforms particularly aimed at facilitating the induction of strong T cell responses [41] [42] [43].

There has also been increasing recognition that such delivery platforms pose numerous advantages, including relative ease of generation, genetic manipulation, scale-up and manufacture; capacity for relatively large inserts and potential for multiantigen *in vivo* delivery; stability; as well as circumventing the need for recombinant protein production and coadministration of reactogenic adjuvants [44]

[45] [46].

1.3.1 DNA based vaccines

A significant advantage of DNA vaccines is their ability to evoke both humoral and cell-mediated immune responses. Following transfection of DNA vaccine into myocytes and antigen presenting cells, the host-synthesised antigen is presented to both major histocompatibility complex class I (MHC I) and MHC II proteins. Antigen-loaded APCs travel to the draining lymph node where they present peptide antigens to naive T cells. Here, activated antigen-presenting CD4+ T-helper cells activate B cells, resulting in both arms of the immune system targeting the plasmid-encoded antigen [47]. The vaccine protein can also be taken up by scavenger cells such as macrophages and DCs and presented without cross-presentation on the exogenous Class II pathway. DNA vaccines can encode multiple immunogenic epitopes, and their construction can be rapid, cheap and large-scale, making them a serious alternative vaccine platform.

The intramuscular route is the most common route of immunisation for DNA vaccines, with myocytes being the predominant cell type transfected [48]. Electroporation is among the most effective preclinical delivery systems for DNA vaccines to increase the transfection efficiency of target cells. Electroporation can involve delivering an electric current at the same time as the DNA injection, or

involve penetrating probes that target the muscle to deliver an electric pulse. The current hypothesis is that electroporation forms transient membrane pores due to the transmembrane voltage, and this membrane destabilisation facilitates the movement of macromolecules in the extracellular medium to the intracellular milieu, though the exact mechanism of DNA translocation (electrophoretic facilitation versus passive diffusion) is debated [49]. *In vivo* expression levels improve markedly using electroporation and can improve both cellular and humoral responses [50] [51] [52].

A few potential concerns of DNA vaccination include the theoretical risks of integrating into cellular DNA resulting in insertional mutagenesis/chromosomal instability, the development of autoimmune disorders against patient DNA, and the risk of transfer of antibiotic resistance conferred by the plasmid gene [53] [54] [55]. The low immunogenicity of DNA vaccines along with the low levels of T cell and B cell memory elicited in humans are being addressed with the use of novel formulations, delivery systems and their use in prime-boost strategies [47].

1.3.2 Adenovirus Vected Vaccines

Recombinant viral vectors have also been explored as vaccine delivery platforms, particularly aimed at inducing strong T cell responses through their direct infection of cells leading to the loading of target antigens onto MHC class I molecules or through the induction of cross-presentation of secreted antigens [56].

Adenoviruses are dsDNA viruses (34-43 kb) belonging to the Adenoviridae family. They are an attractive vaccine platform due to their thorough characterisation and ease of manipulation [57]. For vaccine vector use, Adenoviruses have been made replication-defective for human cells by deleting the E1 and E3 regions. The E1 region codes for viral proteins essential for the initiation of viral replication, while the E3 region is involved in immunomodulation [58] [59], and such deletions have allowed for the insertion of up to ~6 kb of foreign DNA into the viral vector.

The majority of Adenovirus vectored vaccines have been based on the human serotype 5 adenovirus (HuAd5), with this vector able to induce strong B and T cell responses against multiple different antigens [60] [61] [62] [63]. HuAd5 has a broad tropism, infecting a variety of dividing and non-dividing cells through the Coxsackievirus and adenovirus receptor (CAR), and can be applied systemically as well as through mucosal surfaces. Their thermostability and ability to be produced to high titres under GMP conditions has facilitated their clinical use and they have already been used in 5 independent HIV vaccine trials, proving to be safe and immunogenic in humans [64] [65] [66] [67]. The vector's safety was thrown into doubt following an HIV trial in which a non-significant trend for an increased HIV infection rate in immunised volunteers who were seropositive for HuAd5 before vaccination was found [68]. Further analyses have revealed no durable relationship between vaccination and increased HIV infection, yet the claims have however

damaged the reputation of this promising vaccine delivery platform.

HuAd5 has been an invaluable tool in pre-clinical vaccine screenings, yet the high prevalence of pre-existing immunity to HuAd5 within the human population has reduced their immunogenicity and thus their use clinically [69]. To overcome this, rare human adenovirus serotypes (HuAd35) have been explored, as well as the use of Chimpanzee adenoviruses, which are not neutralised by human sera [70], [71].

1.3.3 Poxvirus Vectored Vaccines

Orthopox viruses are large, enveloped dsDNA viruses, which replicate in the cytoplasm. They can accommodate >25 kb of foreign DNA, making them attractive as vaccine vectors. As mentioned previously, smallpox remains the only human disease to date that has been eradicated, and the cross-reactivity within the Orthopoxvirus genus allowed the vaccinia virus to be used to vaccinate millions of people during the WHO's smallpox eradication campaign [72]. However, as vaccinia is replication competent, safety concerns resulted in the development of replication incompetent vaccinia virus. The virus' 570 passages through chick embryo fibroblast cells (CEFs) resulted in a loss of over 15% of the vaccinia genome, resulting in a highly attenuated version of the vaccinia virus. This attenuated strain, unable to replicate in almost all mammalian cells, was named modified vaccinia virus Ankara (MVA) [73]. MVA's use as a vaccine against smallpox in over 120,000 people reveals an excellent

safety profile and strengthens its potential as a vaccine vector for other diseases.

MVA has conserved the ability to activate robust cellular MHC class I- and II-restricted CD8+ and CD4+ T cell responses against recombinant antigens. MVA vectors have never been used for vaccination against *Chlamydia trachomatis*, but have been successfully used to target infectious diseases including malaria, HIV and TB [74], [75], [76]. MVA expressing the Mycobacterium tuberculosis antigen 85A (MVA85A) has been used in Phase I/II clinical trials as a boost to the BCG prime, with the MVA85A vaccine inducing potent CD4+ T cell responses [77], and MVA vectors used in malaria vaccines have induced high frequencies of CD8+ T cells [78].

1.3.4 Recombinant Protein Vaccines

Recombinant protein vaccines typically consist of protein antigens produced in a heterologous expression system, such as bacteria, yeast or eukaryotic cells. Recombinant protein vaccines are based on the concept that humoral immune responses mounted towards an infection are often targeted to regions on the surface of protein antigens, known as epitopes. These immunogenic proteins are then expressed using heterologous expression systems. Vaccinating with just the most immunogenic protein(s) induces a more targeted immune response and eliminates the risk of active infection that may occur with live attenuated or incomplete inactivated vaccines [79].

Based on their composition, recombinant protein vaccines predominately invoke humoral immune responses and antibody-based immunity. There are a number of successful, commercial recombinant protein vaccines where antibody based immunity is paramount, including the recombinant cholera toxin B in the vaccine Dukoral®, and the recombinant hepatitis B surface antigen produced in yeast in the vaccine RECOMBIVAX HB®.

1.3.4.1 Adjuvants

Adjuvants, or “the immunologist’s dirty little secret” [80], are formulations of chemical compounds or molecules added to recombinant protein vaccines to enhance their immunogenicity or to alter their immunogenic profile. Adjuvants are added to recombinant proteins as they lack the intrinsic immunogenic properties found in live, attenuated, killed whole organism or even virally vectored vaccines. In addition to directing immune responses against target antigens, adjuvants can prolong vaccine induced immune responses, as well as increasing the breadth and quality of immune responses they induce [81], [82], [83].

Two specific adjuvants are used in this thesis: MF59 and monophosphoryl lipid A (MPLA). MF59 is the only purely emulsion-based adjuvant currently licensed for use in humans and is an oil-in-water emulsion consisting of squalene, produced by

Novartis, and used in Europe in an influenza vaccine [84]. It has been shown to lead to a broader more balanced IgG1/IgG2a response in mice and when used as the adjuvant in a Phase 1 H5N1 influenza vaccine trial resulted in the persistence of the protective antibody response [85]. MF59 was thought to function through an antigen depot effect like alum, however recent microarray data have shown MF59 up-regulates genes associated with antigen presentation and leukocyte migration [86], and acts in an inflammasome-independent manner [87].

Monophosphoryl lipid A (MPLA) is the only toll-like receptor (TLR) agonist licensed for use in humans. MPLA is an agonist for TLR4, and is a part of the Fendrix HBV vaccine produced by GSK. All TLRs apart from TLR 3 signal through the adaptor molecule MyD88, whereas TLR3 signals through TRIF. TLR 4 is unique in its ability to signal through both adaptor molecules. MPLA is a modified analogue of the Lipid A moiety of Lipopolysaccharide (LPS) which is itself too toxic for use in humans, and has been shown to induce potent antibody and Th1-type immune responses [88] [89].

1.3.5 Prime-Boost Regimes

Single immunisations or sequential immunisations utilising the same vaccine often do not induce protective immune responses against pathogens that may require both a cellular and humoral immune response. Owing to this, prime-boost regimes

can be undertaken in which multiple vaccine vectors expressing the same transgene are administered sequentially [90]. The prime-boost strategy has mainly been employed to enhance T and B cell responses induced by viral vectored vaccines in an attempt to avoid anti-vector immunity induced against the priming or prior boosting vector. Studies have revealed a differential ability of viral vectors to prime and boost humoral and cellular immune responses. In particular MVA fails to prime antibody responses but can efficiently boost adenovirus primed antibody responses, whereas adenovirus can both prime and boost antibody responses [91].

1.4 Chlamydia

The obligate intracellular Gram-negative bacteria of the genus *Chlamydia* cause ocular, genital and respiratory infections. Human ocular infections of *C. trachomatis* cause trachoma, the world's most common preventable blinding disease [92], with an estimated 150 million people infected worldwide and 6 million visually impaired or blinded [93]. Genital infections by *C. trachomatis* are of notable global concern. The WHO rates genital chlamydial infections as the most common bacterial sexually transmitted disease (STD) worldwide, with over 90 million of the 500 million total yearly global cases of STDs being *C. trachomatis* [94]. Economically, in the USA, over \$3 billion is spent each year on an estimated 4 million reported clinical cases of genital chlamydial infections [95].

C. trachomatis infects the single-cell columnar epithelial cells of the endocervix of women and the urethra of men. Within these epithelial cells, Chlamydia undergoes a unique biphasic developmental cycle (Figure 1.1). The infectious elementary body (EB) is endocytosed by mucosal epithelial cells before being surrounded by endosomal membranes to form an inclusion. Within this inclusion, the EB differentiates into the more metabolically active reticulate body (RB), which then divides by binary fission. Within 40-48 hours the RBs redifferentiate into infective EBs, before inclusion exocytosis releases the EBs to infect neighbouring cells. Intracellular RBs can form a non-replicating persistent state if under stresses such as penicillin exposure, but revert after the removal of the stress.

Sexually transmitted *C. trachomatis* infection is an important public health concern due to its adverse effects on reproduction. Pelvic inflammatory disease (PID), ectopic pregnancy and tubal factor infertility (TFI) are major complications of untreated female genital chlamydial infection, with these complications due to scarring of the fallopian tubes and ovaries caused by the infecting Chlamydia. PID is the infection of the upper female genital tract, including the uterus, fallopian tubes and ovaries [96]. Furthermore, *C. trachomatis* infection facilitates HIV transmission [97], and may be a co-factor in human papilloma virus induced cervical neoplasia [98].

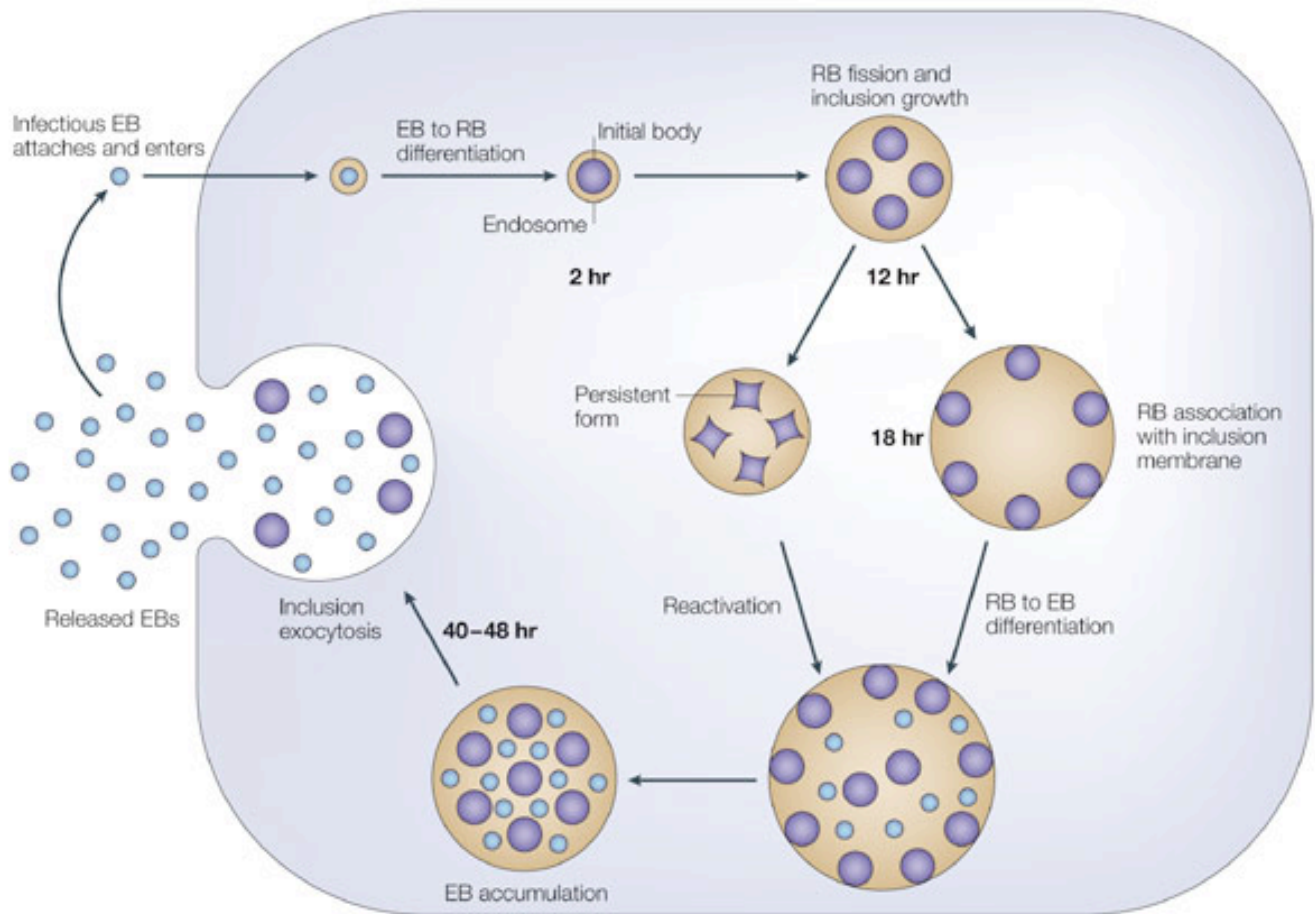


Figure 1.1. *Chlamydia trachomatis*' biphasic developmental cycle.

Adapted from Robert C. Brunham & J. Rey-Ladino [99]. *C. trachomatis* resides within a specialised vacuole termed an 'inclusion' which provides a permissive environment for the replication of *C. trachomatis*. Within the inclusion, the EB differentiates into the RB which divides by binary fission before dedifferentiating back into EBs. These EBs are released from the inclusion to infecting neighbouring cells

Although early detection of infection can lead to successful treatment with antibacterial agents (in particular doxycyclines), the high proportion of asymptomatic infections (70-90% in women, and 30-50% in men) often result in severe and sometimes irreversible complications presenting as the first symptoms of an infection [100] [101]. Up to 40% of untreated chlamydial genital infections lead to PID, and overall 11% of women with PID develop TFI and 9% develop ectopic pregnancies [102]. *C. trachomatis* screening and treatment programs have been set up in the United Kingdom, the United States, Australia, Canada, Finland, Norway and Sweden, yet in all of these countries *Chlamydia* rates have appeared to increase in the face of these programs [103]. These increased rates could be due to improvements in *Chlamydia* testing technology, but another hypothesis is that by identifying and treating chlamydial infections early, the infected individuals may not develop protective immunity to re-infection. This “arrested immunity hypothesis” suspects that those treated are still susceptible to subsequent infection, and they likely rejoin their previous sexual networks and become reinfected [103]. Therefore, of the different prevention and control strategies being developed against *Chlamydia*, the current medical opinion is that immunisation is likely to be the most reliable, effective and economical approach in controlling chlamydial infections, its transmission and its associated complications [92].

1.4.1 Chlamydia and the immune system

Both human and animal studies have been conducted to study the type of immunity required for protection against *Chlamydia*. To analyse the innate and adaptive responses to infection with *C. trachomatis*, the mouse model of vaginal infection, using the murine chlamydial strain *C. muridarum*, has often been used. *C. muridarum* is commonly used as an analogue to *C. trachomatis*, though there are notable differences in their pathology. *C. muridarum* is naturally a gut pathogen of mice, not typically a genital infection, and mice usually clear experimental genital infections with this strain within 4 weeks, whereas *C. trachomatis* infections in humans can persist for months or years [104]. Due to these pathological differences, we will use *C. trachomatis* in the mouse model as demonstrated by Olsen *et al.*, (2010) [105], Peterson *et al.*, (1999) [106] and Ramsey *et al.*, (1999) [107]. Oculogenital serovars of *C. trachomatis* can successfully infect the murine genital tract, with the resulting infection closely mimicking the features of the uncomplicated and self-limiting infection that occurs in most women [108].

Immunity to *C. trachomatis* likely involves protective T cell and B cell immunity in the genital tract mucosa [99]. T cells are known to be key mediators of protection against *Chlamydia* infection. Nude mice were shown to be unable to control infection, indicating the need for T cell mediated immunity [109]. Furthermore, depletion of either CD4+ or CD8+ T cells leads to more severe primary *Chlamydia* infections [110]. The adoptive transfer of CD4+ T cells from *C. muridarum* pre-

infected mice resulted in the protection of naive mice from *C. muridarum* challenge [111], with CD4⁺ Th1 clones specific for *Chlamydia* resulting in protective immunity in nude mice while CD4⁺ Th2 clones did not induce protection [112], [113]. The adoptive transfer of *Chlamydia* specific CD8⁺ T cells also conferred protection against *Chlamydia* challenge, although it was not as effective as CD4⁺ T cells, indicating a stronger role for CD4⁺ T cells. Th1 cytokines, specifically IFN- γ and IL-12 are essential to induce a protective response. IFN- γ knockout mice exhibit greater dissemination of infection and for longer periods, with a proportion failing to resolve their genital tract disease [114], while IL-12 depleted mice display minimal bacterial clearance for a month post-infection [115].

The role of humoral immunity in *C. trachomatis* protection is less clear. Infected B cell deficient mice develop immunity to *Chlamydia* genital infection normally, indicating B cells are not necessary for the generation of protective immunity [116]. However *C. muridarum* pre-infected mice depleted of CD4⁺ and CD8⁺ T cells are resistant to secondary *Chlamydia* genital infection, suggesting that optimally matured B cells and antibodies can be protective against a secondary infection [117]. In addition, passive immunisation of *Chlamydia* infected mouse serum to B cell deficient mice depleted of CD4⁺ T cells yielded protection to *C. muridarum* genital infection [118]. Taking these data into account, it is currently believed that humoral immunity can provide protection against *Chlamydia* reinfection, but this effect is often masked by the superior protection afforded by cell-mediated immunity.

1.4.2 Animal models for Chlamydial disease

Mice are the most commonly used animals in genital chlamydia infection studies. They are commonly adopted due to their ease of handling, low cost, and the availability of genetic knockout strains [119]. With the female mouse genital tract being susceptible to infection with both *C. trachomatis* and *C. muridarum*, there are two established mouse models for genital Chlamydial infection [120] [121].

Intravaginal inoculation with *C. muridarum* in mice results in a genital tract infection closely resembling an acute genital *C. trachomatis* infection in women [120]. The infection ascends to the uterine horns and oviducts resulting in hydrosalpinx and infertility, which are also usual post-infection pathologies in women [122] [123]. In contrast, intravaginal inoculation with *C. trachomatis* in mice results in a mild genital tract infection that resolves quickly and is unable to ascend the genital tract. Hydrosalpinx or infertility is only induced when high *C. trachomatis* doses are administered directly into the uterus or uterine horns [124].

These pathological differences between the *C. muridarum* and *C. trachomatis* mouse models make it difficult to define which model best represents chlamydial infection and pathogenesis in women [125]. The similarity in pathogenesis following *C. muridarum* infection and women with post-chlamydial sequelae makes the *C. muridarum* model useful for the study of Chlamydia pathogenesis, but as it doesn't mimic the chronic stages which are responsible for disease in humans it's appropriateness for the study of *C. trachomatis* infections in women is debated [126]

[127]. Murine *C. trachomatis* genital infection mimics in many ways both the course and the outcome of infection in the majority of women – the asymptomatic, self-limiting infection only rarely resulting in severe sequelae [128]. The choice of infectious agent therefore strongly depends on which aspect of Chlamydia infection one wishes to investigate or mitigate against.

Guinea pig inclusion conjunctivitis (GPIC) infection of the guinea pig genital tract closely resembles a genital *C. trachomatis* infection [129], infecting epithelial cells of the cervix yet frequently ascending and resulting in endometritis or salpingitis [130]. This genital infection can also be transmitted sexually and perinatally, like in human *C. trachomatis* infections [131] [129], and the immunity to reinfection is only short-lived; again analogous to humans [132]. However, the lack of Guinea pig specific reagents makes studying Guinea pig immunology difficult and restrictive in comparison to the murine models.

The pig has been investigated as a large animal model for the studying of *C. trachomatis* genital infections. The majority of genes expressed in porcine female reproductive tissues have been found to be expressed in human genital tissues [133], with pigs furthermore naturally susceptible to four Chlamydial species (though not *C. trachomatis*). *Chlamydia suis* is used as an alternative to *C. trachomatis* in the pig model, yet *C. suis* causes only vulval discharge as opposed to PID or tubal infertility pathologies and thus isn't the best model for human *C. trachomatis* infection [134].

Pig-tailed macaques are prime models for genital chlamydial infections, with similar genital reproductive tract anatomies and physiologies to human women, including the vaginal microflora and their 28-30 day menstrual cycle [135]. Furthermore, pig-tailed macaques are naturally susceptible to genital tract infections with human strains of *C. trachomatis*, not requiring any hormonal pretreatment [136]. Repeated *C. trachomatis* infections can cause tubal scarring and tubal obstruction in pig-tailed macaques, similar to pelvic inflammatory disease in women, and thus are an appealing animal model for the treatment of *C. trachomatis*-induced PID [137] [138]. The practical disadvantages including the high costs, the required expertise, and the required housing facilities for primates have limited the use of pig-tailed macaques as an animal model for chlamydial diseases.

1.5 Chlamydia vaccines

Chlamydia vaccine development has been on going for more than 50 years. The first attempts at human and animal *Chlamydia* vaccines began with the use of inactivated or live, attenuated whole organism preparations. Though offering a degree of protection, their high cost and complexity of production along with the presence of immunopathological antigens have hampered their clinical use. These first generation *C. trachomatis* vaccines were subsequently superseded by second-generation, subunit-based vaccines against *C. trachomatis*.

1.5.1 Live attenuated vaccines

The first vaccines used against *Chlamydiaceae* were live attenuated. Strains were attenuated by the passaging of a wild-type strain in different cell types or by chemical mutagenesis, inducing mutations resulting in avirulent Chlamydia. The live attenuated vaccines could elicit both humoral and cellular immunity as they were replication competent, thus processing and presenting antigens in a very similar way to the natural infection [139]. However, the potential reversion to the virulent wild-type strain resulting in disease or persistent infection, as well as the possible spread of live Chlamydia in the environment makes live attenuated Chlamydia vaccines unlikely to be allowed for use in humans.

Though unsuccessful in trials in the 1960s, there has been a recent re-exploration in the use of live attenuated chlamydia against genital *C. trachomatis* infection. Mice intranasally immunised with viable *C. trachomatis* serovar E exhibited significant, but not complete protection upon a vaginal challenge [106]. In addition, mice vaccinated intranasally with live *C. muridarum* elicited widely disparate levels of protective immunity to genital tract challenge [140].

1.5.2 Inactivated/Killed vaccines

Following live vaccine attempts, research switched towards the use of inactivated or

killed Chlamydia, with inactivation by heat or chemical (formalin) treatment. Their major disadvantage is their inability to replicate, thus requiring revaccination and the use of adjuvants. Their replication deficiency also makes them poor inducers of cell-mediated immunity though capable of inducing humoral immunity [141]. In a rare study on inactivated/killed chlamydia vaccines against genital *C. trachomatis* infection, neither intranasal nor intraperitoneal immunisation with 1×10^6 IFU of UV inactivated *C. trachomatis* serovar E elicited protection against genital infection [106].

The 1960s saw unsuccessful attempts to develop inactivated and live vaccines against trachoma in both human and nonhuman primates models [142] [143] [144]. Although these vaccines were capable of reducing disease in some individuals, they did enhance disease in others resulting from an enhanced delayed-type hypersensitivity (DTH) response. Following these studies, the use of whole chlamydial organisms for developing human vaccines was essentially abandoned [145].

1.5.3 Subunit vaccines

Subunit vaccines are safer, with the inability to revert to a virulent form, and can avoid the presence of undesirable, immunopathological antigens. However, like inactivated vaccines, they are poor inducers of cell-mediated immunity, and the use of adjuvants is required.

Subunit vaccine research has mainly focused on the major outer membrane protein (MOMP) following its identification as a structurally and immunologically dominant protein accounting for 60% of the chlamydial outer membrane [146]. However its efficacy as a vaccine candidate is chequered throughout the literature. Chlamydial outer membrane complex (COMC) preparations of *C. muridarum* induced significantly protective immunity in mice against a genital challenge, while purified MOMP alone preparations did not [147]. However, the same research group subsequently immunised with a purified and refolded preparation of MOMP adjuvanted with Freund's that did provide a significant level of protection in mice against genital challenge [148]. Studies have revealed that some preparations can induce more protection than others, probably due to the differences in extraction methods, which may influence conformationally important epitopes of MOMP. Due to their high expense and the difficulty in growing chlamydia in bulk, purified chlamydial proteins are not commercially viable vaccine candidates. This has lead to interest in recombinant MOMP (rMOMP) preparations made in prokaryotic expression systems. Transcutaneous immunisation with rMOMP adjuvanted with cholera toxin and CpG resulted in enhanced clearance of *C. muridarum* in mice following genital challenge [149]. In addition *C. muridarum* rMOMP adjuvanted with *Borrelia burgdorferi* Outer surface protein (Osp) A induced significant protection in mice against *C. muridarum* genital infection [150].

Though most recombinant vaccines have been based on MOMP, other novel vaccination strategies have used the secreted protein, chlamydial protease-like activity factor (CPAF). rCPAF and IL-12 intranasally administered to mice resulted in

significantly reduced chlamydial shedding following genital *C. muridarum* challenge as well as protection against hydrosalpinx (distally blocked fallopian tube filled with serous fluid) [151]. In 2011, Finco *et al.* (Novartis Vaccines) screened 120 *C. trachomatis* proteins for their immunogenicity to induce both CD4+/IFN- γ + and antibodies [152]. Using protein arrays screened with sera from *C. trachomatis*-infected patients to identify antibody-inducing antigens, and stimulating splenocytes from *C. trachomatis*-infected mice to identify CD4+/IFN- γ + inducing antigens, they revealed five antigens eliciting both types of responses. These antigens were MOMP, IncA, OmcB, HtrA and a hypothetical protein (CT372).

1.5.4 Third Generation Chlamydia vaccines

Third generation Chlamydia vaccines can include the use of DNA, Adenovirus and MVA based vaccines. The MVA vector has not been used as vaccine platforms for genital *C. trachomatis* infections to date, and neither has DNA vaccination adjuvanted with electroporation. Only one Adenovirus vectored vaccine against *C. muridarum* has been published to date, and conferred significant protection as a prime to a CPAF protein boost [153], but has yet to be used for *C. trachomatis*. A MOMP-based DNA vaccine encoding the MOMP gene of *C. muridarum* elicited only modest immune responses, and no protection was established against infection or disease in the mouse model [154]. Recently a DNA vaccine based on *C. trachomatis* MOMP administered to the vaginal mucosa of pigs induced cellular immune responses, and resulted in significant protection although infection was not

completely cleared [155]. As previously discussed, DNA, Adenovirus and MVA based vaccines are currently being utilised against malaria [90] [91], tuberculosis [76] [77] and HIV-1 [156] [157] and are some of the most promising recently developed vaccine platforms.

1.6 Thesis aims and outlines

An efficacious vaccine for *C. trachomatis* is still lacking. However, infection-induced immunity in both animal models and humans indicates a strong role for CD4+ Th1-biased immune responses. It is of timely interest to explore whether the use of third generation vaccines, which include DNA, Adenovirus and MVA based vaccines, used in heterologous prime-boost regimens can induce such CD4+ Th1 biased immune responses against Chlamydia.

Hypothesis: Heterologous prime-boost regimens utilising MOMP-based vaccines will induce a protective response against Chlamydia trachomatis infection.

1.6.1 Aims

- To computationally encompass the amino acid variability within the Chlamydial antigen MOMP by a consensus and a mosaic based vaccine approach.
- To induce humoral and cellular immunity against the MOMP antigen following heterologous prime-boost vaccinations with DNA, Adenovirus, MVA and recombinant protein based vaccines.
- To elucidate MOMP-based heterologous prime-boost vaccine regimens that protect against vaginal *C. trachomatis* infection in two alternate mouse models.
- To reveal the mechanism by which the protective vaccine regimen(s) induce the *C. trachomatis* infection protection.
- To assess whether the heterologous prime-boost vaccine regimens utilising these third generation vaccines can induce Chlamydial specific antibody responses on the murine ocular mucosa.

1.6.2 Outline

Chapter 2 describes the materials and methods used throughout this study.

Chapter 3 describes and assesses the consensus approach versus the mosaic approach to the MOMP antigen design, and details vaccine construction and expression alongside the *in vivo* titration of the DNA, Adenovirus and MVA vaccines.

Chapter 4 investigates the humoral and cellular immunogenicity of the four vaccines in heterologous prime-boost vaccine regimens, as well as the immunogenicity following mucosal immunisation with recombinant protein, Adenovirus and MVA.

Chapter 5 investigates the protective ability of the vaccines in heterologous prime-boost regimens against genital *Chlamydia trachomatis* infection in two mouse strains. The mechanism of protection is investigated.

Chapter 6 investigates the optimal vaccine regimen for the induction of chlamydial specific antibodies on the murine eye. The induction of potentially trachoma relevant antibodies on the murine eye is investigated.

Chapter 7 summarises the results of the thesis and discusses future directions

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Table 2.1 details all commercially available reagents used in this study

Material	Supplier	Catalogue No.
0.45 µm syringe filter	Sartorius Stedim	16555
100 µm cell strainers	Becton Dickinson Ltd	340638
15 ml centrifuge tubes	Corning	734-0452
3 mm glass Beads	Merck	1.04015.0500
50 ml centrifuge tubes	Corning	734-0453
60 mm petri dishes	Jencons	391-1917
70 µm cell strainer	Becton Dickinson Ltd	352350
ACK lysing buffer	Lonza	10-548E
Agarose	Sigma Aldrich	A9539
Amersham ECL Western Blotting System	GE Healthcare	RPN2108
Ampicillin	Sigma Aldrich	A0166
Anti-adenovirus polyclonal antibody	Millipore	AB1056
Anti-Chlamydia trachomatis MOMP-HRP	ABcam	Ab68509
Anti-mouse CD3e PE	eBioscience	12-0031-85
Anti-mouse CD4 APC	eBioscience	17-0041-83
Anti-mouse CD8a eFluor 605NC	eBioscience	93-0081

Anti-mouse IFN- γ PeCy7	eBioscience	25-7311-82
Anti-mouse IgA-HRP	Southern Biotech	1165-05
Anti-mouse IgG-HRP	Southern Biotech	1030-05
Anti-mouse IgG1-HRP	Southern Biotech	1144-05
Anti-mouse IgG2a-HRP	Southern Biotech	1155-05
Anti-mouse IL-2 perCP Cy5.5	eBioscience	45-7021-82
Anti-mouse Kappa light chain	AbD serotec	105001
Anti-mouse Lambda light chain	AbD serotec	106001
Anti-mouse TNF- α FITC	eBioscience	11-7321-82
Anti-rabbit IgG (H+L) Alexa Fluor 594	Invitrogen	A11012
BioMix Red	Bioline	BIO-25006
Bovin Serum Albumin (BSA)	Sigma Aldrich	A7906
Caesium Chloride	Melford Laboratories Ltd	MB1006
CHO cells	ATCC	CCL-61
Coomassie brilliant blue R-250 solution	Bio-Rad	161-0436
Cytofix/Cytoperm fixation/permeabilisation kit	Becton Dickinson Ltd	555028
DAB Peroxidase (HRP) substrate kit	Vector Laboratories	SK-4100
Depo Provera	Pfizer	NHS Pharmacy
Dibasic sodium phosphate	Sigma Aldrich	S3264
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D8418
Dried milk powder	AppliChem, Inc.	A0830
Dulbecco's Modified Eagles Medium (DMEM)	Sigma Aldrich	D6546
Endofree Plasmid Giga-Kit	QIAGEN	12391
Endotoxin free water	QIAGEN	1039498

Ethanol	Sigma Aldrich	E7023
Expand High Fidelity PCR System	Roche	11-732-641-001
Fine tip rayon Dryswab	MWE Co. Ltd.	MW113
Flat-bottom tissue culture plates, 6-well	Becton Dickinson Ltd	35-3046
Flat-bottom tissue culture plates, 96-well	Corning	353072
GelPilot DNA loading dye, 5x	QIAGEN	239901
Gentamicin solution [10 mg/ml]	Gibco	15710-049
GSTrap 4B	GE Healthcare	28-4017-45
Heat Inactivated Foetal Bovine Serum (FBS)	Gibco	10500
IPTG	Melford Laboratories Ltd	MB1008
Isoflurane-Vet	Merial	Imperial CBS
Kanamycin monosulphate	Melford Laboratories Ltd	K0126
L-glutamic acid	Duchefa Biochemie	G0707
L-glutamine (200 mM)	Gibco	25030-024
Lipofectamine	Invitrogen	11668-019
Luria Bertani broth powder	Sigma Aldrich	L3022
McCoy cells	ATCC	CRL-1696
Methanol	Sigma Aldrich	42105
MinElute gel extraction kit	QIAGEN	28606
Monobasic sodium phosphate	Sigma Aldrich	S3139
Mouse IFN- γ ELISpot plus Kit (ALP)	Mabtech	3321-4APT-4
Mouse IgA kappa	Sigma Aldrich	M1421
Mouse IgG	Southern Biotech	0107-01
Mouse IgG1 kappa	Sigma Aldrich	M9269
Mouse IgG2a kappa	Sigma Aldrich	M9144

Myjector U-100 Insulin needles, 0.3 ml	Terumo	BS30M2913
Novex 4-12% Tris-Glycine Protein Gels	Invitrogen	WT4121
Novex Sharp PreStained Protein standard	Invitrogen	LC5800
Novex Tris-Glycine SDS Running buffer, 10x	Invitrogen	LC2675
NUNC Maxisorp 96-well plates	Thermo Scientific	439454
One Shot TOP10 chemically competent <i>E.coli</i>	Invitrogen	C4040
Pen/strep [100U penicillin 100 µg strep]	Gibco	15140-122
Phosphate Buffered Saline (PBS)	Gibco	14190-094
Plasmid Midi kit	QIAGEN	12145
PreScission Protease	GE Healthcare	270843
Protease Inhibitor Cocktail Set	Calbiochem	539131
Proteinase K	QIAGEN	1017738
PVA foam (2 mm x 20 mm)	HUNT	HMD536
QIAprep Spin Miniprep kit	QIAGEN	27104
Restriction enzymes	New England Biolabs	Various
RPMI-1640 medium	Sigma Aldrich	R0883
S.O.C. medium	Invitrogen	15544-034
Sucrose	Sigma Aldrich	S-0389
SureBlue TMB peroxidase substrate	KPL	52-00-00
SYBR Safe	Invitrogen	S33102
T-Rex-293 cells	Invitrogen	R710-07
T4 DNA Ligase	New England Biolabs	M02025
TMB Stop solution	KPL	50-85-06
TrackIt DNA ladder	Invitrogen	10488-058

Tris-acetate-EDTA, 10x	Fisher Scientific	BP1335-1
Tris-Glycine SDS protein loading buffer	Invitrogen	LC2676
Tween-20	Fisher Scientific	337-500
U bottom plates, 96 well	Jencons	734-0027
Urea	Sigma Aldrich	U4883

Table 2.1. Reagents used throughout the study

2.1.2 Solutions

2x Adenovirus storage Buffer: 10 mM Tris, pH8.0, 100 mM NaCl, 0.1% BSA and 50% glycerol.

Complete Medium: 500 ml Dulbecco's Minimal Essential Media (DMEM) was supplemented with 5 ml pen/strep (100 U penicillin, 100 µg streptomycin), 5 ml L-glutamine (200 mM) and 50ml of heat inactivated foetal calf serum (FCS) (10%).

Complete Chlamydia Medium: 500 ml Dulbecco's Minimal Essential Media (DMEM) was supplemented with 5 ml gentamycin (10 mg/ml), 5 ml L-glutamine (200 mM) and 50 ml of heat inactivated foetal calf serum (FCS) (10%).

FACS buffer: 2.5 g (0.5%) Bovine Serum Albumin (BSA) added to 500 ml PBS.

PBS/Tween (PBST): 250 µL (0.05%) Tween-20 added to 500 ml PBS.

SPG buffer: 75 g sucrose, 87 ml 0.2 M dibasic sodium phosphate, 13 ml 0.2 M monobasic sodium phosphate, 0.72 g L-glutamic acid, adjusted to 1 L with dH₂O and pH adjusted to 7.4.

Tris-acetate-EDTA (TAE) buffer: Made up from 50x concentrate with dH₂O

Vaginal/Ocular Extraction buffer: 1.5 g sodium chloride, 20 µL of 10% sodium azide solution in 100 ml Dulbecco's Phosphate Buffered Saline (DPBS).

2.2 Methods – Molecular Biology

2.2.1 Agarose gels

DNA products were separated by 1.5% agarose gel electrophoresis (100 V, 45 min) in 1x Tris-acetate-EDTA (TAE) buffer. The agarose gel was stained with SYBR Safe, and a DNA ladder was loaded and 5 µL of 6x loading dye was added to each sample before loading on the gel. DNA was visualised on a transilluminator and the band size compared against the DNA ladder.

2.2.2 Restriction Digests

DNA restriction digests were carried out as per restriction enzyme manufacturers instructions, with incubations of 1 hr at 37°C. 20 µL reactions were performed as below, yet double digests were performed with 2 µL of each enzyme:

2 µL	Total NEB Restriction enzyme (10 000 U/ml)
2 µL	NEB restriction buffer (10x)
X µL	Plasmid DNA (~1 µg)
upto 20 µL	dH ₂ O

2.2.3 Ligation Reactions

DNA fragments were excised from agarose gels and purified using Qiagen MinElute gel extraction kits. Ligations were carried out into TOPO vectors, at r.t. for 20 min.

Ligation reactions were performed as:

1 μ L	Plasmid DNA (TOPO vector)
X μ L	Insert DNA (5 ng)
1 μ L	Salt solution
3 μ L	dH ₂ O

2.2.4 Cloning into DNA vaccine plasmid pcDNA3.1

Antigen sequence was amplified directly from the GeneArt plasmid by PCR. The PCR program was 98°C for 30 s, with 35 cycles of (98°C for 10 s, 54°C for 30 s, and 72°C for 45 s), followed by 72°C for 10 min. This PCR product was separated on a 1.5% agarose gel and the DNA extracted and purified from the gel by a QIAGEN MinElute gel extraction kit. The purified PCR product was topoisomerised ligated into the TOPO pcDNA3.1 plasmid (Invitrogen) according to manufacturers instructions prior to transformation into TOP10 competent cells (Invitrogen). The sequence insertion was initially checked by restriction digest and then confirmed by sequencing (MWG Eurofins).

2.2.5 Homologous recombination into HuAd5 (pAL1112)

The pAL1112 plasmid is the human Ad5 (HuAd5) genome deleted for E1 (461-3519 bp) and E3 (28131 – 30800 bp) regions in a single copy vector. The CMV promoter in this plasmid is repressible by tet repressors, enabling the vector to be grown in 293TREx cells without expression from the CMV promoter. This allows the cloning of toxic genes or genes which may otherwise interfere with the Ad replication as they are not constitutively being expressed.

The antigen sequence was amplified from the GeneArt construct by PCR using primers with 80 bp overhangs to enable regions of homology to the CMV promoter and polyA (Table 2.2). The PCR used Roche expand HiFi including buffer 2 (containing Mg), and the entire reaction was run on a 1.5% agarose gel. Correctly amplified PCR products were gel purified using GFX columns (GE Healthcare) and eluted into 30 µL of elution buffer.

SW102 *E.coli* were grown at 32°C before inducing the lambda red proteins by incubating at 42°C for 15 min. SW102 bacteria contain a defective phage expressing lambda red genes which mediate homologous recombination between DNA stretches as short as 50 bp. These lambda red genes are under temperature sensitive control, remaining turned off at 32°C but induced at 42°C.

10 ng of the purified antigen sequence PCR product with the nucleotide overhangs was added to a 25 µL aliquot of the freshly induced competent SW102s and transferred to pre-cooled 0.2 cm cuvettes. The DNA and cells were electroporated at 2.50 kV and the bacteria recovered in 5 ml LB for 4 hr at 32°C at 200 rpm. This

extended recovery allowed the loss of *sacB* DNA and proteins from the cells within which the recombination occurred. 50 μ L of the transformed bacteria was plated onto LB with 5% sucrose and chloramphenicol and grown at 30-48 hr at 32°C. Due to the *amp/sacB/lacZ* cassette, the bacteria transformed with successfully recombined genome and antigen insert appeared white amongst negative blue colonies.

Individuals white colonies were picked into 5ml LB plus chloramphenicol and grown overnight at 32°C. DNA was extracted using the QIAgen spin miniprep kit before diagnostic restriction digest. 8 μ L of purified DNA with 1 μ L NEB restriction buffer (10x) and 1 μ L BamH1 reaction was incubated at 37°C for 1 hr before loading the entire 10 μ L reaction onto a 1.5% agarose gel. Following this diagnostic restriction digest, non-recombined HuAd5 reveals bands of 18, 11, 7.7, 2.5, 1.7, 0.8 and 0.6 Kbp while successfully recombined vectors lose the bands at 2.5 and 1.7 Kbp. Successful homologous recombination of the antigen sequence into the HuAd5 vector was then confirmed by sequencing from the CMV promoter and the CMV polyA (Table 2.3) (MWG Eurofins).

Primer	Sequence
CMV Promoter (For)	5'-AACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAG AAGACACCGGGACCGATCCAGCCTGGATCCATGAAGAAGCTGCTGAAGAG-3'
CMV PolyA (Rev)	5'-GGCGTGACACGTTTATTGAGTAGGATTACAGAGTATAACATAGAGTATA ATATAGAGTATACAATAGTGACGTGGGATCCTTATTAGAAGCGGAACTGGG-3'

Table 2.2 Primers used for the addition of homologous ends

Primer	Sequence
CMV Promoter (For)	5'- AATGTCGTAACAACTCCG -3'
CMV PolyA (Rev)	5'-ACCTGATGGTGATAAGAAG -3'

Table 2.3 Primers used for the sequencing of the recombined HuAd5 plasmid

2.2.6 Cloning into MVA shuttle vector

To generate an MVA expression MOMP, the gene was cloned into the MVA-GFP-TD shuttle plasmid kindly provided by The University of Oxford Viral Vector Core Facility. Gene sequences were extracted from pcDNA3.1 by Kpn1-HF and Not1-HF by double digest. The shuttle vector was also digested with Kpn1-HF and Not1-HF. DNA fragments were separated on a 1.5% agarose gel and gel extracted using a QIAgen MinElute gel extraction kit. DNA from the MVA-GFP-TD backbone was ligated to the MOMP insert DNA overnight at 15°C and successful ligation was confirmed by an analytical restriction digest. This was further confirmed by sequencing (MWG Eurofins). MVA shuttle vectors were sent to The University of Oxford Viral Vector Core Facility where they were transfected and the virus propagated and purified for our use.

2.2.7 Bacterial transformation

A vial (50 µL) of either chemically competent TOP10 *Escherichia coli* (*E. coli*) (Invitrogen) for sub-cloning or BL21 *E. coli* (Invitrogen) for protein expression were incubated on ice for 30 min with 2 µL of DNA. Cells were then heat shocked at 42°C for 45 s and incubated on ice again for 2 min. 300 µL S.O.C. media added to the vial and incubated at 37°C for 1 hr at 200 rpm prior to plating. Bacteria were plated at either 200 µL or 20 µL volumes onto LB agar containing the relevant selection antibiotic. Plates were incubated at 37°C overnight before picking colonies.

2.2.8 Colony PCR

Individual colonies were picked into 200 μ L LB medium. An insert specific PCR is carried out with the reaction conditions:

13 μ L	BioMix Red
1 μ L	Forward Primer (5 μ M)
1 μ L	Reverse primer (5 μ M)
1 μ L	Colony in LB media (DNA)
9 μ L	dH2O

2.2.9 Plasmid DNA preparation

Bacteria containing the correct construct were amplified by overnight incubation at 37°C at 250 rpm in 150 ml LB medium plus selection antibiotic. 1 ml of the overnight was removed and mixed with 300 μ L glycerol to form a bacterial stock before long time storage at -80°C. The remaining bacterial overnight was purified by endofree Qiagen plasmid maxiprep kits and the DNA concentration measured by Nanodrop (Thermo Scientific). DNA was stored at 4°C for short-term use, or -20°C for longer periods.

2.2.10 GST-tagged protein purification

The *C. trachomatis* serovar D plasmid encoded protein 3 (Pgp3) sequence cloned

into a glutathione S-transferase (GST)-fusion protein expression vector was kindly provided by Dr. Guangming Zhong (University of Texas Health Science Center at San Antonio). To initiate Pgp3 protein production, bacterial stocks were picked into LB broth plus carbenicillin. This was shaken at 37°C at 300 rpm until the OD600 measured 0.8 on Nanodrop before IPTG was added (200 µM) to induce expression. The culture was then reduced to 30°C with 200 rpm shaking for 3 hr. Cells were harvested by centrifugation (6000g, 15 min) and the bacterial pellet resuspended in Tris buffer. The bacteria were lysed by five freeze thaw cycles before centrifugation at 5000g for 30 min at 4°C. The clarified lysate was then loaded onto GST affinity chromatography columns (GSTrap, GE Healthcare) as per manufacturers instructions. Loaded columns were washed with 5 column volume of PBS before the Pgp3 protein was cleaved and eluted from the column with PreScission Protease (GE Healthcare). Purified Pgp3 protein was visualised on Coomassie gels exhibiting the correct size, and was negative for the GST-tag by anti-GST western blots.

2.2.11 SDS PAGE & Western Blotting

An equal volume of Tris-Glycine SDS protein loading buffer (Novex) was added to the sample and boiled at 95°C for 5 min. The sample was then loaded on the gel. Samples were run on 4-12% Tris-Glycine protein gels (Novex) using a Mini-Protein II cell (Bio-Rad) at 120 V constant voltage in 1x Tris-Glycine SDS Running buffer (Novex). Proteins were visualised by Coomassie brilliant blue staining solution (Bio-Rad).

After separation of the proteins on the gel with discontinuous reducing SDS-PAGE,

they were transferred to nitrocellulose membrane by semi-dry electrophoresis. Transfer was achieved by applying a 20 V constant voltage for 20 min. Immunodetection was performed according to [158]. Briefly, after transfer of proteins onto the nitrocellulose membrane, unoccupied protein-binding sites were blocked by incubating the membranes with 5% milk powder in PBST for 2 hr at room temperature with agitation. Membranes were then incubated for 1 hr with agitation at room temperature with the primary antibody diluted in the blocking buffer at a concentration according to the supplier's recommendation. The membranes were washed three times each for 5 min with PBST. Bound antibodies were visualised by Amersham ECL detection (GE Healthcare), or by DAB Peroxidase (HRP) Substrate Kit (Vector Labs).

2.2.12 Genotyping IFN- γ KO mice

To determine the genotype of IFN- γ KO mice (bred in-house), ear punches were collected from IFN- γ KO and WT (C57BL/6) mice. To extract the DNA, 20 μ g of Proteinase K (Qiagen) diluted in digestion buffer (50 mM Tris pH 8.0, 2 mM NaCl, 10 mM EDTA, 1% SDS) was added to each ear punch in a clean Eppendorf and incubated at 55°C for 30 min. 180 μ L of dH₂O was added to each tube and samples heated to 99°C for 5 min to denature the Proteinase K. Samples were then stored at 4°C until use. PCR reactions were carried out using the BioMix Red (Bioline) in a final reaction volume of 25 μ L. The mixtures of PCR reagents were prepared as below.

13 μ L	BioMix Red
1 μ L	Forward primer (5 μ M)
1 μ L	Reverse primer (5 μ M)
3 μ L	Template DNA (4 ng/ μ L)
7 μ L	dH ₂ O

PCR mix was thermal cycled in a Techne TC-412 (Techne) at cycling conditions: 95°C for 30 s, 35 cycles of (95°C for 10 s, 60°C for 30 s, 72°C for 60 s), 72°C for 10 min before held at 4°C indefinitely. Primers used are outlined in Table 2.4.

Primer	Sequence
IFN- γ For	5'- TGCATCTTGGCTTGCAGCTCTTC-3'
IFN- γ Rev	5'- GGGTTGTTGACCTCAAACCTGGCA-3'

Table 2.4 Primers used for genotypic analysis of IFN- γ KO and WT C57BL/6

PCR products were run as outlined in 2.2.1.1. using a 1.5% agarose gel. IFN- γ KO samples were expected to have no visible bands, while wild type have an expected band around 2000 bp. All animals used in experiments were determined to be KOs.

2.2.13 Droplet digital PCR (ddPCR)

The ddPCR primers targeted the *C. trachomatis* gene *omcB* with the colour Hex as well as the *C. trachomatis* cryptic plasmid on FAM. ddPCR reaction mixtures were 20

µL volumes with final concentrations of 1x ddPCR Supermix (Bio-Rad), 0.2 µM each primer and probe and sample DNA of approximately 3 ng. Droplet generation and droplet reading for ddPCR were carried out according to the manufacturer's instructions using Bio-Rad reagents, and under the supervision of Dr. Chrissy H. Roberts, London School of Hygiene and Tropical Medicine, London. Raw ddPCR data were collected on the Bio-Rad QX100 instrument using Quantalife software (Bio-Rad).

2.3 Methods - Molecular Virology

2.3.1 Adenovirus Transfection and Purification

The HuAd5 genome containing the antigen sequence insert construct was confirmed by sequencing (MWG Eurofins). 2×10^6 293TREx cells were seeded into a T25 flask 24 hr prior to transfection. 4 µg of recombinant HuAd5 vector was diluted into 100 µL DMEM without FCS or antibiotics and 40 µL Polyfect was added before incubating at room temperature for 10 min. To this DNA complex, 1 ml complete medium was added and transferred to the confluent 293TREx cells. 293 cells are permissive for the production and amplification of replication-incompetent adenovirus, as the cells provide the E1 and E3 gene products *in trans*. Cells were incubated at 37°C, 5% CO₂ in a humidified incubator. Cells were monitored daily for cytopathic effect (CPE), with fresh media added every 2-3 days. When complete CPE was visible, the cells were harvested by cell scraping, and centrifuged at 500g at 4°C for 10 min.

Adenovirus was released from the cells by four freeze-thaw cycles in a dry ice/methanol bath and centrifuged at 500g at 4°C for 15 min to obtain a cleared viral supernatant. After rounds of large-scale amplification of the adenovirus, the cleared viral supernatants were transferred to a volume of 8 ml with 4.4 g ultrapure CsCl. The CsCl solution was transferred to 12 ml polyallomer tubes suitable for Beckman SW 41 Ti rotor and centrifuged at 176000g for 18-24 hr at 10°C. The purified virus appeared as a narrow opaque white band and the virus fraction was collected by an 18G needle through the side of the tube. The extracted virus was mixed with 2x adenovirus storage buffer and frozen as aliquots at -80°C.

Virus yield was determined by single cell immunostaining. Purified recombinant adenovirus was titered following serial dilution and infection of 293TREx cells. Infected cells were fixed in ice-cold acetone/methanol and incubated at -20°C for 10 min. Primary antibody staining was with goat anti-Adenovirus antibody (Chemicon, Cat No. AB1056) followed by secondary antibody staining with donkey anti-goat hrp (ABcam). For the detection reagent, metal enhanced DAB substrate (Pierce, Cat. No. 34065) was added to the fixed antibody stained cells and incubated for 10 min at room temperature. Adenovirus infected cells showed brown staining and the number of stained cells was counted before calculating plaque-forming units (PFU) per ml.

2.4 Methods - Immunology

2.4.1 Animals

All procedures were carried out in accordance with the terms of the UK Animals (Scientific Procedures) Act Project Licence (PPL 70/7457) and were approved by the Ethical Review Board of Imperial College. All mice were housed in the Central Biomedical Services (CBS) facility at St Mary's Hospital under Specific Pathogen Free (SPF) conditions. Female BALB/c (H-2d) (Harlan, UK) and B6C3F1 (Charles River, Germany) were 6-8 weeks old at commencing of experiments. IFN- γ KO mice (C57BL/6 background) were kindly provided by Dr John Tregoning (Imperial College London).

Mice were anaesthetised prior to required procedures using the inhalation anaesthetic Isoflurane (2-3.5%) carried by oxygen (1-2 L/min).

2.4.2 Route and dose

Intramuscular (i.m.) vaccinations were administered in a volume of 50 μ L into the *musculus tibialis* using 26G needles.

Intravenous (i.v.) injections were administered in a volume of 100 μ L into the lateral tail vein using a 26G needle. To encourage vein dilation, mice were incubated for > 10 min at 38°C in a heatbox prior to injection.

Intraperitoneal (i.p.) injections were administered in a volume of 100 μ L using a 26G needle.

Subcutaneous (s.c.) injections were administered into the scruff of the neck in a volume of 50 μ L using 26G needles.

Intranasal (i.n.) vaccinations were administered under anesthesia in a volume of 20 μ L by Gilson pipette.

Intravaginal (i.vag.) vaccinations were administered under anaesthesia in a volume of 20 μ L by positive-displacement pipette (Gilson).

Electroporation was with 5 mm electrodes at the injection site immediately after immunisation, using an ECM 830 Square Wave Electroporation System (BTX) with pulses of 100 V, positive and negative polarity at a rate of one pulse per second with each pulse lasting 50 ms.

2.4.3 Vaccine preparations

DNA vaccines were prepared in sterile, endotoxin free PBS, and dosed on concentrations determined by Nanodrop at absorbance 260 nm. Viral vectored vaccines were prepared in sterile, endotoxin free PBS. Viral vectors were dosed on PFU (plaque forming units) to ensure equivalent doses between subsequent preparations. Protein vaccines were prepared in sterile, endotoxin free PBS, and mixed 1:1 with the adjuvant MF59 (Novartis). MOMP concentration was determined by NanoOrange (Invitrogen), with Pgp3 and PmpD concentration determined by

Nanodrop at absorbance 280 nm.

2.4.4 Specific IgG, IgA and isotype ELISA

Blood was collected from tail veins into microcentrifuge tubes and allowed to clot for 1 hr at room temperature before centrifugation at 13000 rpm for 10 min. Serum was removed, transferred into clean microcentrifuge tubes and stored at 4°C until use.

To detect antigen-specific IgG responses, 72 wells of the 96 well Nunc-Immuno Maxisorp Plates (Thermo Scientific, UK) were coated with 0.5 µg/ml of protein in PBS, with 24 wells for the standard curve coated with 1:3200 anti-Ig Kappa/Lambda (AbD serotec) overnight at 4°C. Plates were washed four times with PBST and blocked with PBS/BSA for at least 1 hr at 37°C. Plates were washed again as before. Sera were diluted 1:100, 1:1000, 1:10000 in PBS/1% BSA and plated in triplicate. Mouse IgG standard (Southern Biotech) was diluted from 1000 ng/ml at 1:5 dilutions and plated in triplicate onto the anti-Ig Kappa/Lambda coated wells. Plates were incubated for 1 hr at 37°C and washed again as before. Anti-mouse IgG-HRP (Southern Biotech) was diluted 1:4000 in PBS/BSA and added for 1 hr at 37°C. Following a final wash, TMB (KPL) was added as a developing substrate, with the colorimetric reaction stopped after 5 min with TMB Stop solution (KPL). The optical density (OD) was read within 10 min at 450 nm using a KC4 spectrophotometer. Serum antibody concentrations were calculated off the standard curve present on each plate.

This procedure was carried out for antigen-specific IgA responses, except with the

alteration of using a mouse IgA standard (Sigma Aldrich) and detecting with anti-mouse IgA-HRP.

This procedure was carried out for antigen-specific IgG1 and IgG2a responses, except with the alteration of using a respective mouse IgG1 or IgG2a standard (Sigma Aldrich) and detecting with a corresponding anti-mouse IgG1-HRP or anti-mouse IgG2a-HRP.

2.4.5 Total IgG, IgA ELISA

For total IgG and IgA ELISAs, the exact procedure as 2.2.3.4 was carried out, with the exception that all the wells of the 96 well Nunc-Immuno Maxisorp Plates (Thermo Scientific, UK) were coated with 1:3200 anti-Ig Kappa/Lambda (AbD serotec).

2.4.6 Avidity ELISA

The avidity indices of serum samples were determined by their antibody-antigen binding resistance to 8 M urea. Serum samples were pre-diluted to give an OD_{450 nm} readout between 1.0 and 1.5 in an ELISA and were added to MOMP coated plates. Plates were then washed three times with either PBST or 8 M urea in PBST, before incubating with anti-mouse IgG-HRP. Samples were developed with TMB as described above. The avidity index was calculated as the percentage of urea treated OD_{450 nm}/ PBS-T OD_{450 nm}. Antisera with index values exceeding 50% were ascribed as high avidity, 30–50% were ascribed intermediate avidity and <30% were ascribed

low avidity.

2.4.7 Peptides

MOMP derived peptides used in cellular assays were commercially synthesised by JPT Peptide Technologies GmbH. Peptide lengths were 15mers, with 96 peptides covering the whole MOMP sequence. Peptides were dissolved in DMSO at a concentration of 20 mg/ml and stored at -80°C. Pools of peptides were made from these stocks and stored at a final concentration per peptide of 1 mg/ml.

2.4.8 Splenocyte preparation

Mice were sacrificed by cervical dislocation and spleens removed by dissection. Spleens were macerated in complete RPMI medium through a 70 µm cell strainer and centrifuged (500g, 5 min). Supernatants were discarded and pellets resuspended in 4 ml ACK lysis buffer for 5 min before the addition of 5 ml PBS/2.5% FCS. Cells were recentrifuged (500g, 5 min) and resuspended in 5 ml PBS/2.5% FCS.

Splenocytes were counted using a Scepter 2.0 handheld automated cell counter (Millipore) and resuspended to appropriate concentrations in complete RPMI medium for either ELISpot or intracellular cytokine staining.

2.4.9 IFN- γ ELISpots

IFN- γ ELISpots were carried out to quantify the number of IFN- γ secreting splenocytes under antigen specific peptide stimulation. Pre-coated mouse IFN- γ ELISpot plates by MABtech were used as per manufacturers instructions. Briefly, plates were blocked with complete media for at least 30 min at room temperature. Splenocytes were re-suspended at 5×10^6 cells/ml, and 50 μ L of cells plated in duplicate. Peptides were added at a final concentration of 5 μ g/ml, while ConA was added at a final concentration of 5 μ g/ml for the positive control wells, and complete media alone was added to the negative control wells. Plates were incubated at 37°C, 5% CO₂ for 18 hr. Plates were washed five times with PBS. Detection antibody (R4-6A2-biotin) at 1 μ g/ml was added at 100 μ L/well for 2 hr at room temperature. Plates were washed a further five times with PBS. Streptavidin-ALP (1:1000) in PBS was added at 100 μ L/well and incubated for 1 hr at room temperature. Plates were washed a further five times with PBS before the substrate solution BCIP/NBT-plus was added at 100 μ L/well. The plates were developed until distinct spots emerged. ELISpot plates were counted using the AID plate reader and software (Autoimmun Diagnostika, Germany) with counts then visually confirmed.

2.4.10 Intracellular cytokine staining (ICS)

ICS was carried out to analyse the percentage of cytokine producing CD4⁺ and CD8⁺ T lymphocytes from the spleens. Splenocytes were stimulated with peptides at a final concentration of 1 μ L/ml per peptide for 6 hr at 37°C, 5% CO₂ in the presence of

Brefeldin A. Following stimulation, plates were stored at 4°C overnight. Plates were centrifuged to pellet the cells, the supernatant discarded and the cells resuspended in anti-mouse CD16/CD32 (Fc block) for 15 min. Cells were washed in FACS buffer and surfaced stained with anti-CD3e PE, anti-CD4 APC and anti-CD8a eFluor605NC for 30 min. After permeabilisation using Cytfix/Cytoperm cells were washed and stained intracellularly with anti-IFN- γ PeCy7, anti-TNF- α FITC and anti-IL-2 perCP Cy5.5 for 30 min. Samples were washed and resuspended in 1.5% PBS Formalin before being acquired on a BD FORTESSA and analysed using FloJo (TreeStar Inc.). Within the analysis, background responses from unstimulated cells were subtracted from stimulated responses. Following analyses, the polyfunctionality of the CD4+ or CD8+ T cells were represented graphically using the software SPICE (Mario Roederer, National Institute of Health).

2.4.11 CD4+ T cell depletion

mCD4+ T cells were depleted using an anti-mCD4 GK1.5 (Rat IgG2b) mAb purchased from BioXcell (Catalog BE0003-1). Mice were injected i.p. with 500 μ g of mAb diluted in PBS on days -1 and +1 with respect to day of challenge on day 0. The degree of CD4+ T cell depletion was assessed by flow cytometry using anti-CD3e PE, anti-CD4 APC and anti-CD8a eFluor605NC in the PBMCs, splenocytes and vaginas of depleted mice and undepleted control mice on day +2 with respect to day of challenge.

2.4.12 Ocular sampling

Ocular sampling was performed at identical time points as tail bleeds. 10 µL of ocular extraction buffer (Chapter 2.1.2) was pipetted onto each murine eye and subsequently absorbed with 2 mm x 5 mm PVA swabs. The swabs were then incubated with an additional 50 µL of ocular extraction buffer at room temperature before centrifugation at 500 g for 5 min through a 0.45 µm filter. The eluent was harvested and stored at 4°C until analysis.

2.5 Chlamydia

2.5.1 Strains

C. trachomatis E-Bour was supplied by the American Tissue Culture C (ATCC). *C. trachomatis* D/UW3-cx was kindly provided by Dr. R. Carabeo, Imperial College London. Both *C. trachomatis* strains were provided with sequence verification of *ompA* and were stored as Elementary Bodies (EBs) by cryopreservation in SPG buffer at -80°C.

2.5.2 Maintenance of Chlamydia

The frozen stocks of Chlamydia EBs were immediately transferred to 37°C and gently

agitated until thawed. The EBs were diluted to an appropriate concentration in sterile SPG buffer such as there is infection at a multiplicity of infection of 1-10 EBs/host cell. The culture medium was removed from the McCoy cells and an appropriate amount of diluted EBs added to each well or flask. The tissue culture plates were then centrifuged at 900g for 1 hr at room temperature. Complete Chlamydia media was then added to the infected cells and the cells incubated at 37°C, 5% CO₂ for 24 hr. The infection efficiency was checked by examining inclusion formation by phase-contrast microscopy.

2.5.3 Purification of EBs

The culture medium from *C. trachomatis*-infected McCoy monolayer cells was decanted and replaced with 5 ml SPG. The infected cells were dislodged from the flask with disposable sterile cell scrapers and transferred to chilled screw-cap microcentrifuge tubes containing three 3 mm glass beads. The cell mixture was then vortexed for 1 min to allow the beads to tear open the infected cells. The cell suspension was centrifuged at 100g for 5 min at 0°C, and the supernatant carefully aspirated and kept. The supernatant was subsequently analysed by dynamic light scattering (DLS) on a Malvern Zetasizer to confirm the purity of the EB solution (Appendix Figure 9.1).

2.5.4 Titering Infectious forming units by Indirect Immunofluorescence

McCoy's were seeded in 96 well tissue culture plates with complete medium and incubated for 24 hr at 37°C, 5% CO₂ until confluent. The EB aliquot to be titered was quickly thawed and kept on ice before the EBs were diluted over ten-fold ranges (10¹ to 10⁻⁶) in SPG buffer to a volume of 350 µL. The culture medium was removed from the monolayers and 100 µL of each dilution added to three wells. The tissue culture plates were then centrifuged at 900g for 1 hr at room temperature. Complete Chlamydia media is then added to the infected cells and the cells incubated at 37°C, 5% CO₂ for 24 hr. After incubation the medium was then gently aspirated and 100 µL of 100% methanol added to each well for 10 min at room temperature. After the methanol-fixation, the cells were washed 3 times with PBS before primary antibody staining with rabbit anti-MOMP serum (1:150 dilution). This primary antibody was incubated at room temperature for 1 hr before washing the cells three times with PBS. Goat anti-Rabbit H+L–Alexa594 was added as the secondary antibody to the cells for 1 hr at room temperature (1:1000 dilution) before another three washes with PBS. Fixed and stained cells were stored in PBS at 4°C until fluorescent microscopy. Using a hand-held counter, all fluorescent inclusions per well were counted, requiring between 50 and 500 inclusions for the optimal dilution. Averaging the total inclusion number over the three replicates and back calculations provided the inclusion forming units (IFU) per ml of the EB aliquot.

2.5.5 Intravaginal *C. trachomatis* challenge

Purified *C. trachomatis* EBs were thawed quickly on ice, dissolved in SPG buffer to a concentration of 4×10^7 IFU/ml, and kept chilled on ice. Seven days prior to intravaginal infection, mice were injected subcutaneously with 2 mg of DMPA (Depo-Provera, Pfizer). For intravaginal infection mice were anaesthetised, and 10 μ L of the EB solution pipetted into the mouse vagina with a positive displacement pipette.

2.5.6 Infectivity Assay

The infectivity assay follows the same procedure of 2.2.4.4 with the alteration in the sample preparation prior to infection. The rayon swabs following murine vaginal sampling were stored in 500 μ L SPG buffer, prior to mechanical disruption of the swab by vortexing glass beads. The SPG buffer was then aliquoted to 120 μ L volumes before storage at -80°C . The individual aliquots were quickly thawed on ice, and treated a neat, 10^{-1} and 10^{-2} dilutions in volumes of 100 μ L SPG buffer before being centrifuged onto McCoy monolayers.

2.5.7 Neutralisation Assay

A neutralisation assay was carried out to assess the ability of antibodies raised following immunisations to inhibit the infection of *C. trachomatis* EBs into cells (neutralise the elementary bodies). Two weeks following final immunisation, the mice were bled by tail snips, and the serum isolated. Each serum sample was tested

at 1:10, 1:20, and 1:40 dilutions, dissolved in SPG buffer. 100 µL of diluted sera was pre-incubated with 100 µL of 10^4 IFU/ml *C. trachomatis* EBs for 30 min at 37°C with gentle rocking. The mixture was then centrifuged onto confluent McCoy cells in 96 well tissue culture plates and titered as in 2.2.4.4. Mean neutralisations were calculated as the average reduction in inclusion forming units for each regimen sample (N=8) compared to the mean number of inclusions from the naïve serum dilutions.

2.6 Bioinformatic analyses

The MOMP consensus antigen was created using Consensus Maker v2.0.1 (<http://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html>). The 'Advanced' tool was used to make a consensus of blocks of sequences in an alignment using the following parameters. The minimum number of sequences for consensus was set to 3, with no consensus of consensus. The unanimous value was set to 1.00, with a majority value of 0.50, using the most common character at each alignment point. Nucleotides were set to AGCTU-, and the character set for the making of the consensus included ARNDCEQGHILKMFSTYWV- amino acids. The majority value was incrementally increased to find the most stringent consensus.

The MOMP mosaic antigen was created using Mosaic Vaccine Designer (<http://www.hiv.lanl.gov/content/sequence/MOSAIC/makeVaccine.html>). The test

sequences from which the mosaic was designed from were pasted into the Input box. The cocktail size was set to 1, with epitope length of 9 (to allow the software to cover Class I and II), with the rare threshold value of 1. Default 'Run settings' included a Max Runtime of 5 hr, a population size of 200, with 10 cycles, stall factor of 10, internal crossover probability of 0.5 and iteration and random seed values of 0.

The proportion of all epitope-length peptides from the test sequences matched by the consensus or the mosaic antigen was calculated with the Epitope Coverage Assessment Tool EPICOVER (<http://www.hiv.lanl.gov/content/sequence/MOSAIC/epicover.html>). The settings included a nominal epitope length of 9 (to allow the software to cover Class I and II), with a maximum amino acid mismatch of 2 in order to score, and a minimum of 3 occurrences of a potential epitope in the test set to consider for coverage. Precision was set to 4 decimal places, and the subsets were defined on the first character in the sequence names (the serovar designation).

A guide tree of the *ompA* serovar E variants and the amino acid properties were analysed within the Align section of UniProt (<http://www.uniprot.org/>). Following the alignment of the *ompA* serovar E variant sequences in FASTA format, a guide tree is constructed via a UPGMA cluster analysis of the pairwise alignments (CLUSTAL) [159]. The alignment also allows the visualisation of specific amino acid properties via a menu, annotating regions of the protein with similar properties.

2.7 Statistics

All statistical analyses were carried out using Prism 6.0 (Graphpad, USA). Normality of the data distribution was assessed using the Kolmogorov Smirnov normality test. For non-parametric data the Kruskal-Wallis test with Dunn's multiple comparison post-test was used to compare more than two groups, or the two-tailed Mann-Whitney test to compare two groups. For parametric data, a one-way ANOVA was used for multiple comparisons, with Bonferroni's multiple comparison post-test for comparison of specific groups. $P \leq 0.05$ was considered significant (* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ and **** $p \leq 0.0001$). The log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test were used to compare shedding (survival) curves between two groups. Correlations were tested using the Pearson product-moment correlation test.

Chapter 3

***Chlamydia trachomatis* Major Outer Membrane Protein**

(MOMP) antigen design

3 *Chlamydia trachomatis* Major Outer Membrane Protein (MOMP) antigen design

3.1 Introduction

Chlamydia vaccine research has mainly focused on the Major Outer Membrane Protein (MOMP) as the primary vaccine antigen and some results have been encouraging while others rather disappointing. In 1997, Pal *et al.* found that a chlamydial outer membrane complex (COMC) preparation of *C. muridarum* could significantly protect mice against a genital challenge [147] and then some years later the same team demonstrated that a purified and refolded preparation of the *C. muridarum* MOMP conferred a significant level of protection against a genital challenge [148]. Furthermore, the immunisation of mice with purified *C. muridarum* MOMP could induce neutralising antibodies [160]. Igietseme *et al.* prepared a MOMP-ISCOM vaccine with MOMP extracted from *C. trachomatis* serovar D, and immunised mice cleared a vaginal infection with one week [161]. These studies show that some MOMP preparations can induce more protection than others. This is most likely due to the difference in extraction methods, which can influence the conformation and/or presentation of epitopes that may be necessary for protection. The immunogenic refolded, purified MOMP preparations however are very expensive to make and there are major issues in growing chlamydia in bulk,

rendering these types of vaccines commercially unviable [145].

It is now possible to produce high yields of bacterial proteins by recombinant DNA technology, which costs less and is technically more efficient, and MOMP is now generally made by this recombinant technology. However, the expression of full-length rMOMP in *E. coli* is often toxic and it is difficult to produce rMOMP with conformationally relevant epitopes [162]. rMOMP has however been used in an attempt to elicit *C. trachomatis* protection. The transcutaneous immunisation with rMOMP adjuvanted with cholera toxin and CpG resulted in the enhanced clearance of *C. muridarum* following intravaginal challenge in mice [149]. Furthermore in a comparison between *C. muridarum* recombinant MOMP and native MOMP, the rMOMP did elicit a protective immune response in mice against an intranasal challenge, however the degree of protection was not as robust as immunisation by nMOMP [163].

More recently, Novartis vaccines carried out a large proteomic screen of over 120 *C. trachomatis* proteins for their ability to induce both CD4⁺/IFN- γ ⁺ T cell responses and antigen-specific antibodies [152]. Using protein arrays screened with sera from *C. trachomatis*-infected patients to identify antibody-inducing antigens, and by stimulating splenocytes from *C. trachomatis*-infected mice to identify CD4⁺/IFN- γ ⁺ inducing antigens, they discovered only five antigens eliciting both types of responses. MOMP was one of these five antigens.

As a collection, all these studies strengthen the argument to use MOMP as the antigen throughout our vaccine studies. Moreover, as some of these vaccine

platforms have yet to be used in chlamydia vaccine research, let alone in heterologous prime-boost regimens, it is appropriate to use an established chlamydial antigen in order to be able to compare against previous research.

MOMP, encoded by the *ompA* gene, is one of the predominant proteins at the surface of both EB and RB forms, making up 60% of total outer membrane protein [146]. MOMP has a molecular mass of ~40 kDa, and has been published as having functions as a structural protein [164], a general porin [165] [166] [167], and a chlamydial cytoadhesin [168] [169]. The most accepted function of MOMP is that of a porin, serving as a general diffusion porin being strongly size-selective but not ion-selective [170]. Despite years of research, the real structure of MOMP is still unknown. Using secondary structure prediction methods, variable domains (VDs) were located on the outer loops and exposed on the surface, consistent with immunogenicity and infectivity data (Figure 3.1) [171] [172].

The breakthrough in cloning and sequencing of the *ompA* gene of *C. trachomatis* revealed extensive DNA sequence variation and correlated with observed molecular weight variation [173] [174] [175]. In this respect *C. trachomatis* was different to *C. pneumoniae* where there is comparatively less variability in MOMP [176]. The variation of *C. trachomatis* MOMP is mostly isolated to four variable segments or domains (termed VS or VD 1 to VD4), which include subspecies- and serovar-specific antigenic determinants [171]. The typing of *C. trachomatis* is based on the serological differentiation of antigenic epitopes on MOMP into 19 human *C. trachomatis* serovars (A to K, Ba, Da, Ia, Ja, L1 to L3, and L2a). Based on amino acid similarities, these serovars are grouped into the following serogroups or classes: B

class (B, Ba, D, Da, E, L1, L2 and L2a), C class (A, C, H, I, Ia, J, Ja, K, and L3), and intermediate class (F and G) [177] [178] [179]. Generally, type specific antibodies show specificities for one of these serovars only; sub-species specific antibodies show specificities for the class to a varying extent; and species specific antibodies show specificities for the whole *C. trachomatis* species [180] [181].

In our vaccine design we sought to use a MOMP antigen that encompassed the broadest coverage of these different serovar groups and classes and thus have a strong translational potential. We investigated both a consensus and a mosaic antigen approach to encompass the amino acid variability of MOMP. Mosaic vaccine antigens are composite proteins, optimised to include the maximum number of potential T cell epitopes from a set of samples. The mosaics are generated from natural sequences and resemble natural proteins but systematically include common (and exclude rare) potential epitopes. The mosaic approach has been extensively analysed for HIV proteins, but can be applied to any variable protein and to other pathogens [182]. Consensus-based vaccines rely on a centralised antigen designed to reduce sequence diversity by using the most common amino acid at each position of the protein. Consensus vaccines are designed to reduce the genetic differences between the vaccine and the primary isolate and increase the breadth of immune response [183].

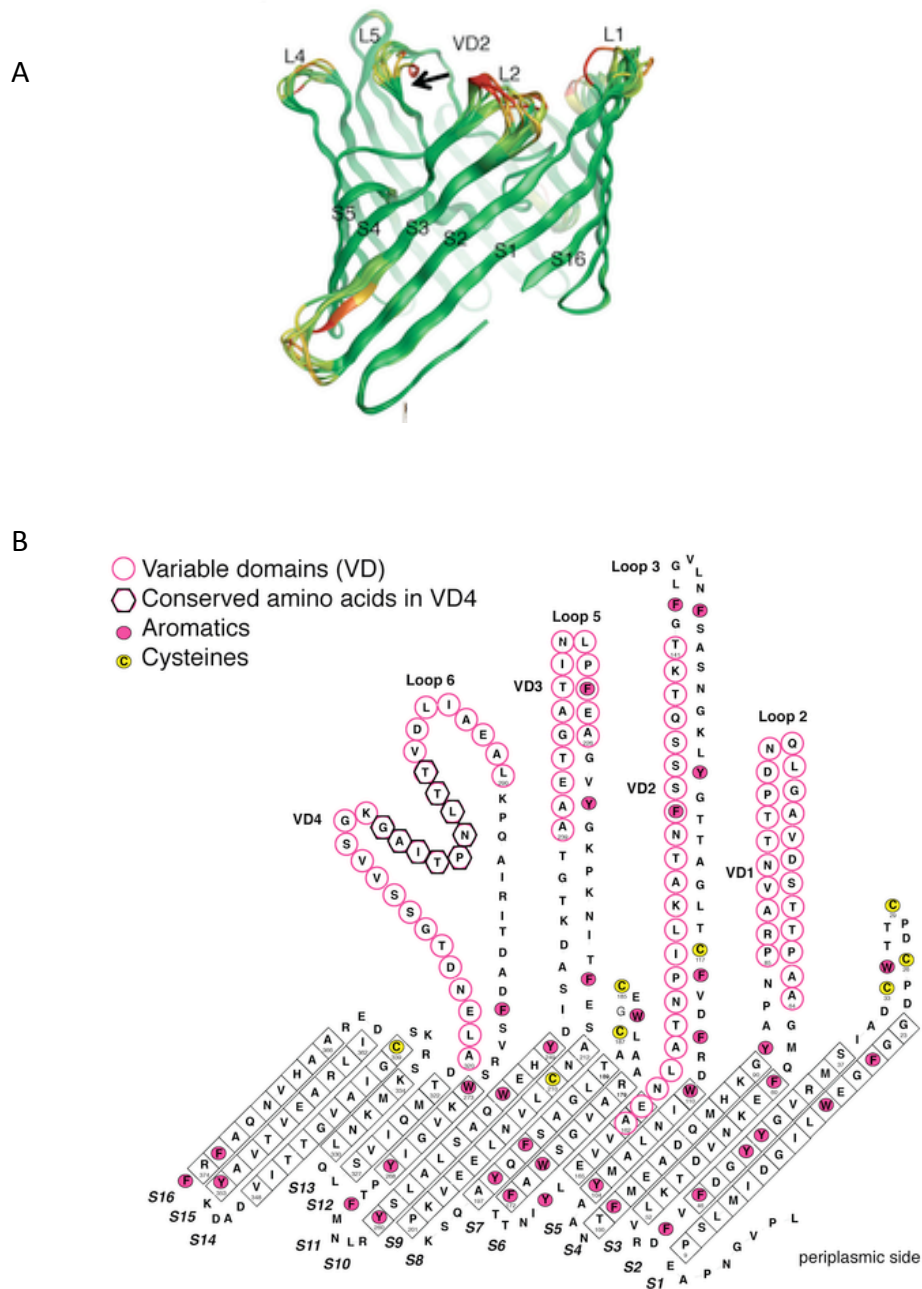


Figure 3.1. *Chlamydia trachomatis* MOMP structure

(A) Backbone and (B) predicted topology of *C. trachomatis* serovar C MOMP monomer. Figures adapted from Feher *et al.*, 2013 [184]. The backbone is a superposition of 10 low energy monomer structures, with ribbon colours illustrating the relative deviations of the heavy atoms to the average structure. The predicted topology of the monomer indicating variable domains, loop structures and aromatic/cysteine amino acids.

The aim of this chapter is to computationally assess whether the amino acid variability within the Chlamydial antigen MOMP can be encompassed within a consensus or a mosaic based vaccine. In this chapter consensus and mosaic approaches will be bioinformatically analysed to assess which approach provides the greater coverage of worldwide circulating MOMP sequences. Based on this analysis the optimal antigen will be expressed from four differing vaccine platforms; DNA, HuAd5, MVA, and recombinant protein and used as tools to investigate what anti-MOMP immune responses can be induced, and subsequently determine what anti-MOMP immune responses are required for protection against Chlamydia.

Aim

- To computationally assess whether the amino acid variability within the Chlamydial antigen MOMP can be encompassed within a single antigen.

Objective

- Consensus and mosaic approaches will be bioinformatically analysed to assess which approach provides the greater coverage against worldwide circulating MOMP sequences.

3.2 Results

3.2.1 Genital *Chlamydia trachomatis* serovar prevalence worldwide

Though MOMP-based vaccines have induced strong, near protective immunity in animal models, there is significant diversity in the amino acid sequence of the protein (encoded by *ompA*), and the serovar typing of *C. trachomatis* is based on this MOMP variability. To date there isn't a known worldwide prevalence of specific urogenital *C. trachomatis* serovars, therefore a literature review was undertaken to investigate this. Pubmed searches for the terms "chlamydia trachomatis serovar prevalence" and "ompA trachomatis prevalence" were undertaken in October 2011. Research articles were only included if they stated the serotype and the serotypes percentage prevalence or relative prevalence to other serovars. Unless otherwise stated, only serovar prevalence's from genital *C. trachomatis* samples were included, albeit from either male or female subjects. From this PubMed search in October 2011, only 13 publications described the country or regional serovar-specific prevalence. A subsequent PubMed search in November 2014 provided an additional 5 publications describing country/regional serovar-specific prevalence's. Serovar E clearly revealed itself to be the most prevalent in the majority of cases, accounting for 49% of cases in Australia (2011), 46% in Buenos Aires, Argentina (2010), and 41% in Holland (2000). Overall, in 10 out of the 13 *C. trachomatis* serovar surveys published, serovar E emerged as the most prevalent (Figure 3.2). In a subsequent 5 publications since October 2011, 4 of the 5 publications revealed serovar E to be the most prevalent serovar in their studied regions [185] [186] [187] [188].

3.2.2 Encompassing MOMP variability in a consensus and mosaic vaccine antigen

However, there is amino acid variability in the protein MOMP between, and within, serovars. To account for the amino acid variability in MOMP within serovar E, a consensus approach was undertaken using over 1500 serovar E *ompA* sequences obtained worldwide and generously provided by Prof. Joao Gomes (NIH, Portugal). These sequences were surveyed from a total of 5026 *C. trachomatis* strains isolated in 33 distinct geographic regions from five continents [189]. These samples were sourced from both hetero and homosexual patients from general practices, family planning centres, obstetrics and STD clinics. They found 49 *ompA* variants within their serovar E samples, and these sequences were used for an *in silico* generation of a consensus sequence. The Los Alamos Consensus Online Program (<http://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html>) was used on the 49 variant sequences, which aligns the 49 sequence reads and calculates the majority amino acid at each position before providing a consensus identity. The consensus sequence calculated had a 61% identity, and matched the circulating strain E-Bour. However, from an amino acid guide tree of the 49 variants, there was significant clustering of CalE samples. After blocking for these 11 CalE variants, the consensus still remained as the strain E-Bour but its coverage identity dramatically rose to 92.1%. The consensus' epitope coverage against the variant sequences of serovars D, E, F and G was analysed by EPICOVER (Figure 3.3A).



Country (Year)	D	E	F	G	H	I	J	K
Amsterdam (Holland) (1995) [190]								
Australia (2008) [191]		49%	22%	6%				6%
Buenos Aires (Argentina) (2010) [192]	19%	46%	15%					
China (2007) [193]	11%	27%	23%	12%				
Greece (2010) [194]								
<i>Hafner</i> (2008) [195]								
Holland (2000) [196]	12%	44%	22%					
Hungary (2009) [197]	34%	22%	19%	9%				
Italy (2009) [198]	20%	25%	16%	19%				
Japan & Thailand (1990s) [199]	32%	18%	17%					
Papua New Guinea (2007) [200]	30%	21%	25%		20%			
Russia (2009) [201]	10%	34%		26%			8%	
Seattle (USA) (1988-1996) [202]	13%	32%	18%					

Figure 3.2. *C. trachomatis* serovar prevalence's worldwide.

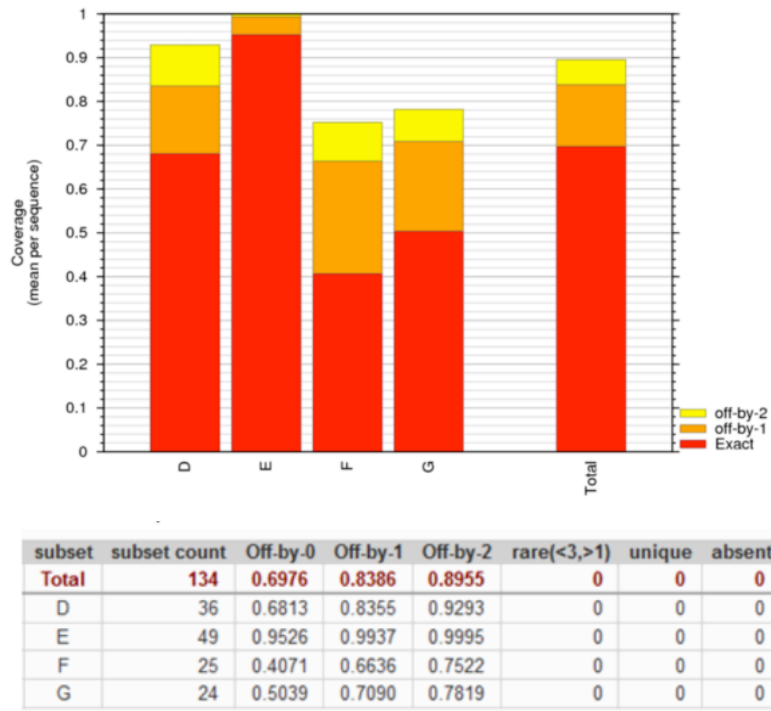
The three most prevalent serovars for the studied countries are ranked in order of prevalence. When percentages are unavailable, colour coding represents the relative prevalences, with dark red (most prevalent) to yellow (4th most prevalent). Studies available after October 2011 are not included in this figure, but are discussed in the text.

EPICOVER calculates the proportion of all epitope-length peptides in the test set that are matched by some peptide or peptides in each designed antigen. EPICOVER analyses at a nominal epitope length of 9, for which the program is optimised to analyse both Class I and II epitopes; allows up to 2 maximum amino acid mismatches; and requires a minimum of 3 occurrences of the epitope from the epitope test set to consider it as part of the coverage. The consensus has an exact epitope coverage of 95.26% against the 49 serovar E variants, and a coverage of 68.13% against the 36 serovar D variants, with an overall total epitope coverage of 69.76% against the 134 variants of serovar D, E, F and G together. For epitopes off by 1 amino acid or 2 amino acids, the coverage percentages are greater, as expected. There are advantages to the consensus sequence of MOMP matching that from a wild-type circulating strain (E-Bour). Firstly it ensures that the MOMP will have a natural, functioning protein structure, and secondly it allows for homologous challenges using actual *C. trachomatis* strain E-Bour elementary bodies. To further confirm the functional relevance of the consensus MOMP sequence, a Uniprot alignment analysis was carried out with the consensus MOMP sequence against the other 38 serovar E variant sequences. This alignment revealed no negative charge, aliphatic, aromatic or physical size site differences between the sequences, changes that may influence the protein folding, the transmembrane anchoring during production or the functionality of the mature protein.

As a comparison to the consensus approach, a mosaic vaccine strategy was investigated. Mosaic vaccines use natural sequences to generate a small number of mosaic sequences that include the maximal diversity of potential T cell epitopes

from circulating sequences. This method was first applied to HIV antigens due to the large genetic diversity in the virus, but has not yet been applied to any *C. trachomatis* protein. A mosaic protein is an artificial recombinant protein designed from a set of reference protein sequences so that every constituent peptide is found in some place in the set of input proteins. A mosaic vaccine was designed (<http://www.hiv.lanl.gov/content/sequence/MOSAIC/makeVaccine.html>) from the MOMP variant sequences of both D and E serovars, and the epitope coverage assessed by EPICOVER (Figure 3.3B). The mosaic provided exact epitope coverage of 76.31% against serovar E variants, 64.82% against serovar D variants, and 70.81% against all four serovars. The mosaic vaccine's overall epitope coverage is comparable to that of the consensus vaccine, but the lower coverage for the most prevalent serovar E and particularly the lack of a native MOMP structure given the expression difficulties experienced by researchers in the past argued against the mosaic approach for this work. From this evidence I was confident to use the consensus MOMP sequence as the antigen in the vaccines developed throughout this project. The nucleotide and amino acid sequence of the consensus MOMP antigen is provided in the Appendix (Figure 9.6 & Figure 9.7).

A



B

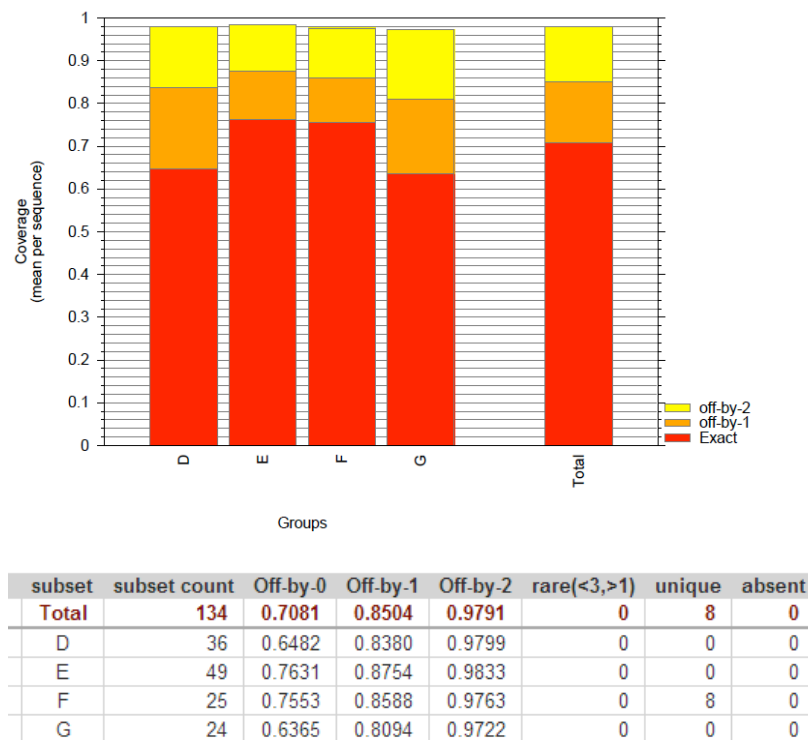


Figure 3.3. EPICOVER analysis of MOMP consensus and MOMP mosaic antigens.

Epitope coverage (EPICOVER) analysis of a 9-mer window matching for the computationally designed A) Consensus and B) Mosaic MOMP antigens. Mean coverage of either the consensus or mosaic MOMP antigen against the 134 variant MOMP sequences (provided by Gomes, NIH Portugal),

grouped by serovar, represented graphically and tabulated. Mean coverage represented as either exact epitope matches (off-by-0) or by 1 or 2 amino acid mismatches. Mean epitope coverage against serovars D, E, F and G (Total) are very similar between consensus MOMP and mosaic MOMP (69.7% vs 70.8, respectively). Consensus approach therefore adopted due to the greater conservation of the MOMP 'native' structure. Software freely available by The Los Alamos National Laboratory.

3.2.3 Vaccine development

3.2.3.1 Vaccine construction

The full methodologies and details on the constructions of the DNA, HuAd5, MVA and recombinant MOMP vaccines are described in Chapters 2.2 and 2.3. Briefly, the human codon optimised consensus MOMP sequence was synthesised by GeneArt, and cloned into the plasmid pcDNA3.1 to form the DNA vaccine. For the construction of the HuAd5 vaccine, the same synthesised consensus MOMP sequence was homologously recombined into the E1 and E3 deleted HuAd5 genome plasmid, pAL1112. Successfully recombined clones were transfected into 293TREx cells to propagate HuAd5 virus. The synthesised consensus MOMP was successfully cloned into the MVA-GFP-TD shuttle plasmid before being sent to The University of Oxford Viral Vector Core Facility where the plasmids were transfected and the MVA virus propagated and purified. For the recombinant MOMP protein, an *E. coli* codon optimised consensus MOMP sequence was synthesised by GeneArt and cloned into the bacterial expression plasmid pET101, but this protein expressed at too low a yield in *E. coli*. Therefore a closely matched recombinant MOMP (*C. trachomatis* D/UW/Cx) was used instead, kindly provided by Dr. Frank Follmann (Statens Serum Institut).

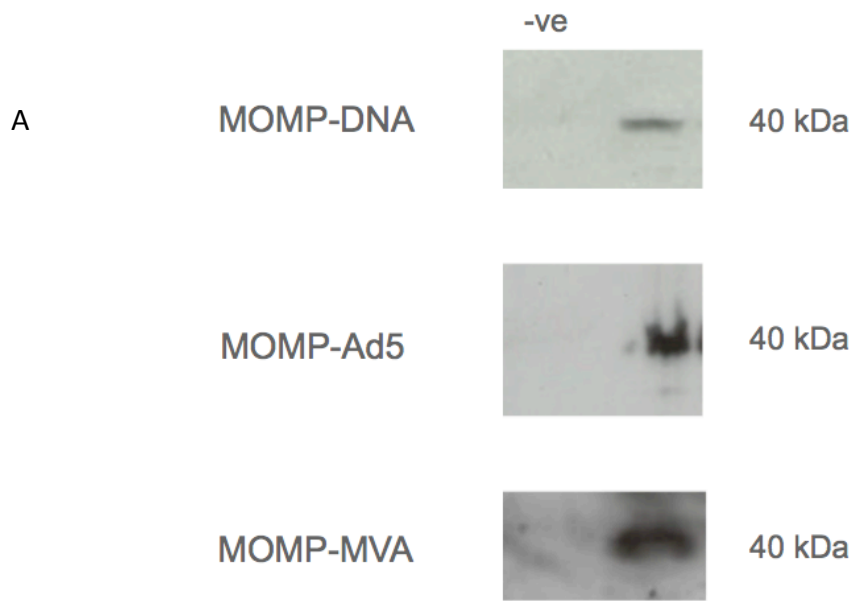
3.2.3.2 Antigen expression from DNA, HuAd5 and MVA vaccines

Expression of the consensus MOMP antigen from the DNA, HuAd5 and MVA based vaccines was confirmed by polyclonal anti-MOMP Western blots (Figure 3.4). MOMP-DNA was transfected into 293TREx cells, and the cell lysate and supernatant analysed 24 hr post transfection by anti-MOMP western blot. MOMP was expressed at the correct molecular weight (40 kDA) in the cell lysate following MOMP-DNA transfection. CHO cells were infected with MOMP-HuAd5, the cell lysate and supernatant analysed 24 hr post infection by anti-MOMP western blot and MOMP was found to be expressed at the correct molecular weight (40 kDA) in the cell lysate. TREx-293 cells were infected with MOMP-MVA, and MOMP was expressed in the cell lysate 24 hr post infection.

3.2.3.3 Recombinant protein expression plasmid

To prepare recombinant MOMP protein we used an inducible prokaryotic expression system. Consensus MOMP recombinant protein could not be expressed to high enough yields in the *E. coli* expression system for use as a vaccine. Induction and expression of the consensus MOMP recombinant protein was compared against a serovar D MOMP recombinant protein (kindly provided by Dr. Frank Follmann, Statens Serum Institut). Under identical induction conditions, the serovar D MOMP recombinant protein was expressed to a higher yield (Figure 3.4B, Lane 3) than the consensus MOMP recombinant protein (Figure 3.4B, Lane 6), which yielded usable amounts for vaccination studies. Therefore the recombinant protein from serovar D

MOMP was used throughout this thesis as the recombinant protein vaccine. It is unclear how the eight amino acid differences between the consensus MOMP and the serovar D MOMP could result in such different yields in the *E. coli* expression system.



E. coli expression

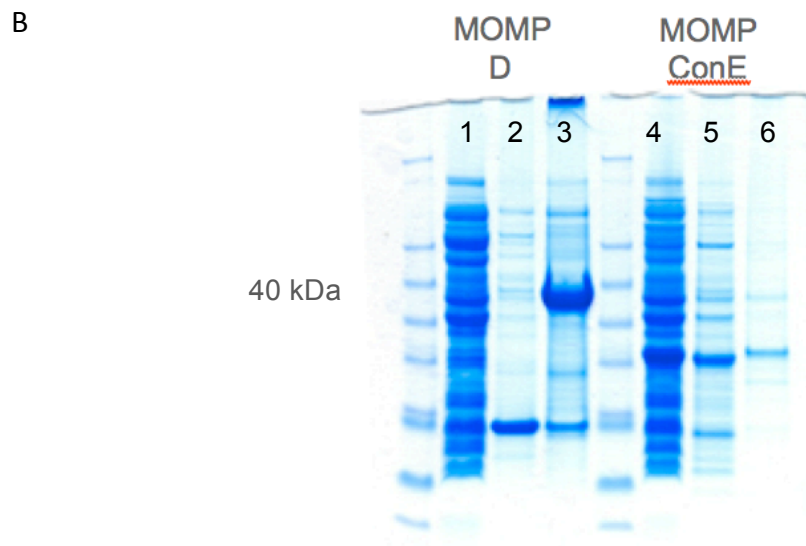


Figure 3.4. MOMP expression from (A) DNA, HuAd5 and MVA vectored vaccines and the (B) production of recombinant MOMP from *E. coli*.

MOMP expression from the DNA, HuAd5 and MVA vectored vaccines confirmed by polyclonal anti-MOMP Western Blot. Full-length consensus MOMP protein detected at a molecular weight of 40 kDa. MOMP production from *E. coli* was confirmed by Coomassie. Coomassie gel reveals poor consensus MOMP yield following expression in *E. coli* in comparison to the MOMP serovar D antigen. Lanes 1 & 4 are non-purified soluble proteins expressed following induction, lanes 2 & 5 are flow through proteins (unbound) following HIS-purification of the soluble protein fraction, and lanes 3 & 6 are eluted proteins (bound) following HIS-purification of the soluble protein fraction.

3.2.4 DNA, HuAd5 and MVA vaccine *in vivo* dose titration

To determine the appropriate dose for these novel untested vaccine constructs, an *in vivo* dose titration was performed for each one in turn. The immunisation and sampling schedule for the *in vivo* dose titrations are represented in Figure 3.5 and for each vaccine type (DNA, HuAd5, and MVA) the MOMP-specific serum IgG concentration and the MOMP-specific T cell IFN- γ production was measured (Figure 3.6).

All DNA immunisations were delivered intramuscularly with electroporation as described in Chapter 2.4.2. A dose of 0.5 – 50 μg was evaluated for induction of MOMP-specific IgG (Figure 3.6A). There were significantly higher serum MOMP-specific IgG concentrations following vaccination with 50 μg DNA doses compared to 0.5 μg doses or unvaccinated naïve controls ($*p \leq 0.05$, one way ANOVA with Bonferroni's multiple comparison post-test), with a mean concentration of 13.8 $\mu\text{g/ml}$ MOMP-specific IgG following four 50 μg DNA immunisations with electroporation. The same dose titration was assessed for induction of cellular responses (Figure 3.6A). There were significantly greater MOMP-reactive IFN- γ SFU from splenocytes from mice vaccinated with 50 μg DNA doses ($*** p \leq 0.0005$) or 5 μg DNA doses ($*p \leq 0.05$) compared to unvaccinated naïve controls (Kruskal-Wallis with Dunn's multiple comparison test). Four 50 μg DNA immunisations with electroporation measured a median of 335 IFN- γ SFU/ 10^6 splenocytes, with the 5 μg dose providing a median of 179 IFN- γ SFU/ 10^6 . As there were no statistical differences between 50 μg and 5 μg doses, we decided upon a workable dose of 10

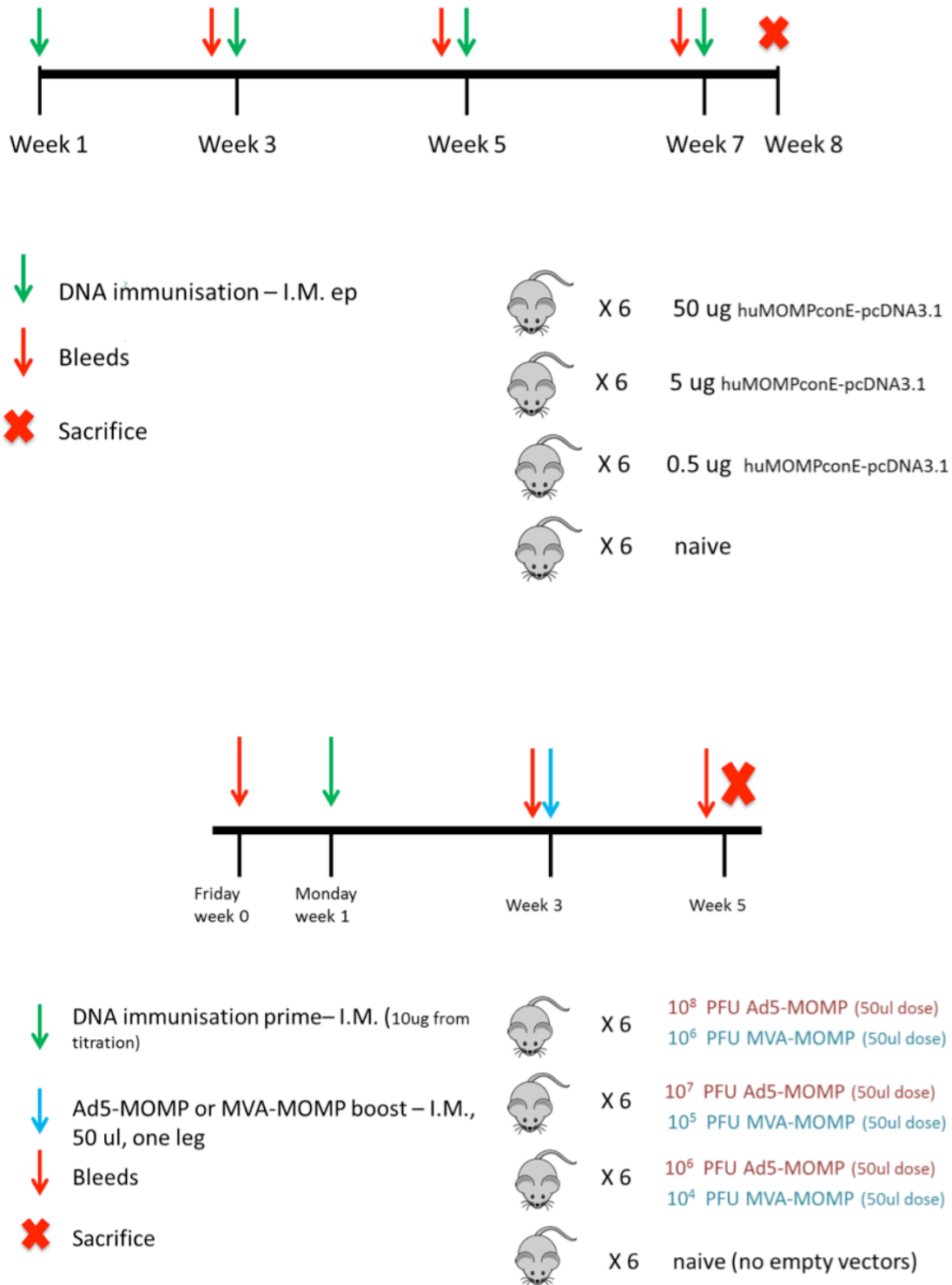


Figure 3.5. Vaccination and sampling schedule for the *in vivo* titration of DNA, HuAd5 and MVA vectored vaccines expressing MOMP.

The DDDD regimen for the dose titration of the DNA vaccine in BALB/c mice (Top) and the DA and DM regimen for the dose titration of HuAd5 and MVA expressing MOMP (Bottom). Immunisations, bleeds and sacrifices are indicated on the regimen schedules. Immunisation volume, route and vaccine concentrations are presented, alongside mice numbers per group.

µg which is often used for DNA immunisations adjuvanted with electroporation (Dr. Paul McKay, personal communication).

For the HuAd5 construct a dose titration of $10^6 - 10^8$ PFU was performed. The intramuscular HuAd5 immunisation was the boost to a DNA prime, with the vaccination and sampling schedule presented in Figure 3.5B. The HuAd5 was a boost to a DNA prime instead of just a HuAd5 prime alone to allow dose effect differences to be distinguishable, and a HuAd5 prime-HuAd5 boost was avoided in order to circumvent any potential vector immunity issues. A dose titration of $10^6 - 10^8$ PFU was evaluated for the induction of MOMP-specific IgG (Figure 3.6B) There were significantly higher serum MOMP-specific IgG concentrations following vaccination with 10^7 PFU or 10^8 PFU HuAd5 compared to unvaccinated naïve controls ($*p \leq 0.05$, one way ANOVA with Bonferroni's multiple comparison post-test), with a mean concentration of 2.7 µg/ml MOMP-specific IgG for 10^7 PFU and 3.5 µg/ml MOMP-specific IgG for 10^8 PFU HuAd5. A dose titration of $10^7 - 10^8$ PFU was assessed for the induction of cellular responses (Figure 3.6B). There were significantly greater MOMP-reactive IFN-γ SFU from splenocytes from mice vaccinated with 10^7 PFU or 10^8 PFU HuAd5 compared to unvaccinated naïve controls ($*p \leq 0.05$, Kruskal-Wallis with Dunn's multiple comparison test), with median values of 246 and 267 IFN-γ SFU/ 10^6 splenocytes for 10^7 and 10^8 PFU HuAd5 respectively. We decided upon a HuAd5 dose of 10^7 PFU for future immunisations as 10^7 PFU was the lowest dose to yield both significantly greater humoral and cellular responses compared to the naïve controls.

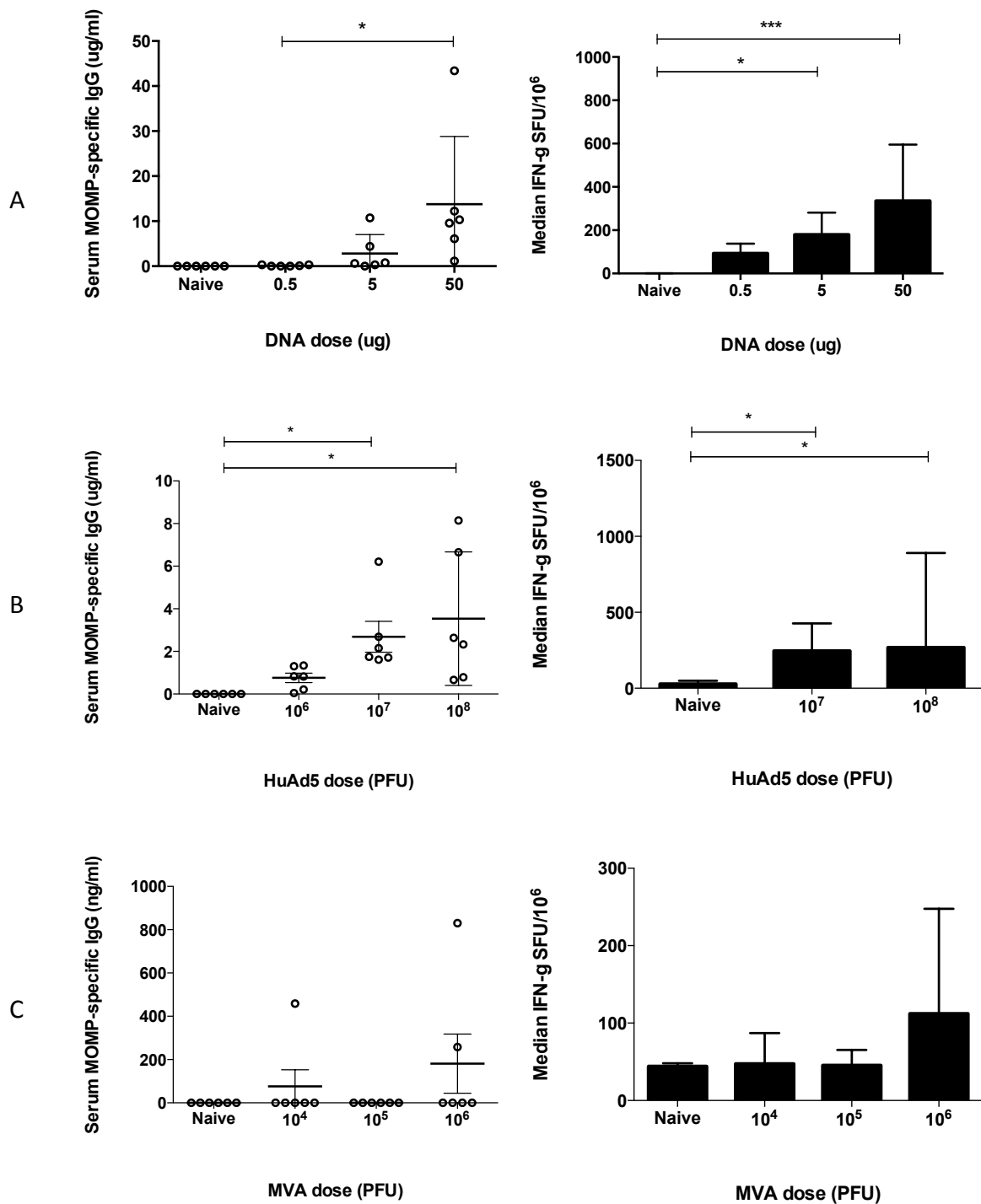


Figure 3.6. *In vivo* titration of DNA, HuAd5 and MVA expressing MOMP.

MOMP-specific IgG from serum were assessed (n=6 BALB/c per group) one week after DDDD regimen for DNA titration (A), or DA or DM for HuAd5 (B) and MVA (C) titration. Antibody results are expressed as group means (+SEM) ($\mu\text{g/ml}$ or ng/ml).

* $p \leq 0.05$, one-way ANOVA with Bonferroni's multiple comparison post-test. These vaccinated BALB/c mice were sacrificed one week post final immunisation and splenocytes were assessed by IFN- γ ELISpot for MOMP-reactive T cells using 15-mers overlapping by 11 amino acid. Graph is expressed as group medians (SFU/million antigen stimulated cells (+ interquartile range)) * $p \leq 0.05$ and *** $p \leq 0.0005$ by Kruskal-Wallis with Dunn's multiple comparison test.

For the MVA construct a dose titration of $10^4 - 10^6$ PFU was performed. As for the HuAd5 titration, the intramuscular immunisation was the boost to a DNA prime (Figure 3.5B). A dose titration of $10^4 - 10^6$ PFU was evaluated for the induction of MOMP-specific IgG and MOMP-reactive IFN- γ SFU from splenocytes (Figure 3.6C). None of the MVA doses induced significantly greater serum MOMP-specific IgG concentrations than the unvaccinated naïve controls, with the highest MVA dose of 10^6 PFU inducing a mean concentration of only 181 ng/ml serum MOMP-specific IgG. In addition, none of the MVA doses induced significantly greater MOMP-reactive IFN- γ SFU from splenocytes compared to unvaccinated naïve controls. However, the highest dose of 10^6 PFU did induce the highest median with 112 IFN- γ SFU/ 10^6 . As 10^6 PFU was the only dose to induce any measurable humoral and cellular response, it was chosen as the workable dose for future MVA immunisations.

3.3 Discussion

The majority of currently available successful vaccines induce host responses against antigens that are highly conserved in the targeted pathogens. Diphtheria, tetanus, and pertussis vaccines confer protection by inducing neutralising antibodies to the conserved bacterial toxins that are the major virulence factors. The *Hemophilus influenzae* B vaccine induces effective responses to conserved epitopes in the sugar structure of the bacterial capsular polysaccharide [203]. However, new strategies are required to be successful against the more intractable pathogens, particularly those with a high degree of antigenic variation.

To the best of our knowledge, no one has tried to deal with the variability in the amino acid sequence of MOMP serovars within a single protein antigen. Groups have attempted to encompass the variability through the use of a several MOMP-based vaccines formulated together. A polyvalent MOMP based vaccine approach was used against *Chlamydia pecorum* in koalas, co-immunising with MOMP A and MOMP F. MOMP A, MOMP F and MOMP G were able to induce systemic and mucosal immune responses in the healthy, *Chlamydia*-free koalas, but these animals were not challenged [204]. Another potential approach to contend with the genetic diversity of *C. trachomatis* serovars is to use non-variable Chlamydial antigens. The polymorphic membrane protein D (PmpD) is a species-specific antigen that elicits pan-neutralising antibodies [205]. If produced and formulated in such a way as to be a protective vaccine antigen, it could potentially provide cross-serovar protection to

C. trachomatis [206].

It is important to recognise that concentrating vaccination strategies onto a select few serovars, such as E and D in this case, can potentially result in serovar/serotype replacement at a population scale. Although serotype replacement is a possible issue when multiple serovars are in circulation, serotype replacement was not detectable after the introductions of the *Haemophilus influenzae* type b (Hib) conjugate vaccines. Serotyping of *H. influenzae* carriage in over 360 families in the UK found no evidence of increased carriage of non-b *H. influenzae* following vaccination [207]. In contrast, pneumococcal conjugate vaccine studies have shown substantial evidence of serotype replacement [208]. Since the introduction of the heptavalent conjugate pneumococcal vaccine (PCV7), the pneumococcal serotype distribution as well as the patterns of antibiotic resistance has changed. Serotyping of *Streptococcus pneumoniae* strains from across 16 Massachusetts communities between 2001 and 2004 revealed non-PCV7 serotypes increasing from 34% to 55%, indicating either serotype replacement or serotype switch in which strains express a new capsular serotype to evade host immunity [209]. Given that the *ompA* genes of Chlamydiae have recombination hotspots and regions that can transpose between types, serotype replacement is a potential worry in Chlamydia vaccine design and would have to be investigated and followed-up in population settings.

The use of consensus antigens has had some success for particular diseases in animal studies. The failure in protection with current HBV vaccines in 15% of individuals is due to the genetic divergence among different HBV genotypes and the immune

escape mutations that occur within the surface antigen HBsAg. To avoid these issues, Obeng-Adjei *et al.* designed a novel consensus-based approach to vaccine design for HBV. Their DNA vaccine based consensus was based on the HBV core (HBcAg), with codon and RNA optimisation for maximum expression. The consensus vaccine was able to induce strong antigen-specific T cell responses in C57BL/6 mice, as well as a high titre antibody response capable of recognising a native HBcAg though there were no challenge studies to assess protective ability [210]. A consensus H5N1 influenza hemagglutinin (HA) antigen was designed from circulating 2006 strains and inserted into a DNA plasmid. Mice immunised intramuscularly (via electroporation) with this consensus H5N1 HA antigen elicited neutralising antibodies against various H5N1 viruses across five clades. Moreover, this vaccine in mice conferred complete protection from H5N1 virus challenges against clades 1 and 2.2, and significant protection against clade 2.1 [211]. Rhesus macaques were vaccinated with a polyvalent mixture of purified HIV-1 trimerised consensus Env-gp140 proteins encompassing clades A, B, C and E, and compared against a single consensus Env-gp140 representing group M. Both vaccines elicited anti-Env-gp140 IgG antibodies, and both vaccines elicited neutralising antibodies against the HIV-1 SF162p4 isolate. However, these consensus-vaccinated monkeys were not protected against SHIV SF162p4 challenge [183]. Inovio Pharmaceuticals, Inc., recently revealed positive interim results from a phase I study of a consensus based H5N1 DNA vaccine. The VGX-3400X study, utilising Inovio's SynCon® consensus design technology, demonstrated vaccine-elicited high antigen-specific binding antibody concentrations and a four-fold rise in geometric mean titres in an HAI assay against six different

H5N1 virus strains. This, they argue, is an initial demonstration of the potential to generate 'universal' immune responses in humans.

The mosaic approach to vaccine antigen design has been pursued most diligently in HIV-1 vaccine research. When administered to monkeys, mosaic vaccines elicited stronger responses, covering more epitopes and with greater cross-reactivity than single immunogens [212] [213]. Furthermore, bivalent HIV-1 mosaic antigens have shown protection against acquisition of infection with the difficult to neutralise SHIV SF162p3 virus in rhesus macaques. Adenovirus/poxvirus and adenovirus/adenovirus vector-based vaccines expressing HIV-1 mosaic Env, Gag, and Pol afforded a significant reduction in the per-exposure acquisition risk following repetitive, intrarectal SHIV-SF162P3 challenges, with protection correlating with vaccine-elicited binding, neutralising, and functional non-neutralising antibodies [156]. The mosaic approach has not been published for any other pathogen except HIV-1 to the best of our knowledge.

From our EPICOVER and proteomic analyses, the consensus MOMP provided greater epitope coverage against the most prevalent circulating *C. trachomatis* strains and the consensus provided a nearer-native MOMP structure in comparison to the mosaic antigen. Therefore we decided to use the consensus MOMP sequence as the antigen in the vaccines developed throughout this project.

We carried out an *in vivo* titration of the DNA, HuAd5 and MVA vectored MOMP vaccines to determine the optimal inoculation dose to elicit maximal cellular and

humoral immunity. The dose we chose for the DNA vaccine with electroporation was 10 µg DNA. This is a dose comparable to other DNA vaccines, including those against Merkel cell polyomavirus [214], against HIV [157], and against tumors in mice [215]. From the *in vivo* titration, there were no significantly different antibody or IFN-γ ELISpot induction between the 5 µg and 50 µg doses, and for comparability to other DNA vaccines we decided upon 10 µg DNA with electroporation (Dr. Paul McKay, personal communication). Our optimal dose for HuAd5-MOMP immunogenicity, delivered intramuscularly, was 1×10^7 PFU. This is the same dose as is used in the adenovirus-vectored vaccine against Venezuelan equine encephalitis virus in BALB/c mice [216], and comparable to dosages used to provide protective immunity to botulism [217] and as a sublingual immunisation to SARS [218]. Finally, our optimal dose for MVA-MOMP was 1×10^6 PFU – the same dose as used in pre-erythrocytic stage malaria vaccines in BALB/c mice [219] and in pre-clinical HIV-TB pediatric vaccines in BALB/c mice [220].

There were difficulties in expressing the consensus MOMP in the *E. coli* expression system, with consistently low yields. Expression of MOMP in heterologous systems such as *E. coli* has also previously proved to be highly problematic, since the protein tends to misfold and aggregate [221]. These difficulties have been experienced by other groups and in other expression systems including *Arabidopsis thaliana* [222]. To avoid the difficulties in expressing full length MOMP, Kalbina *et al.* formed a chimeric MOMP polypeptide, minimizing the number of hydrophobic amino acids from the transmembrane regions to thus increase the protein's solubility. We also attempted this chimeric approach, but with low yields. It was only the full length

MOMP with 8 amino acid alterations, made in collaboration with the Statens Serum Institute (Copenhagen, Denmark) that provided workable yields of MOMP protein for immunisations and immunogenicity assessments. It is uncertain exactly how these amino acid alterations affected the MOMP yields; especially as the amino acid sequence matched a natural circulating Chlamydial strain. This may be due to the expression of MOMP within *E. coli*, with post-translational modifications potentially differing from those in Chlamydia. Even single amino acid alterations within the *C. trachomatis* MOMP protein can drastically affect the protein yields (Dr. Frank Follmann, personal communication) and may affect the protein folding, the pore formation or the integrity of the full length 393 amino acid protein.

Overall this chapter sought to computationally assess whether the amino acid variability within the Chlamydial antigen MOMP could be encompassed within a consensus or a mosaic based vaccine. We bioinformatically attempted to encompass the variability of the chlamydial antigen MOMP, with the consensus approach providing greater epitope coverage and native structure than a mosaic antigen. DNA, HuAd5 and MVA based vaccines were successfully constructed and their optimal immunisation dose determined through *in vivo* titrations. A recombinant consensus MOMP was poorly expressed from *E. coli*, so a closely related MOMP serovar D antigen was adopted. This toolbox of MOMP-based vaccines will allow us to investigate which vaccine-induced immune responses are protective against *C. trachomatis* genital infections in the murine model.

Chapter 4

**Multi-component vaccines against *Chlamydia trachomatis*
elicit varying immune outcomes following heterologous
prime-boost immunisations**

4 Multi-component vaccines against *Chlamydia trachomatis* elicit varying immune outcomes following heterologous prime-boost immunisations

4.1 Introduction

Established vaccines currently in clinical use predominantly act by the induction of antibodies, yet stimulating strong cellular immunity has proved harder to achieve. New arrays of alternative antigen delivery systems have the potential to induce cell-mediated immunity, such as DNA vaccines, recombinant viral vectors, protein-in-adjuvant formulations and recombinant virus-like particles. For DNA and recombinant virus vector vaccines, the DNA sequence for the antigen(s) of choice is inserted into an *E. coli* derived purified plasmid or the genome of a double-stranded DNA virus, such as Adenovirus or vaccinia. Host CD4+ and CD8+ responses are then induced from the intracellular synthesis of the transgene, with processing and HLA presentation of class I and II T cell epitopes from the antigen [223].

There has been much optimism about the potential for DNA vaccines as an effective preventative measure for a range of intracellular diseases, including malaria, tuberculosis and HIV. Human studies have confirmed the safety of the approach, along with the ability to elicit antigen-specific CD8+ CTLs [224], though the magnitude of T cell responses were not shown to be sufficient for protection against

malaria challenge. Alongside the development of DNA vaccines, the emergence of recombinant viral vectors as vaccine delivery systems, such as poxviruses and adenoviruses also showed promise as effective inducers of immune responses. The highly attenuated recombinant vaccinia virus MVA revealed to have excellent immunogenicity [225], but when used singly or with repeated administration (homologous boosting) did not produce levels of T cell responses that were protective against malaria [226]. It was therefore logical to try combining different approaches. The initial use of a heterologous prime-boost immunisation approach in HIV-1 vaccine development was based on the following rationale:

“Recombinant envelope glycoproteins, while being able to elicit specific neutralising antibody responses, were unable to elicit cytotoxic T cell responses, and on the other hand, immunisation with recombinant vaccinia expressing HIV-1 antigens could elicit good T cell responses but not high levels of protective antibodies. Therefore combined immunisation including both of these two types of vaccines may be more effective than either immunogen alone” [227].

Priming the immune system against a target antigen and subsequently boosting antigen-specific immune responses with a different distinct immunogen can result in the synergistic enhancement of specific immunity, such as increased number of antigen-specific T cells and the selective enrichment of T cell avidity [228] [229]. Potential mechanisms that contribute to the success of this strategy include T cell immunodominance, the nature of the boosting vectors, and the generation of only low levels of anti-vector immunity [230] [231]. But not all heterologous prime-boost

strategies are successful at producing a synergistic enhancement of T cell responses. Many vector agents are able to prime an immune response, but not all are effective boosters, with the nature of the antigen delivery system determining its ability to boost a cell-mediated immune response. A priming immunisation with a recombinant influenza virus expressing an epitope from a protein of *P. yoelii* followed by a boosting of the same epitope from a recombinant vaccinia virus provided protection in mice, yet the opposite order of immunisation failed to induce protection [232]. And from HIV vaccine research, DNA priming followed by protein-in-adjuvant boosting resulted in enhanced antibody titres, but by an additive rather than a synergistic effect and did not result in greatly enhanced effector T-cell induction [233] [234].

In general DNA plasmids are excellent priming agents but relatively ineffective as boosting agents, while MVA and adenovirus strains are capable of either priming or boosting when used in heterologous regimens. Many groups have now reported enhanced CD4+ and CD8+ T cell induction by heterologous prime-boost strategies in a range of disease models (tuberculosis, HIV, HPV and Ebola) but is yet to be investigated for *C. trachomatis* [235] [236] [237] [238].

In this chapter we will comparatively assess the induction of humoral and cellular immunity against *C. trachomatis* following heterologous prime-boost vaccinations with DNA, Adenovirus, MVA and recombinant protein based vaccines expressing MOMP. As there is not yet a strong correlate of protection to genital *C. trachomatis*

infection in the mouse model, let alone in humans, these heterologous prime-boost vaccination approaches using regimens encompassing the four differing vaccine platforms described in chapter 3 should elicit a spectrum of immune responses, and potentially engage both the humoral and cellular arms of the immune system. These vaccine constructs and their use application in differing regimens will provide an invaluable tool kit to investigate potential correlates of vaccine induced *C. trachomatis* protection.

Aim

- To elicit a spectrum of immune responses against MOMP, and potentially engage both the humoral and cellular arms of the immune system.

Objective

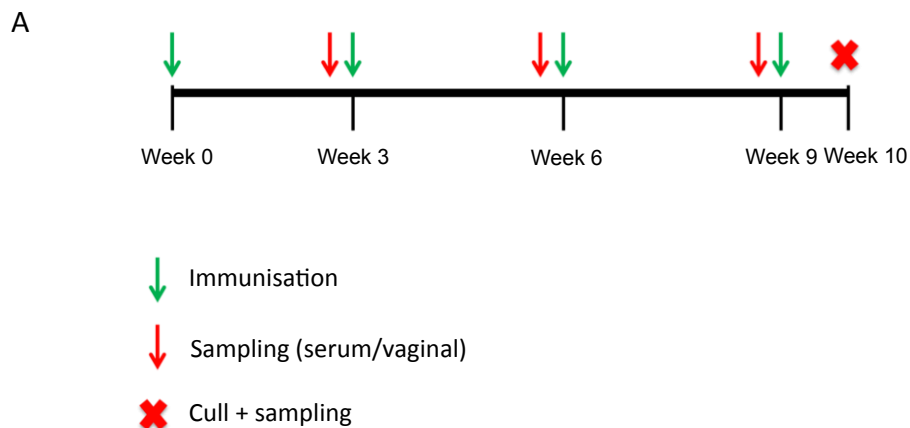
- To comparatively assess the induction of humoral and cellular immunity against MOMP following heterologous prime-boost vaccinations with DNA, Adenovirus, MVA and recombinant protein based vaccines.
- MOMP-specific humoral immunity will be measured by ELISA and ELISA-based avidity assays.
- MOMP-specific cellular immunity will be measured by IFN- γ ELISpot and T-cell intracellular cytokine staining.

4.2 Results

4.2.1 Study Design

The vaccination regimens are abbreviated according to the vaccines and their order in the vaccination schedule. The DNA vaccine with electroporation is hereon referred to as **D**, the human Adenovirus 5 vectored vaccine as **A**, the MVA vectored vaccine as **M**, and the recombinant protein adjuvanted with MF59 as **P**. Therefore, a regimen involving a DNA prime (**D**), followed by a HuAd5 boost (**A**), a subsequent MVA boost (**M**) and a final recombinant protein adjuvanted with MF59 boost (**P**) would be represented as a **DAMP** regimen. Accordingly, a DNA prime with two subsequent DNA boosts all with electroporation is abbreviated to **DDD**. All immunisations, unless otherwise stated, are administered intramuscularly at three weeks apart, with serum and vaginal sampling two weeks after each immunisation. These timings had been previously optimised in our laboratory in order to sample at peak antigen-specific antibody concentrations. Mice were sacrificed one week after the final immunisation to assess cellular responses from splenocytes (Figure 4.1A).

The vaccination regimens tested were based off a matrix-like approach to encompass as many iterations as practically feasible (Figure 4.1B). DNA and HuAd5 vaccines, as discussed in Chapter 4.3, have been previously shown to be good priming agents, while MVA vectored vaccines are poor at priming and this set the basis for a set of the heterologous prime-boost regimens investigated. HuAd5 prime or boosts were subsequently boosted with either MVA or recombinant protein to avoid any risk of anti-vector immunity dampening immune responses. With respect



B

Protein-free Regimens	Protein-boost Regimens
DDDAM	DAMP
DDDA	AMPP
DDDM	DDPP
DDD	APP
AM	PPP
	PP

Figure 4.1. Immunisation and sampling schedule and prime-boost regimens.

The immunisation and sampling schedule for the investigation into the immunogenicity of a four-dose prime-boost vaccination regimen (A). The regimens investigated, categorised as either protein-free or protein-boosted (B).

to the difficulties in producing high yields of recombinant MOMP protein, regimens without any recombinant protein was investigated alongside regimens with recombinant protein boosts. Previous work in our laboratory revealed the need for two-three initial DNA vaccinations to induce sufficient humoral and cellular immune responses in mice and considerations like these shaped the regimens investigated. To contrast regimens consisting of up to five separate immunisations, we considered short regimens within our investigations such as AM and APP.

4.2.2 Antibody immunogenicity of *C. trachomatis* MOMP vaccines in intramuscular heterologous prime-boost regimens

The immunogenicity of a *C. trachomatis* antigen MOMP was assessed in BALB/c mice immunised in heterologous prime-boost regimens with DNA, HuAd5, MVA and protein (+ MF59) based vaccines. Immunisations were administered three weeks apart and serum and vaginal washes sampled two weeks post-final immunisation (Figure 4.2A). Peak MOMP-specific serum IgG concentrations were measured as described in Chapter 2.4.4, with the highest mean concentration afforded by the PPP regimen (mean = 1.27 mg/ml) and the lowest from the DDD regimen (mean = 15 µg/ml).

MOMP-specific serum IgG concentrations from heterologous prime-boost vaccine regimens (columns) were compared against other heterologous prime-boost vaccine regimens (rows) by one-way ANOVAs with Bonferroni's multiple comparison post-

Compared against	Vaccine Regimens										
	DDDAM	DDDA	DDDM	DDD	AM	DAMP	AMPP	DDPP	APP	PPP	PP
DDDAM							**		***	****	
DDDA							***		****	****	*
DDDM							***		****	****	*
DDD							****		****	****	*
AM							***		****	****	*
DAMP									*	****	
AMPP										*	
DDPP										****	
APP											
PPP											
PP										***	

Table 4.1. MOMP-specific serum IgG concentration comparisons.

One-way ANOVA with Bonferroni's multiple comparison post-test analysis of MOMP-specific serum IgG concentrations elicited following heterologous prime-boost vaccine regimens. Column regimens eliciting significantly greater IgG concentrations than row regimens are marked with the significance, * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ and **** $p \leq 0.0001$.

tests and are presented in Table 4.1. Regimens (columns) inducing significantly greater MOMP-specific serum IgG concentrations than other regimens (rows) are signified by their degree of significance (* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ and **** $p \leq 0.0001$). This indicates that it is the final two protein (+ MF59) immunisations that significantly increase MOMP-specific serum IgG concentrations above prime-boost regimen without two protein boosts. The MOMP-specific IgG kinetics following the differing vaccination regimens are provided in the Appendix (Figure 9.4 & Figure 9.5).

Alongside serum measurements, MOMP-specific vaginal IgG concentrations were measured following the heterologous prime-boost vaccine regimens. With a one-way ANOVA and Bonferroni's multiple comparison post-test analysis, PPP (*, $p \leq 0.05$) induced significantly higher MOMP-specific vaginal IgG than DDDAM, DDDA, DDDM, DDD, AM or the naïve group. This indicates that only the PPP regimen induced significantly higher vaginal MOMP-specific IgG concentrations, compared to naïve controls and regimen without protein boosts (Figure 4.2B).

Next we utilised an avidity assay (as described in Chapter 2.4.6) as a means to differentiate and qualitatively evaluate the humoral responses (Figure 4.2C). MOMP-specific serum IgG avidities are grouped low, medium and high based upon the % avidity index. Low is classified as <30%, medium is 30-50%, and high avidity are >50%. All protein free regimens (DDDAM, DDDA, DDDM, DDD and AM) had mean avidity indices of <30%, with DDDM having the lowest mean avidity index (mean = 17.5). In addition, the DAMP and PP regimens had low mean avidity indices, with these

regimens only having a single protein (+MF59) boost. The regimens with two protein (+MF59) boosts (AMPP, DDPP and PPP) all had medium mean avidity indices. The APP regimen was the only regimen to induce high avidity MOMP-specific serum IgG (mean = 51.8).

A correlate for Th1 and Th2 skewing of the immune response in mice is the antigen-specific IgG2a to IgG1 ratio. MOMP-specific serum IgG2a and IgG1 concentrations were measured by ELISA, and the ratios calculated (Figure 4.2D). Mean ratios + SEM are represented. The highest MOMP-specific serum IgG2a:IgG1 ratio was induced following the DAMP regimen (mean = 14.2), with the lowest ratio induced following PP (+ MF59) vaccination (mean = 0.0036). There was a significant statistical difference in the IgG2a:IgG1 ratios between DAMP and PPP (**, $p \leq 0.005$) and DAMP and PP (***, $p \leq 0.0005$, one-way ANOVA with Bonferroni's multiple comparison post-test). Protein only vaccine regimens induced Th2-biased immune environments indicated by IgG2a:IgG1 ratios of less than 1. However, vaccine regimens with a vector-based vaccine prime consistently induced a Th1-biased, cytophilic antibody response as indicated by IgG2a:IgG1 ratios of greater than 1. It has previously been shown that viral vector containing regimens induce a higher and longer lasting cytophilic antibody response [239].

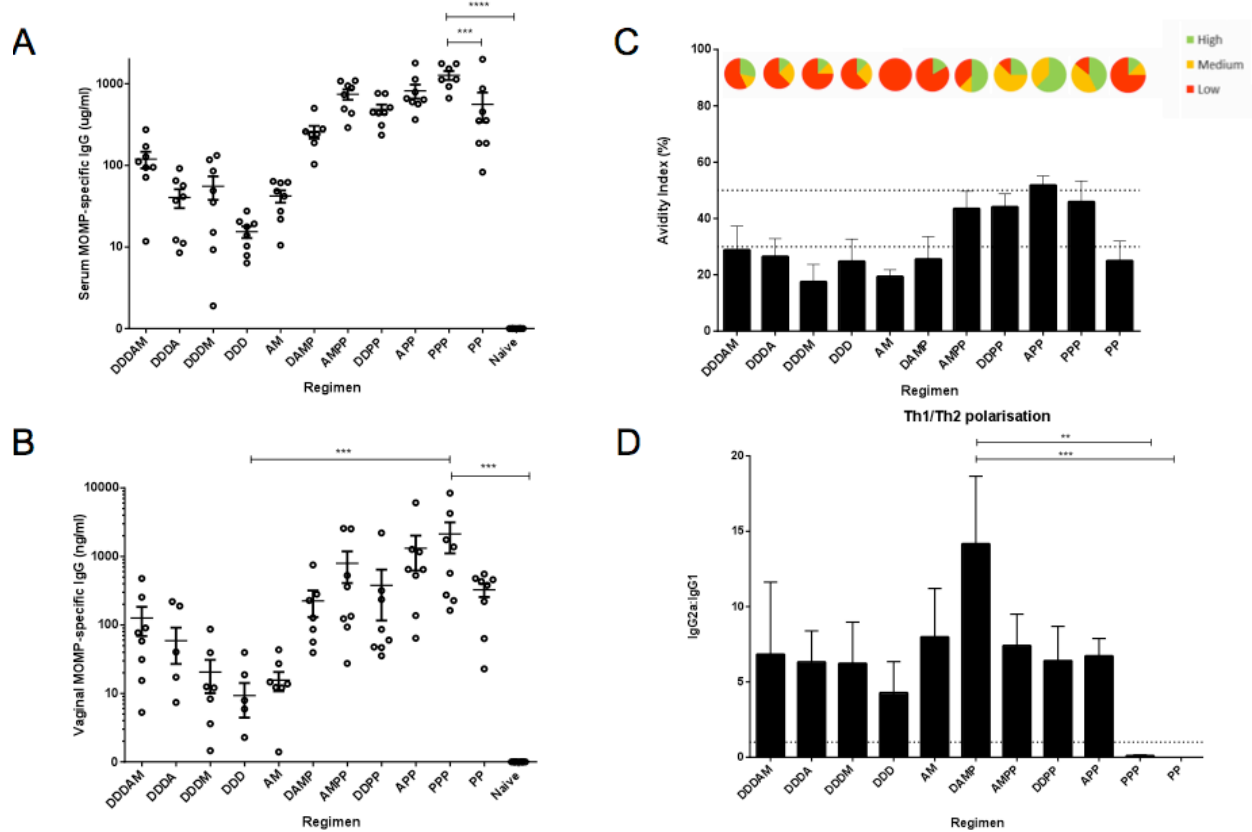


Figure 4.2. Antibody responses following heterologous prime-boost intramuscular vaccination.

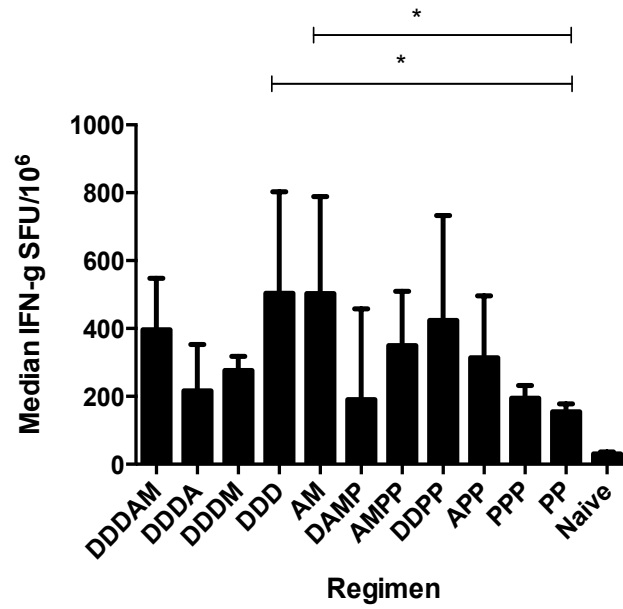
MOMP-specific IgG from serum (A) and vaginal washes (B) were assessed in BALB/c mice (n=8 per group) two weeks after completion of the indicated prime-boost regimens. Antibody results are expressed as group means (+SEM) (µg/ml or ng/ml). The antibody avidity assessment (C) is the corresponding serum MOMP-specific IgG ELISA using non-reducing (PBS) and reducing (8M urea) washes after sample addition. Results are shown as percentage (%) change in binding ($\frac{\text{reducing OD}_{650}}{\text{non-reducing OD}} \times 100$). Serum MOMP-specific IgG2a and IgG1 titres were measured (n=8 per group) two weeks after completion of the indicated prime-boost regimens and the IgG2a:IgG1 ratio represented as group means +SEM (D). * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ and **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni's multiple comparison post-test.

4.2.3 T cell immunogenicity of *C. trachomatis* MOMP vaccines in intramuscular heterologous prime-boost regimens

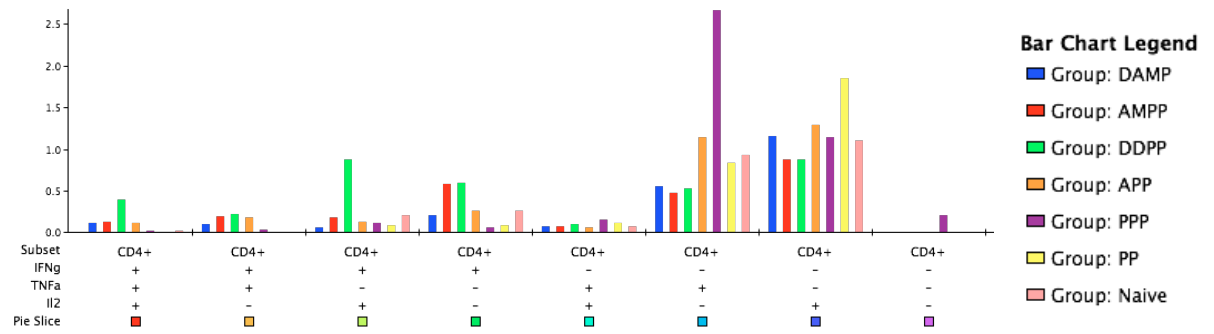
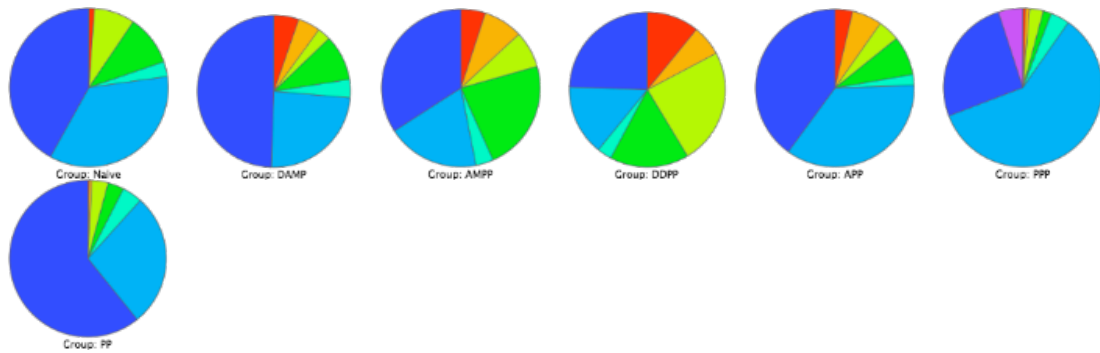
T cell responses were also assessed in BALB/c mice following heterologous prime-boost vaccine regimens with DNA, HuAd5, MVA and protein (+ MF59) based vaccines one week after the final immunisation (Figure 4.3). Splenocytes were re-stimulated with MOMP peptides as described in Chapter 2.4.9. T cell IFN- γ responses were induced by all prime-boost regimens, with the DDD and AM regimens inducing the strongest T cell responses, with a median of 504 and 502 SFU/10⁶ splenocytes respectively. The T cell responses induced by the DDD and AM regimens were significantly higher than those induced in the PP regimen ($p < 0.05$ by Kruskal-Wallis test with Dunn's multiple comparison post-test) (Figure 4.3).

Intracellular cytokine staining was used to assess the polyfunctionality or cytokine response profile of CD4⁺ and CD8⁺ T cells from the heterologous prime-boost vaccinated BALB/c mice. From these analyses it was clear that the CD4⁺ T cells displayed a greater degree of polyfunctionality than the CD8⁺ T cells. Very low percentages, if any, of CD8⁺ T cells expressed all three of the measured cytokines, IFN- γ , TNF- α and IL-2. CD4⁺ T cells from DDPP vaccinated mice exhibited the highest percentage of polyfunctionality, with 0.5% of total CD4⁺ T cells expressing all three cytokines. There was expression of all three cytokines by CD4⁺ T cells from the DAMP, AMPP, DDPP and APP regimens, while there was poor polyfunctionality from the PPP and PP regimens, indicating a greater polyfunctionality of CD4⁺ T cells from vector-based vaccine primes in contrast to protein alone. The PPP regimen did

A



B



C

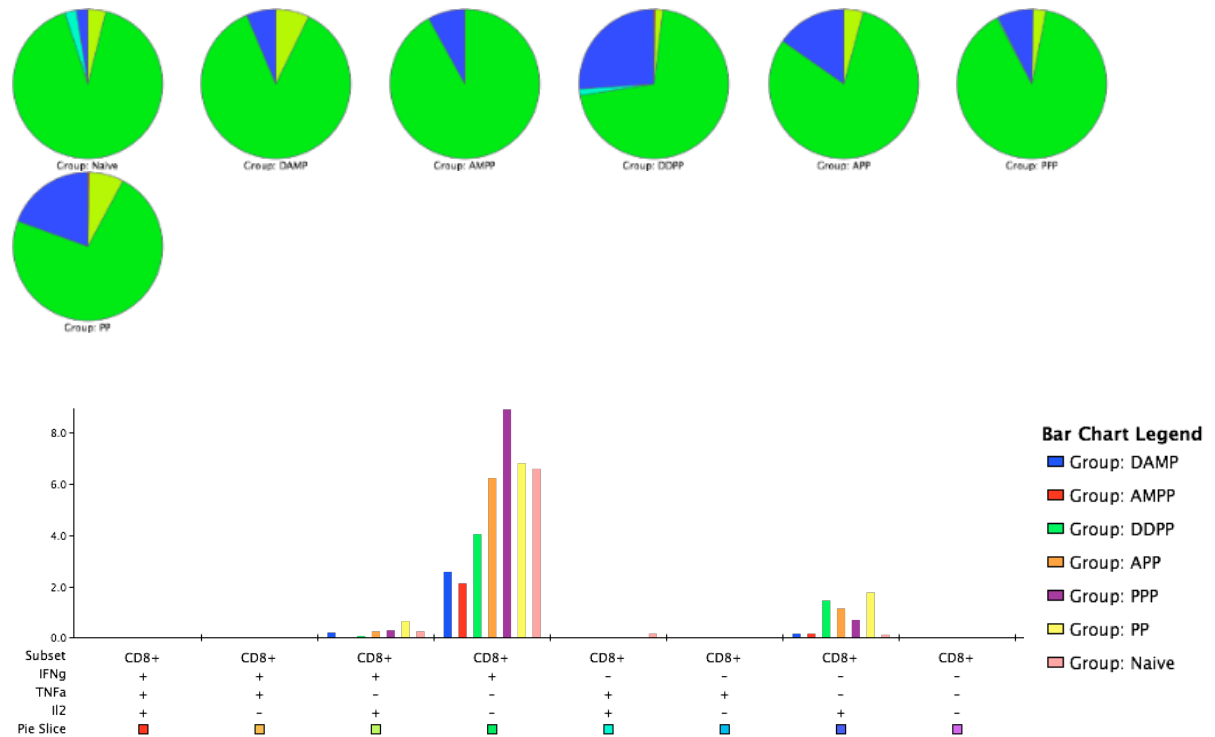


Figure 4.3. Cellular responses following heterologous prime-boost intramuscular vaccination.

Vaccinated BALB/c mice ($n = 8$ per group) were sacrificed one-week post-final immunisation. (A) Splenocytes were assessed by IFN- γ ELISpot for MOMP-reactive T cells using 15-mers overlapping by 11 amino acids. Data is expressed as group medians (SFU/million antigen stimulated cells (+ interquartile range)) * $p \leq 0.05$ by Kruskal-Wallis with Dunn's multiple comparison test. (B and C) MOMP-reactive splenocytes were assessed for polyfunctional cytokine responses of IFN- γ , TNF- α and IL-2. CD4+ (B) and CD8+ (C) T cell populations were gated and single, double or triple cytokine expressing cell percentage enumerated. The percentage cytokine expression of each BALB/c mouse was averaged within the group and expressed as a pie chart of relative proportions of the total cytokine expressing cell population.

induce 2.5% of the CD4+ T cells to produce TNF- α alone following MOMP peptide stimulation. The majority of CD8+ T cells specific to MOMP following peptide stimulation expressed IFN- γ alone, followed by IL-2 alone.

4.2.4 Antibody immunogenicity of *C. trachomatis* MOMP vaccines following mucosal application in prime-boost regimens

The *C. trachomatis* MOMP protein, formulated with the adjuvant monophosphoryl lipid A (MPLA), was administered intranasally into BALB/c mice as a mucosal route of delivery to assess whether enhanced MOMP-specific mucosal immune responses could be elicited. Mucosal immunisation has been previously shown to elicit mucosal immune responses, in particular specific IgA, to other antigens due to mucosal lymphoid linkage [240]. Immunisations were administered three weeks apart and serum and vaginal washes sampled two weeks post-final immunisation (Figure 4.4A). While the third protein intranasal immunisation did not induce significantly higher MOMP-specific serum IgG concentrations, the fourth protein intranasal inoculation significantly boosted MOMP-specific serum IgG concentrations over two (***, $p \leq 0.0005$) and three immunisations (**, $p \leq 0.005$). There was no MOMP-specific serum IgA present (Figure 4.4A).

Concentrations of MOMP-specific vaginal IgG were only detectable following three intranasal immunisations (mean = 23.5 ng/ml) (Figure 4.4B). Significantly greater concentrations (***, $p \leq 0.0005$, mean = 189.4 ng/ml) were induced after the fourth

intranasal immunisation. Concurrently, after four intranasal immunisations detectable concentrations of MOMP-specific vaginal IgA were induced indicating that antigen specific IgA was generated in the mucosal compartments even when absent from the serum (mean = 48.8 ng/ml).

HuAd5 and MVA vectored vaccines expressing the MOMP transgene were used in adenoviral prime-MVA boost regimens (**AM**). Both these viral vectored vaccines can infect through the mucosa, and thus were administered both intranasally (I.N.) and intravaginally (I.Vag.) and compared to intramuscular (I.M.) injections. Immunisations were administered three weeks apart and serum and vaginal washes sampled two weeks post-final immunisation (Figure 4.4C). I.M. AM (mean = 46.4 $\mu\text{g/ml}$) induced significantly greater MOMP-specific serum IgG concentrations than both I.Vag. AM (* $p \leq 0.05$, mean = 0.06 $\mu\text{g/ml}$) and I.N. AM (* $p \leq 0.05$, mean = 3.7 $\mu\text{g/ml}$). I.M. AM was the only route and regimen to induce vaginal MOMP-specific IgG (mean = 34.9 ng/ml) and the specific-IgG concentrations were significantly greater than I.N. AM (* $p \leq 0.05$) and I.Vag. AM (* $p \leq 0.05$). There was no MOMP-specific serum or vaginal IgA present.

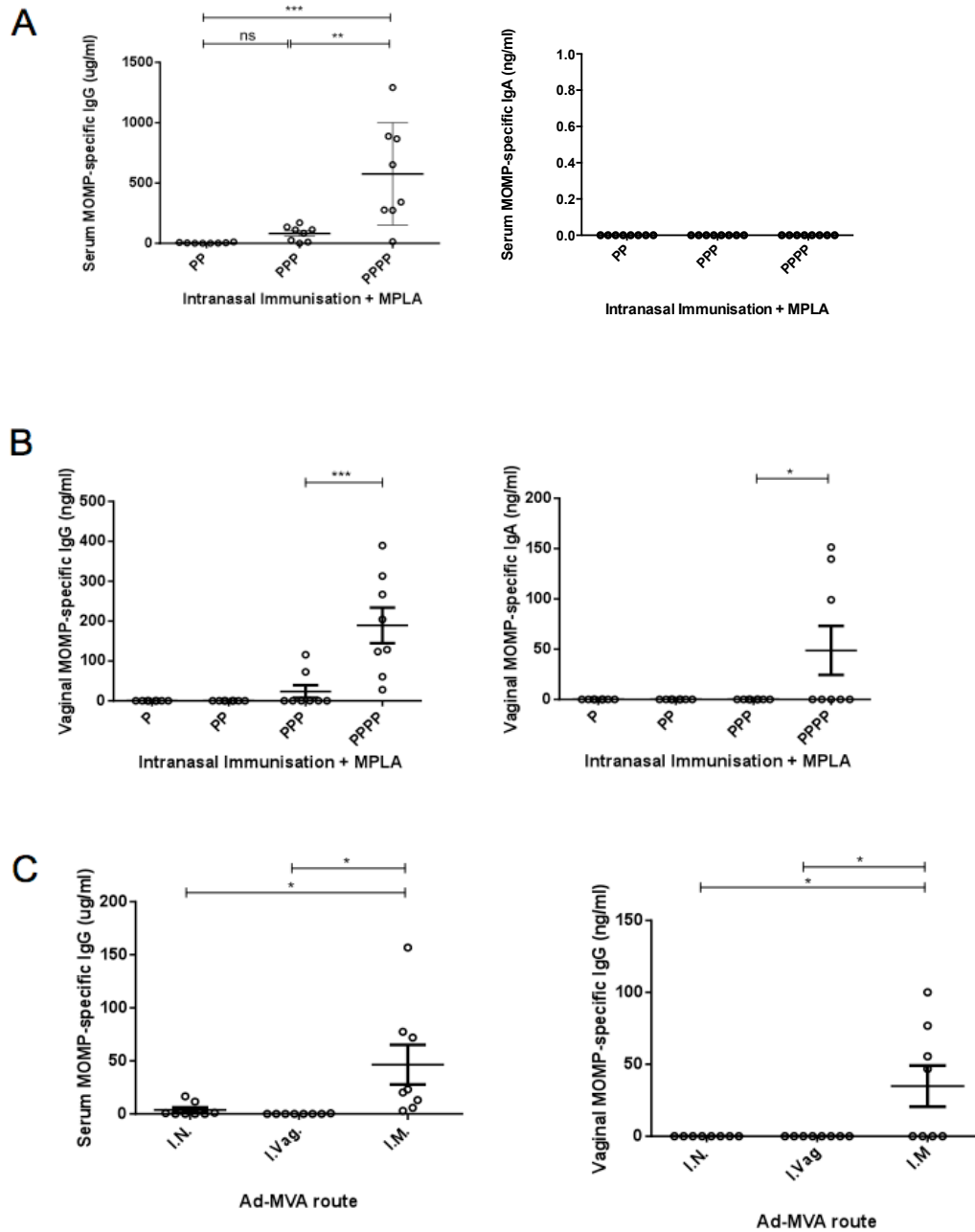


Figure 4.4. Antibody responses following prime-boost mucosal vaccination.

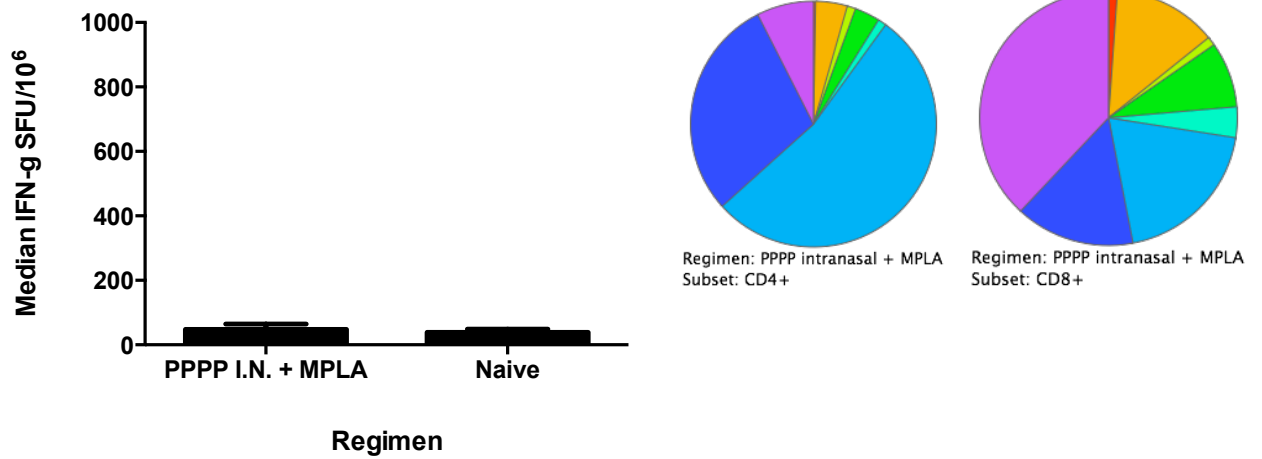
MOMP-specific IgG and IgA from serum (A) and specific IgG and IgA from vaginal washes (B) were assessed in BALB/c mice ($n = 8$ per group) two weeks after each MOMP+MPLA intranasal immunisation. MOMP-specific IgG from serum and vaginal washes (C) were assessed in BALB/c ($n = 8$ per group) two weeks after Adenoviral prime-MVA boost by the intranasal route, the intravaginal route or the intramuscular route. Antibody results are expressed as group means (+SEM) ($\mu\text{g/ml}$ or ng/ml). Statistical significance was assessed by one-way ANOVA with Bonferroni's multiple comparison post-test with $*p \leq 0.05$, $**p \leq 0.005$ and $***p \leq 0.0005$.

4.2.5 T cell immunogenicity of *C. trachomatis* MOMP vaccines following mucosal application in prime-boost regimens

T cell responses were assessed in BALB/c mice following intranasal PPPP (+ MPLA) immunisation one week after the final immunisation (Figure 4.5A). Splenocytes were re-stimulated with MOMP peptides as described previously. Low T cell IFN- γ responses were induced by intranasal PPPP (+ MPLA), with a median of 48 SFU/10⁶ splenocytes, compared to a median of 39 SFU/10⁶ splenocytes for unvaccinated naïve BALB/c. There was no statistical difference between intranasal PPPP (+ MPLA) and unvaccinated controls (Kruskal-Wallis test with Dunn's multiple comparison post-test). Intracellular cytokine staining was used to assess the polyfunctionality or cytokine response profile of CD4+ and CD8+ T cells from the intranasal PPPP (+ MPLA) vaccinated BALB/c mice. MOMP-specific CD4+ T cells were poorly polyfunctional, with 0.6% of MOMP-reactive CD4+ T cells expressing just TNF- α . MOMP-specific CD8+ were polyfunctional for IFN- γ and TNF- α , with 0.4% of the reactive CD8+ cells expressing both together.

T cell responses were also assessed in BALB/c mice following intranasal (I.N.), intravaginal (I.Vag.) and intramuscular (I.M.) adenoviral prime-MVA boost regimens (AM) one week after the final immunisation (Figure 4.5B). Splenocytes were re-stimulated with MOMP peptides as described previously. Low T cell IFN- γ responses were induced by both intranasal and intravaginal routes of immunisation, with a median of 82 and 176 SFU/10⁶ splenocytes respectively. The highest T cell IFN- γ responses were induced by intramuscular AM, with a median of 908 SFU/10⁶

A



B

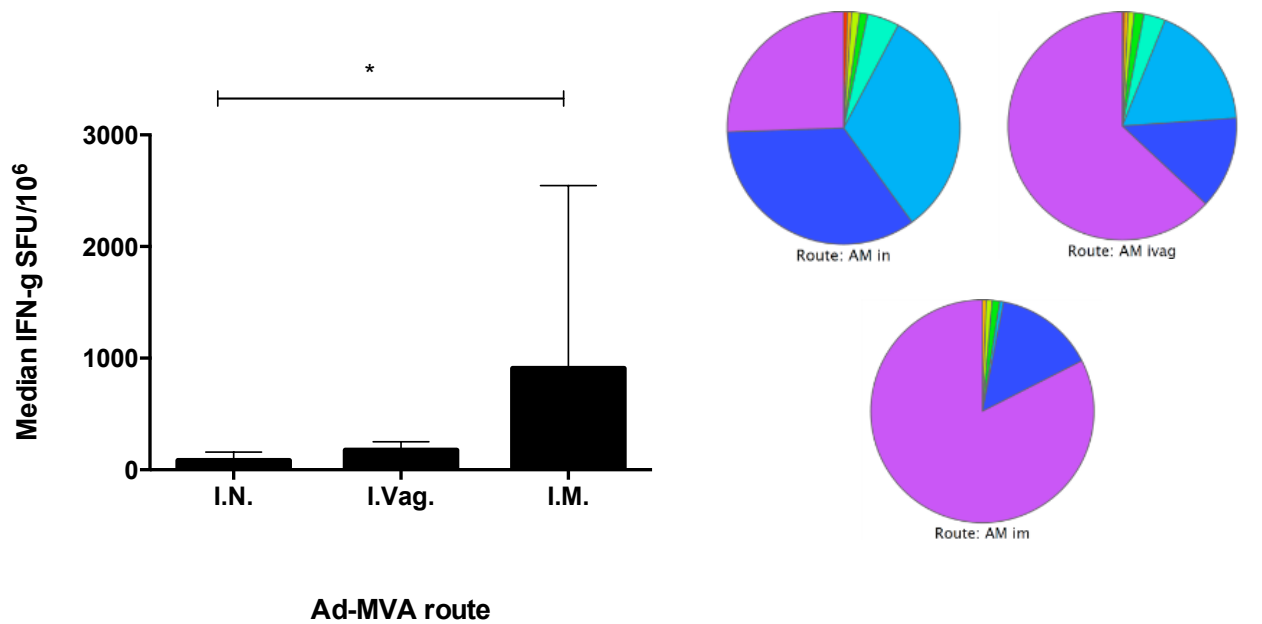


Figure 4.5. Cellular responses following prime-boost mucosal vaccination.

(A) BALB/c mice (n = 8 per group) after four MOMP+MPLA intranasal immunisations were sacrificed one week after the final immunisation. Splenocytes were assessed by IFN- γ ELISpot for MOMP-reactive T cells using 15-mers overlapping by 11 amino acid. Data is expressed as group means (SFU/million antigen stimulated cells (+ SEM)). MOMP-reactive splenocytes were assessed for

polyfunctional cytokine responses of IFN- γ , TNF- α and IL-2. CD4⁺ and CD8⁺ T cell populations were gated and single, double or triple cytokine expressing cell percentage enumerated. The percentage cytokine expression of the stimulated cells from each BALB/c mouse was averaged within the group and expressed as a pie chart of relative proportions of the total cytokine expressing cell population. (B) Identical procedures were carried out on BALB/c mice (n = 8 per group) after adenoviral prime-MVA boost immunisations via alternate immunisation routes including intranasal (I.N.), intravaginal (I.Vag), and intramuscular (I.M.). Pie charts only representing CD4⁺ T cell populations. Table shows cytokine profile provided for each colour represented in the polyfunctionality pie charts.

splenocytes. There was a statistical difference between the intranasal and intramuscular route of immunisation for adenoviral prime-MVA boost regimens (AM) in the induction of T cell IFN- γ responses (* $p \leq 0.05$, Kruskal-Wallis test with Dunn's multiple comparison post-test). Intracellular cytokine staining was used to assess the polyfunctionality or cytokine response profile of CD4+ and CD8+ T cells from the intranasally (I.N.), intravaginally (I.Vag.) and intramuscularly (I.M.) immunised adenoviral prime-MVA boost regimens (AM) in BALB/c mice. MOMP-specific CD4+ and CD8+ T cells were poorly polyfunctional irrespective of the route of immunisation for the AM regimens. CD4+ T cells were monofunctional for either TNF- α or IL-2 following intranasal and intravaginal immunisation, with CD8+ T cells being monofunctional for IFN- γ following intranasal and intravaginal immunisation.

The relative degrees of immunogenicity elicited following the heterologous prime-boost regimens are summarised in Table 4.2.

Regimen	Serum IgG	Vaginal IgG	Vaginal IgA	IFN- γ ELISpot	Polyfunctionality
DDDAM	+	+		++	ND
DDDA					
DDDM					
DDD				+++	
AM				+++	
DAMP	++	++			++
AMPP	+++	+++		++	++
DDPP	++	++			+++
APP	+++	+++			+
PPP	+++	+++			
PP	++	++			
PPPP + MPLA (I.N).	++	++	+		
AM (I.N.)					
AM (I.Vag.)					

Table 4.2. Regimen screening immune outcomes in BALB/c mice.

Relative strength of specific immunogenicities for each regimen represented by +. CD4+ polyfunctionality not determined (ND) for five of the vaccination regimens.

4.3 Discussion

There are only a few heterologous prime-boost regimens that have been tested as potential chlamydia vaccine candidates, with the majority of chlamydia vaccine research concentrating on homologous prime-boost strategies. One heterologous study examined the immune response induced by recombinant adenovirus expressing the chlamydia antigen CPAF (Chlamydia Protease-like Activity Factor) and recombinant CPAF subunit vaccines, adjuvanted with CpG and the immunomodulatory peptide HH2. The heterologous adenovirus prime and protein boost induced a Th1-biased cellular immune response, while the homologous CPAF subunit induced a more mixed Th1/Th17 profile. Despite these disparities in the regimen, both the heterologous and homologous prime-boost regimens conferred significant protection against genital *C. muridarum* challenge in BALB/c and C3H/HeN mice [153]. Another chlamydial heterologous prime-boost vaccination strategy involved DNA priming and protein boosting with the chlamydial protein CT043. This regimen resulted in a 2.6-log reduction in the IFU in a murine chlamydia lung infection model [241].

There have been previous attempts to use DNA vaccines against Chlamydia, though in contrast to our current study none of these have been administered with electroporation. In one study, intramuscular DNA immunisation with either the MOMP gene or the cytosine triphosphate synthetase (CTP) gene was investigated in the *C. trachomatis* mouse pneumonitis (MoPn) lung challenge model. Only the

MOMP DNA vaccine generated serum antibodies to MoPn EBs and reduced the peak growth of MoPn by more than 100-fold following lung challenge [242]. In 2010 and 2011, Schautteet *et al.*, investigated the protective capacity of a DNA vaccine based on *C. trachomatis* MOMP against genital *C. trachomatis* infection in their recently developed pig model. When administering the DNA vaccine to the vaginal mucosa, a cellular immune response was induced alongside significant protection in pigs, yet infection could not be cleared completely [155]. When the DNA vaccine was administered to both the nasal and vaginal mucosa of the pig, cellular and humoral immune responses were induced concurrently, and this immunity afforded significant protection to the pigs against a genital *C. trachomatis* infection [243].

A literature review revealed only one previous use of an adenovirus-vectored vaccine against Chlamydia in the mouse model. This involved priming with the recombinant adenovirus vector expressing CPAF prior to a recombinant protein boost, as described earlier [153]. Lu *et al.*, transfected dendritic cells with recombinant adenovirus expressing the *C. trachomatis* serovar E MOMP gene and observed an increased expression of CD80 and enhanced IL-12 secretion and the increased secretion of IFN- γ from stimulated T cells. When the Ad-MOMP transfected DCs were adoptively transferred intravenously into naive mice, they generated Th1-biased cytokines and mucosal IgA responses specific for *C. trachomatis*. Furthermore, following the adoptive transfer the mice mounted protection against genital tract challenge infection, with lower body mass loss, lower chlamydial loads and less severe pathology [244]. One other viral vector has been utilised, with a poliovirus used to express a MOMP-epitope. A poliovirus hybrid was

constructed from poliovirus type 1 Mahoney, expressing the variable domain I of MOMP of *C. trachomatis* serovar A, and rabbit antisera raised against this hybrid neutralised chlamydia infection *in vitro*. Moreover, this antisera when passively infused into cynomolgus monkeys protected against ocular infection, with no chlamydial culture from the conjunctivae and no clinical disease score post challenge [245]. To the best of our knowledge, neither the MVA viral vector nor any vaccinia-based vector has been used as a vaccine vector against Chlamydia.

Within our studies we investigated the avidity of the anti-MOMP antibodies raised following the differing prime-boost regimens. There can be more to antibody-mediated protection than the peak of vaccine-induced antibody titres, with the quality of such antibody responses, such as their avidity, identified as a potential determining factor of efficacy. For meningococcal C vaccines only high-avidity antibodies are bactericidal, and conjugate vaccines against *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) specifically induce an increase in antibody avidity [246] [247] [248]. Furthermore, low concentrations of passively infused high-avidity antibody can protect experimentally infected animals from these diseases [249] [250], with avidity likely playing a role in the antibody's neutralisation ability [251]. At the date of writing, no publically accessible publications have examined anti-MOMP polyclonal antibody avidities. Our data suggests that two protein boosts are required to push the antibody avidities into a medium to high category as assessed by the urea denaturation avidity ELISA assay, with protein-free regimens

inducing consistently low avidity anti-MOMP antibodies.

The polyfunctionality of the T cells following vaccination were investigated. Polyfunctional CD4⁺ T cells are a characteristic of HIV-controllers [252], and in mouse models, vaccine-induced polyfunctional CD4⁺ T cells correlate with protection against *Leishmania major* infection [253]. Furthermore, human studies have shown that triple positive T cells express the highest levels of cytokines per cell, while single positive T cells express the lowest [254] [255]. Therefore polyfunctional T cells may be superior both in terms of establishing immunological memory as well as in producing high amounts of cytokines [256]. T cell polyfunctionality has only been analysed for one chlamydia vaccine to date. Following subcutaneous immunisation with the chlamydia proteins CT144 and CT823 with the adjuvant AbISCO-100, the CD4⁺ polyfunctionality for IL-10, TNF- α and IL-2 was investigated [257]. T cells from the spleen and mediastinal lymph nodes (MdLN) of mice that had been vaccinated with MOMP co-formulated with differing adjuvants and administered by differing routes were harvested and restimulated with recombinant MOMP antigen. The bulk production of IFN- γ , TNF- α , IL-4, IL-10 and IL-17 from these stimulation cultures was assessed using a Luminex-type assay but the polyfunctionality of individual T cells was not investigated [258].

From our results it is apparent that DNA priming induces the greater CD4⁺ T cell polyfunctionality, with HuAd5 priming also affording some increase in polyfunctional cytokine cellular responses. The observation that DNA priming enhances the

polyfunctionality of antigen-reactive T cells has been demonstrated in other vaccine studies [157]. The PP and PPP regimen, however, induced no polyfunctionality of the CD4+ T cells. For HIV-1, a DNA plus NYVAC vaccine regimen induced T cell responses in over 90% of vaccinees, with only 33% responding to the NYVAC alone regimen. These T cell responses from DNA priming were of a polyfunctional cytokine phenotype for both CD4+ and CD8+ T cells, and durable for up to 72 weeks [259]. For hepatitis C, it was a DNA prime and MVA boost regimen that was capable of activating quadrifunctional T cells (IFN- γ /TNF- α /CD107a/IL-2) in mice, and showed protection in a *Listeria*-NS3-1a challenge model [260].

Another potential correlate of protection that we analysed following the heterologous prime boost regimen was the MOMP-specific IgG isotypes, in particular IgG2a and IgG1. An IgG2a:IgG1 ratio of >1 is associated with a Th1 response, while a ratio <1 is associated with a Th2 response. A significantly greater IgG2a:IgG1 ratio was induced following the DAMP regimen, with a mean ratio of 14. Potential roles of antibody isotypes are discussed in Chapter 5.

We obtained our highest serum and vaginal anti-MOMP IgG concentrations via the PPP regimen, with the recombinant protein adjuvanted with MF59. This regimen induced 1.27 mg/ml serum anti-MOMP IgG and 2.13 μ g/ml vaginal anti-MOMP IgG. As a comparison, a native preparation of *C. trachomatis* MoPn adjuvanted with CpG and Montanide[®] induced a serum specific IgG titre of 409,600 and a vaginal wash IgG

titre of 160 in BALB/c mice [261], while MOMP adjuvanted with cholera toxin and CpG by the transcutaneous route elicited a serum anti-MOMP IgG titre of 400,000 [258]. It is difficult to directly compare values, with our antibody quantification being the more current semi-quantitative assay in contrast to the end-point titres, which are necessarily assessed at the non-linear part of a dilution curve. However, for the HIV-1 antigen gp140, antibody concentrations of 1 µg/ml are equivalent to an end point titre of approximately 10,000 (Dr. McKay, personal communication). This would suggest the antibody levels induced in our studies being much higher than those of these previous studies.

We obtained our highest IFN-γ ELISpot SFU from the splenocytes of BALB/c mice vaccinated with the DDD and AM regimens. The DDD regimen had a median SFU of 504, and AM with a median IFN-γ SFU of 503 /10⁶ cells. Hansen *et al.*, induced 483 IFN-γ SFU/10⁶ cells from their liposomal delivery of MOMP with the adjuvant CAF01 [262], whereas four MOMP MoPn DNA vaccinations in BALB/c mice induced 150 EB-specific IFN-γ SFU/10⁶ splenocytes [263]. Alternative chlamydial antigens have afforded greater IFN-γ ELISpot responses. The PmpG antigen adjuvanted with AbISCO® resulted in over 1000 IFN-γ secreting splenocytes per million cells [264], while CT144 also adjuvanted with AbISCO® induced CT144 specific responses of ~5000 IFN-γ SFU/million CD4+ T cells [257].

It required four intranasal protein immunisations, adjuvanted with MPLA, to induce

mean MOMP-specific vaginal IgA concentrations of 49 ng/ml. None of the intramuscular routes of immunisation nor the mucosal application of HuAd5 or MVA were capable of inducing MOMP-specific vaginal IgA in our studies. Singh *et al.* were capable of inducing MOMP-specific IgA systemically and vaginally by administering the recombinant MOMP protein intranasally with the mucosal adjuvant cholera toxin [265]. Furthermore, *Vibrio cholera* ghosts expressing MOMP and the HSV-2 glycoprotein D were capable of eliciting secretory IgA to both antigens in serum and vaginal secretions following intramuscular immunisation [266]. These studies reveal other ways to elicit antigen-specific IgA, and if greater concentrations could be generated then it could be used to determine whether MOMP-specific mucosal IgA was a vaccine immune correlate of protection.

The AM regimen via mucosal surfaces was a poor inducer of MOMP-specific responses. Neither the AM regimen via the intranasal nor the intravaginal route induced antigen-specific humoral or cellular responses. There are no publications in which the AM regimen is exclusively used on mucosal surfaces, often instead with the adenoviral prime being intramuscular [267]. However, BALB/c mice intranasally immunised with just one shot of rAd expressing the G protein of RSV induced strong serum antibodies [268], and an MVA expressing HIV-1 Env inoculated intranasally induced significant immune responses to the HIV-1 antigen. However, intravaginal inoculation of the MVA-Env was not immunogenic [269]. These successful uses of mucosal immunisations by viral vectors suggests that the poor immunogenicity we

observed may be a MOMP-specific issue, or potentially due to the timing between prime-boost immunisations. Reyes-Sandoval *et al.* observed polyfunctional T cells with strong IFN- γ production following an AM regimen for malarial antigens, with 8 weeks between the prime and boost as opposed to our 3-week interval [91]. This timing may be important for T cell contraction and maturation of the immune response.

We set out to comparatively assess the induction of humoral and cellular immunity against MOMP following heterologous prime-boost vaccinations with DNA, Adenovirus, MVA and recombinant protein based vaccines. Taken all together, this chapter revealed a capability to induce an array of MOMP-specific immune responses, both cellular and humoral, using the four differing MOMP-based vaccines in heterologous prime-boost regimens. Protein boosting significantly increased both serum and vaginal antibody concentrations, while DNA and viral vector alone regimens induced the greatest cellular responses as analysed by IFN- γ ELISpot. Protein boosts increased the avidity of the induced specific-serum antibodies, while CD4+ T cell polyfunctionality was broadest following regimens with DNA or HuAd5 prime. The regimens will be grouped according to the distinct MOMP-specific immune environments they elicit, and in the subsequent chapters taken through into *C. trachomatis* vaginal challenge studies to shed light on the relative contribution of each environment to protective immunity.

Chapter 5

**CD4-dependent protection against *Chlamydia trachomatis*
following heterologous vector prime-protein boost
vaccination in B6C3F1 and BALB/c mice**

5 CD4-dependent protection against *Chlamydia trachomatis* following heterologous vector prime-protein boost vaccination in B6C3F1 and BALB/c mice

5.1 Introduction

Mice are the most commonly used animals to study genital chlamydial infections as the female mouse genital tract is susceptible to infection with both *Chlamydia muridarum* [120] and *Chlamydia trachomatis* [121] strains resulting in the establishment of two murine models; the *C. trachomatis* mouse model and the *C. muridarum* mouse model.

C. muridarum (also termed *C. trachomatis* MoPn) is a natural mouse pathogen that causes pneumonitis and was originally isolated from the lungs of mice [270]. Intravaginal inoculation of *C. muridarum* in mice results in a genital tract infection that closely resembles acute genital *C. trachomatis* infections in women [120]. Mice generally resolve the genital tract infection within 4 weeks, after the infection has ascended to the uterine horns and oviducts and resulted in hydrosalpinx.

Mice can also be genitally infected with human *C. trachomatis* serovars [121]. Intravaginal inoculation with *C. trachomatis* typically produces a mild genital tract infection that resolves relatively quickly and is mostly unable to ascend to the upper genital tract. Only when high doses of *C. trachomatis* are inoculated directly into the uterus or ovarian bursa can *C. trachomatis* infections cause hydrosalpinx or infertility

in the mouse model [124] [271] [272]. Infection by vaginal inoculation normally resolves without complications [273] [274] [275]. Lyons *et al.* argue that the infection of mice with *C. trachomatis* mimics in many ways both the course and outcome of infection in most women – an asymptomatic and self-limiting infection that only rarely results in severe upper genital tract disease [128]. This was a strong rationale for us using the human *C. trachomatis* strains in our murine genital challenge studies.

Genital tract infection of mice with *C. trachomatis* is dependent on the mouse strain used, with C3H mice being more susceptible to infection than other strains. A genital *C. trachomatis* serovar E infection has a significantly longer duration of infection in C3H/HeN mice than in C57BL/6 [273]. C57BL/6, BALB/c and CF-1 mice infected with *C. trachomatis* serovar D resulted in BALB/c mice having significantly shorter durations of infection than either C57BL/6 or CF-1. However, C57BL/6 and CF-1 mice shed significantly less chlamydia than BALB/c mice during the first 2 weeks of infection [108].

We employed two differing mouse strains for our challenge studies: BALB/c and B6C3F1 (female C57BL/6 and male C3H/HeN cross). BALB/c mice have been used in *C. trachomatis* recombinant protein vaccine studies by the Infectious Disease Research Institute (Seattle, USA) and GlaxoSmithKline Biologicals (Seattle, USA) [276]. B6C3F1 mice have recently been adopted by the Statens Serum Institute (Copenhagen, Denmark) for genital *C. trachomatis* vaccine antigen screening. B6C3F1 mice, being a cross between C57BL/6 and C3H mice, provide a balanced Th-profile, unlike BALB/c

mice that have a Th2-skewing background.

In this chapter we aim to assess the protective capacity of MOMP-based heterologous prime-boost regimens against vaginal *C. trachomatis* infection in two differing mouse models and to investigate the mechanism of the vaccine induced *C. trachomatis* protection. Following vaccination and challenge, any protective immunity observed will be investigated further to look at the relative roles of each arm of the immune system. The contribution of CD4+ T cells will be investigated as they have been previously implicated in partially protective chlamydia vaccines and have been shown to confer protection to secondary chlamydial infections [262] [105] [117]. A role of CD8+ T cells in murine genital chlamydial immunity has not been convincingly shown to date, with CD8+ T cell depletions having no significant effect on bacterial loads during primary nor secondary infections [277] [278]. In assessing the potential humoral contribution to protection, the role of antibody will be investigated through serum transfer and their neutralisation ability will be explored.

Aim

- To reveal a MOMP-based vaccination regimen which protects against intravaginal *C. trachomatis* infection in mice.
- To reveal the mechanism by which the MOMP-based vaccination regimen confers intravaginal *C. trachomatis* protection.

Objective

- Assess the protective capacity of MOMP-based heterologous prime-boost regimens against vaginal *C. trachomatis* infection in BALB/c and B6C3F1 mouse strains by measuring the vaginal chlamydial load following heterologous prime-boost MOMP-based vaccination regimens.
- Assess the role of CD4+ T cells in the vaccine-induced protection by depleting them in vaccinated mice, prior to intravaginal *C. trachomatis* infection.
- Assess the role of MOMP-specific antibodies in the vaccine-induced protection by passively infusing serum from vaccinated mice into naïve mice, prior to intravaginal *C. trachomatis* infection.
- Assess the role of MOMP-specific antibodies from the protective vaccine regimen in an IFN- γ -deficient environment by passively infusing serum from vaccinated mice into naïve IFN- γ -receptor knock-out mice, prior to intravaginal *C. trachomatis* infection.
- Assess the role of antibody-mediated neutralisation in the protection conferred by the heterologous prime-boost regimen(s) by performing neutralisation assays from the sera of differentially vaccinated mice.

5.2 Results

5.2.1 *C. trachomatis* serovar D shedding profiles following primary vaginal infection of BALB/c and B6C3F1 mice

Two mouse strains used for the screening of *C. trachomatis* vaccines include BALB/c (IDRI, GSK) and B6C3F1 (SSI, ADITEC). Therefore these two mouse strains were vaginally infected with *C. trachomatis* to investigate any differences in the shedding profiles between the two mouse strains (Figure 5.1). The intravaginal *C. trachomatis* challenge, infectivity assay and titering protocols are detailed in Chapters 2.5.5, 2.5.6 and 2.5.4 respectively.

Mice were infected with 4×10^5 IFU (Dr. Frank Follmann, personal communication) at day 0. At day 3 post challenge, BALB/c and B6C3F1 mice had a median of 2381 and 6500 IFU per swab respectively. With sampling every 3 days for 15 days, the chlamydial load continued to decrease. At the final sampling time point of 15 days post challenge, BALB/c and B6C3F1 mice had a median of 25 and 13 IFU per swab respectively. For each sampling time point there was large variation in the Chlamydial shedding IFUs, resulting in no statistical difference in the chlamydial loads between BALB/c and B6C3F1 mice at any time point (2-tailed Mann-Whitney U test).

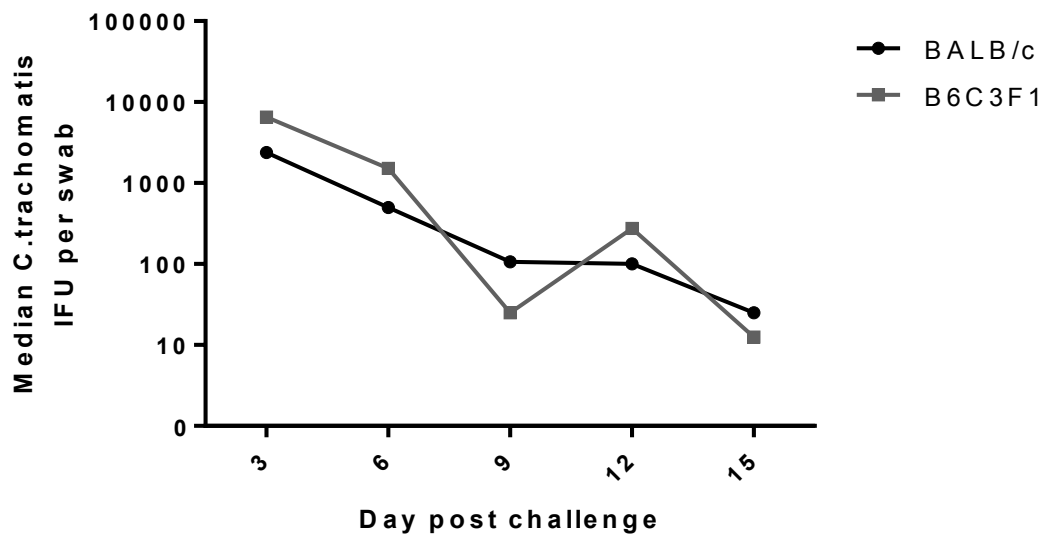


Figure 5.1. *Chlamydia trachomatis* shedding profiles following primary infection in BALB/c and B6C3F1 mice.

Groups of mice (N=5) were intravaginally infected with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx, 7 days following 2 mg/mouse subcutaneous Depo-Provera treatment. Vaginal bacterial loads were sampled post infection and median vaginal bacterial loads compared. * P<0.05, ** P<0.01; *** P<0.001, 2-tailed Mann-Whitney U test.

5.2.2 Intramuscular heterologous prime-boost *C. trachomatis* MOMP vaccine regimens elicit different immune responses in BALB/c and B6C3F1 mice

The *C. trachomatis* antigen MOMP was assessed in B6C3F1 mice immunised in heterologous prime-boost regimens (Table 5.1) with DNA, HuAd5, MVA and protein (+ MF59) based vaccines and compared against their immunogenicity in BALB/c mice (Figure 5.2). Immunisations were administered three weeks apart and serum and vaginal washes sampled two weeks post-final immunisation. The DAMP regimen induced significantly higher MOMP-specific serum IgG concentrations in B6C3F1 mice than in BALB/c mice (***) $p \leq 0.0005$, 2-way ANOVA with Bonferroni's multiple comparisons test), with respective means of 1796 $\mu\text{g/ml}$ and 258 $\mu\text{g/ml}$. AMPP also induced significantly higher MOMP-specific serum IgG concentrations in B6C3F1 mice than in BALB/c (* $p \leq 0.05$, 2-way ANOVA with Bonferroni's multiple comparisons test) with respective means of 1733 $\mu\text{g/ml}$ and 741 $\mu\text{g/ml}$. The regimens AM, DDD, PPP and naïve controls did not significantly differ in their mean MOMP-specific serum IgG concentrations between B6C3F1 and BALB/c mice.

There were no statistically significant differences in MOMP-specific vaginal IgG concentrations between B6C3F1 and BALB/c mice for any of the regimen investigated (2-way ANOVA with Bonferroni's multiple comparisons test).

There were no statistically significant differences in the IgG2a:IgG1 ratios between the B6C3F1 and BALB/c mouse strains for the screen regimens. But like BALB/c mice,

Regimen	Characteristic
DAMP	Highest MOMP-specific IgG2a serum concentration Lowest MOMP-specific IgG1 serum concentration
AMPP	↑ Cellular ↑ Humoral
AM	↑ Cellular ↓ Humoral
PPP	↓ Cellular ↑ Humoral
DDD	↑ Cellular ↓ Humoral
Naive	Non-intervention control

Table 5.1. Challenge Regimen

The prime-boost regimens chosen for challenge experiments in BALB/c and B6C3F1 mice due to the differing MOMP-specific immune environments they induce.

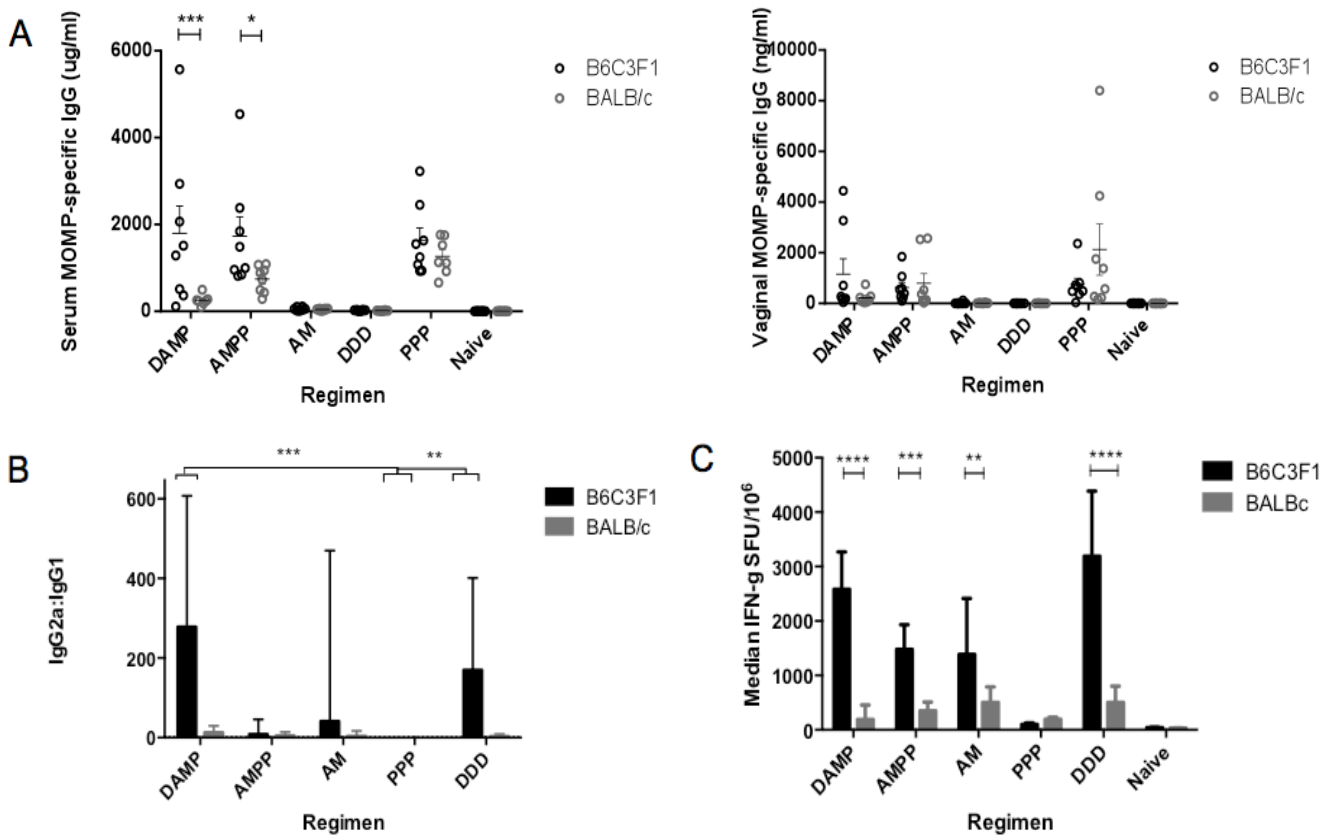


Figure 5.2. Prime-boost vaccine immunogenicity variation between BALB/c and B6C3F1 mice.

MOMP-specific IgG from serum and vaginal washes were assessed two weeks after prime-boost regimens in both BALB/c (n = 8) and B6C3F1 (n = 8) mice (A). Antibody results are expressed as group means (+SEM) ($\mu\text{g/ml}$ or ng/ml). Serum MOMP-specific IgG2a and IgG1 titres were measured two weeks after prime-boost regimens in both BALB/c (n = 8) and B6C3F1 (n = 8) mice and the IgG2a:IgG1 ratio represented as group medians (B). Vaccinated BALB/c (n = 8 per group) and B6C3F1 (n = 8 per group) were sacrificed one week post final immunisation and splenocytes assessed by IFN- γ ELISpot for MOMP-reactive T cells using 15-mers overlapping by 11 amino acid (C). Data is expressed as group medians [SFU/million antigen stimulated cells (+ interquartile range)]. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ and **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni's multiple comparison post-test for (A) and (C). ** $p \leq 0.005$ and *** $p \leq 0.0005$ by Kruskal-Wallis with Dunn's multiple comparison test for (B).

there are significant differences in the IgG2a:IgG1 ratio for the differing regimen in B6C3F1 mice. The DAMP (***) $p \leq 0.0005$) and DDD (** $p \leq 0.005$) regimen induced significantly higher IgG2a:IgG1 ratios than the PPP regimen (Kruskal-Wallis test, Dunn's multiple comparisons test).

T cell responses, analysed 1 week after the final immunisation, were also assessed in B6C3F1 mice following heterologous prime-boost vaccine regimens with DNA, HuAd5, MVA and protein (+ MF59) based vaccines and compared against BALB/c mice (Figure 5.2C). B6C3F1 splenocytes were re-stimulated with MOMP peptides as described previously. T cell IFN- γ responses were induced by all prime-boost regimens, with the DAMP and DDD regimens inducing the strongest T cell responses, with a median of 2580 and 3190 SFU/10⁶ splenocytes respectively. The T cell responses induced by the DAMP (**** $p \leq 0.0001$), DDD (**** $p \leq 0.0001$), AMPP (***) $p \leq 0.0005$), and AM (** $p \leq 0.005$) regimens were significantly higher in B6C3F1 mice than in BALB/c mice (2-way ANOVA with Bonferroni's multiple comparisons test).

5.2.3 *C. trachomatis* protective heterologous prime-boost vaccine regimens in B6C3F1 mice.

Only two of the five investigated regimens provided some protection against vaginal *C. trachomatis* infection in B6C3F1 mice (Figure 5.3). The DAMP regimen significantly reduced chlamydial IFU per swab at day 3 post challenge (median = 6531 IFU/swab) compared to unvaccinated controls (median = 34788 IFU/swab) (* $p = 0.0303$, 2-tailed Mann-Whitney test). The PPP regimen also significantly reduced chlamydial IFU per swab at day 3 post challenge (median = 8095 IFU/swab) compared to unvaccinated controls (median = 34788 IFU/swab) (* $p = 0.0451$, 2-tailed Mann-Whitney test). There was no statistical difference at the later time points of 7, 10 and 14 days post challenge due to the resolution of infection by the unvaccinated naïve controls. The DDD, AM, and AMPP regimens did not significantly reduce chlamydial shedding at any time points sampled post challenge in B6C3F1 mice.

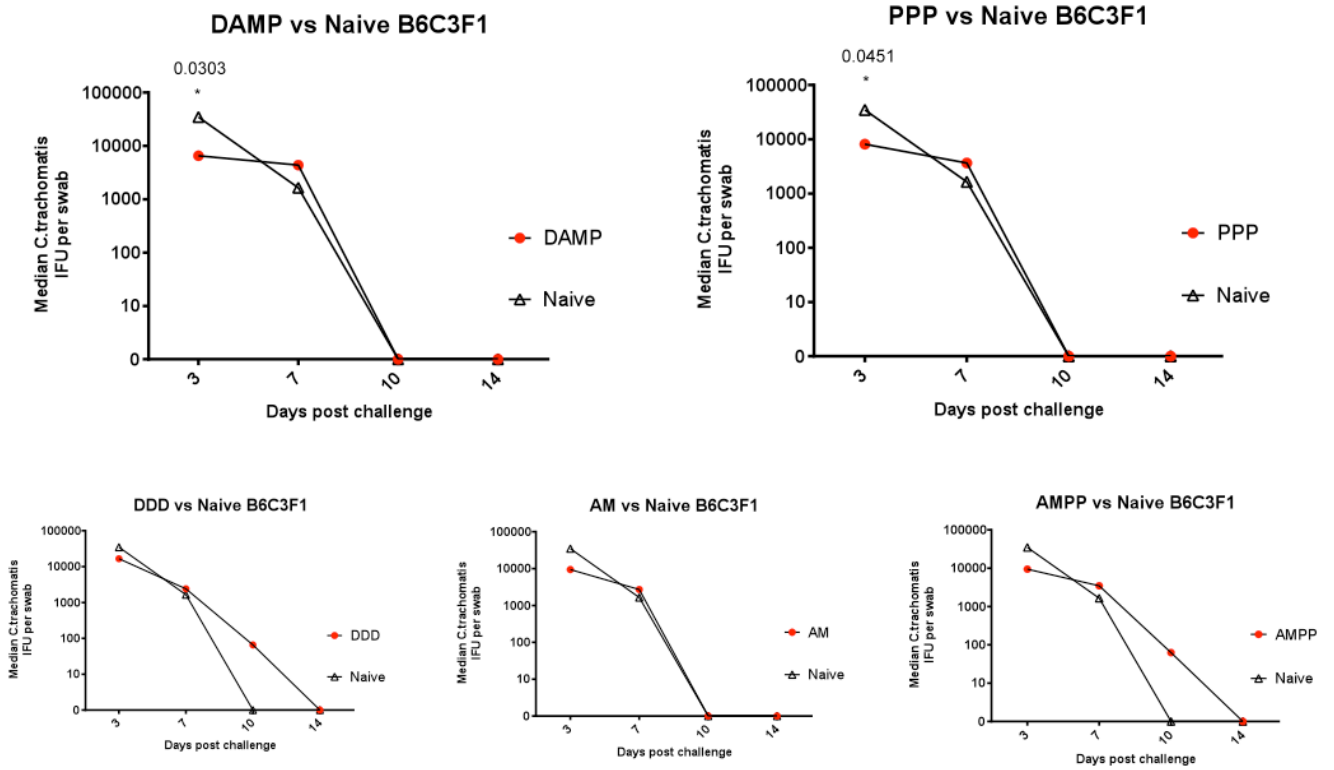


Figure 5.3. Prime-boost regimens *C. trachomatis* protective capacity in B6C3F1 mice.

Six weeks after the final vaccination and 1 week after 2 mg/mouse subcutaneous Depo-Provera treatment, B6C3F1 mice ($n = 5$ to 8) were infected intravaginally with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx. The vaginal vault of each mouse was sampled using individual swabs at 3, 7, 10 and 14 days post challenge, and vaginal Chlamydial loads quantified by infection assay and immunofluorescent microscopy. Median values are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, 2-tailed Mann-Whitney U test.

5.2.4 Percentage of B6C3F1 mice shedding following heterologous prime-boost vaccine regimens

At day 3, 7, 10 and 14 post challenge, the number of B6C3F1 mice shedding *C. trachomatis* was assessed via the infectivity based assay from the vaginal swabs (Figure 5.4). All naïve mice were shedding up to day 7, before a reduction to only 20% shedding at day 10 and day 14. None of the assessed vaccination regimens had a statistically significant difference in the shedding curves compared to the naïve, unvaccinated mice (by either Log-rank (Mantel-Cox) test or Gehan-Breslow-Wilcoxon test). Though not significant, at day 10 post challenge only 20% of naïve B6C3F1 mice were shedding while 71.4% of AMPP vaccinated mice were still shedding.

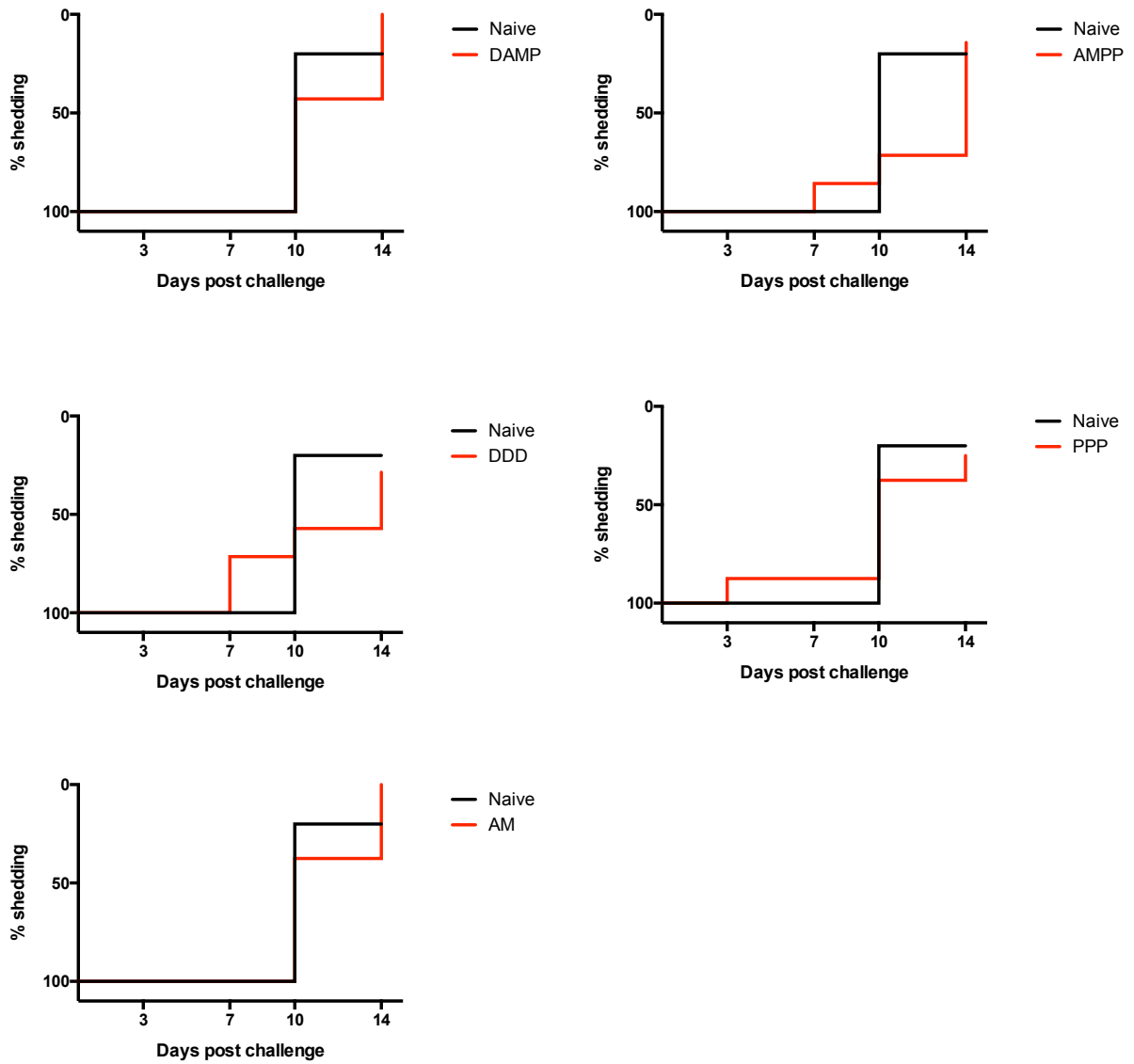


Figure 5.4. Percentage of B6C3F1 mice shedding *C. trachomatis* following vaccination with prime-boost regimens.

Six weeks after the final vaccination and 1 week after 2 mg/mouse subcutaneous Depo-Provera treatment, B6C3F1 mice (n = 5 to 8) were infected intravaginally with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx. The vaginal vault of each mouse was sampled using individual swabs at 3, 7, 10 and 14 days post challenge, and vaginal Chlamydial loads quantified by infection assay and immunofluorescent microscopy. Mice are designated non-shedding when below the limit of detection (6 IFU/swab). Shedding curve differences were analysed by both the Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test.

5.2.5 Assay used to measure *C. trachomatis* shedding: Droplet digital PCR (ddPCR) compared to infectivity assay

Vaginal swabs from days 7 and 14 post challenge were analysed by ddPCR as a comparison to the infectivity based assay from which the shedding data is calculated (Figure 5.5). The ddPCR assay is detailed in Chapter 2.2.13. ddPCR measures exact copies of chlamydial genes (*omcB* in this case) and is independent of the infectivity of the shed bacterium. ddPCR was carried out to investigate whether chlamydial loads remained high even though infectivity diminished as revealed by the infectivity assay. *OmcB* copies followed the same trend as the infectivity assay. At day 7 post challenge, the median copies of *omcB* per swab for the regimens DAMP, PPP and unvaccinated controls were 17094, 12800, and 5272 respectively. At day 14 post challenge, the median copies of *omcB* per swab for the regimens DAMP, PPP and unvaccinated controls were 6, 6 and 6 respectively (lowest limit of detection of the assay). This similar trend between ddPCR chlamydial copies and the chlamydial infectivity assay reveals there is likely to be little immune pressure driving the Chlamydia into a non-infectious latent state, either following vaccination or not.

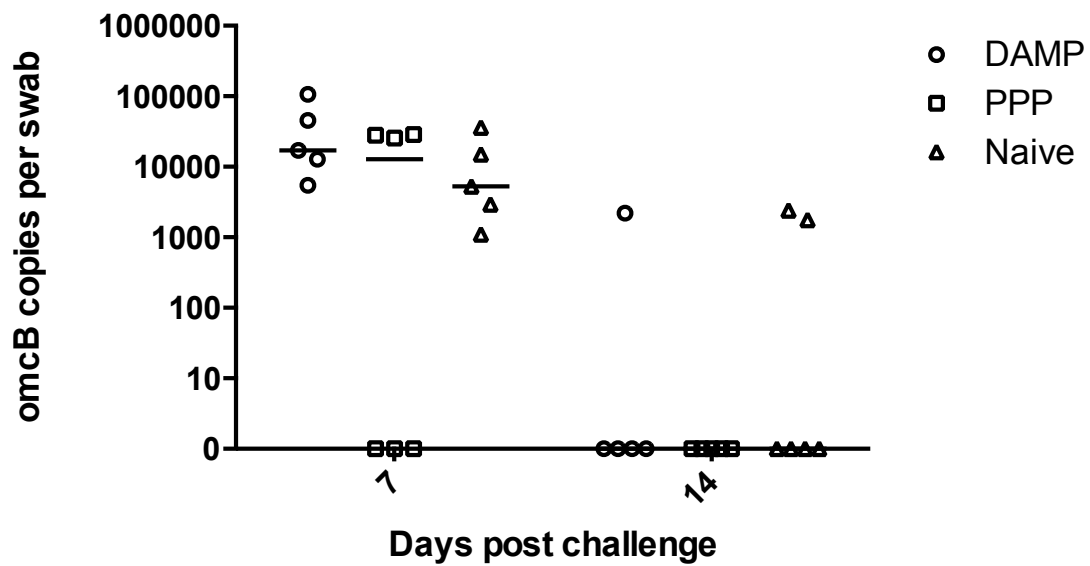


Figure 5.5. Vaginal *C. trachomatis* load post vaccination as determined by droplet digital PCR (ddPCR).

Vaginal samples day 7 and 14 post challenge from B6C3F1 mice (n = 5 to 6 per group) with prior vaccination with the DAMP and PPP regimens alongside unvaccinated controls (naïve) were analysed by ddPCR. Median values are indicated.

5.2.6 *C. trachomatis* protective heterologous prime-boost vaccine regimens in BALB/c mice

Only two of the five investigated regimens provided protection to vaginal *C. trachomatis* infection in BALB/c mice (Figure 5.6). The DAMP regimen significantly reduced chlamydial IFU per swab at day 3 post challenge (median = 354 IFU/swab) compared to unvaccinated controls (median = 22688 IFU/swab) (* $p = 0.0359$, 2-tailed Mann-Whitney test). The AMPP regimen also significantly reduced chlamydial IFU per swab at day 3 post challenge (median = 2563 IFU/swab) compared to unvaccinated controls (median = 22688 IFU/swab) (* $p = 0.0447$, 2-tailed Mann-Whitney test). There was no statistical difference at the later time points of 7, 10 and 14 days post challenge due to the resolution of infection by the unvaccinated naïve controls. The DDD, AM, and PPP regimens did not significantly reduce chlamydial shedding at any time points sampled post challenge in BALB/c mice.

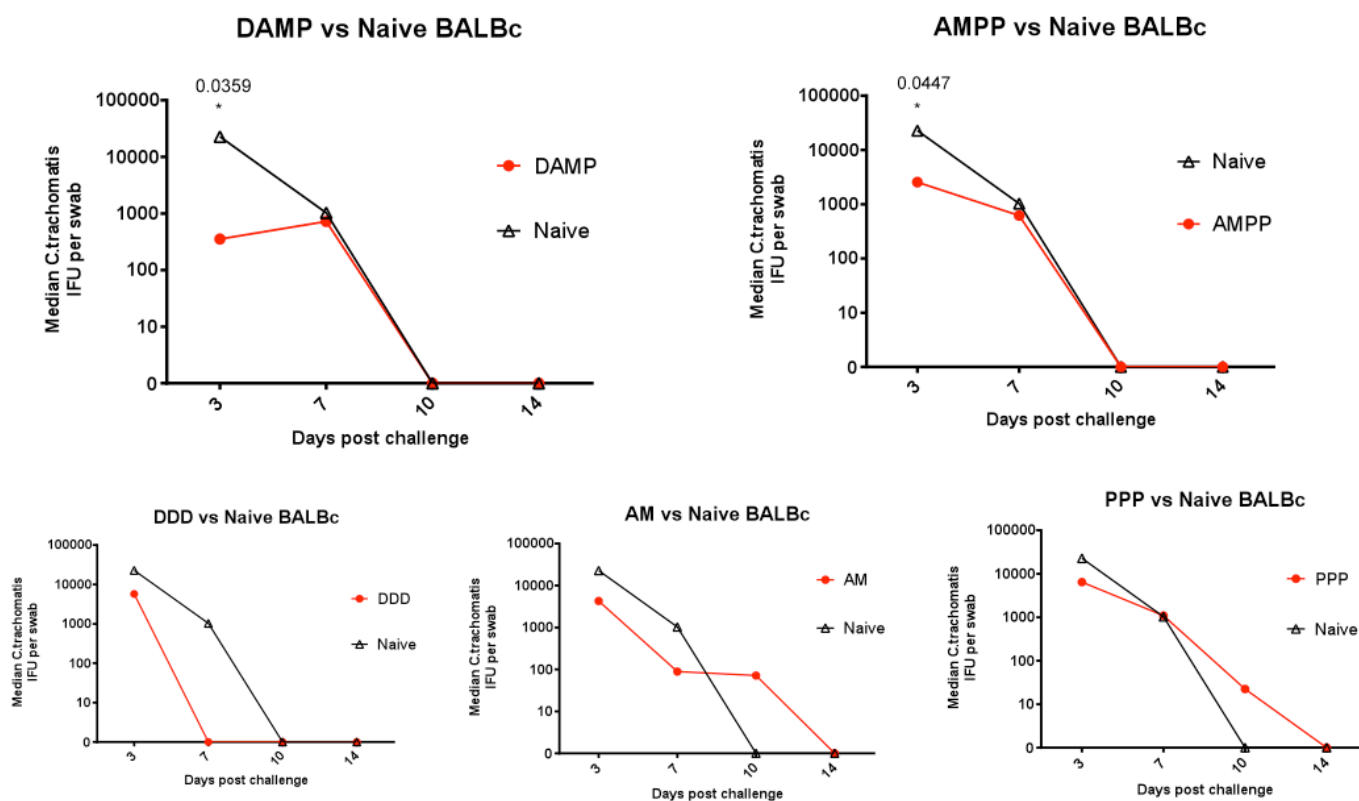


Figure 5.6. Prime-boost vaccine regimens *C. trachomatis* protective capacity in BALB/c mice.

Six weeks after the final vaccination and 1 week after 2 mg/mouse subcutaneous Depo-Provera treatment, BALB/c mice ($n = 7$ to 10) were infected intravaginally with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx. The vaginal vault of each mouse was sampled using individual swabs at 3, 7, 10 and 14 days post challenge, and vaginal Chlamydial loads quantified by infection assay and immunoflorescent microscopy. Median values are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, 2-tailed Mann-Whitney U test.

5.2.7 Percentage of BALB/c mice shedding following heterologous prime-boost vaccine regimens

At day 3, 7, 10 and 14 post challenge, the number of BALB/c mice shedding *C. trachomatis* was assessed via the infectivity based assay from the vaginal swabs (Figure 5.7). 80% of naïve unvaccinated mice were shedding at day 7, with a reduction to only 30% shedding at day 10. None of the assessed vaccination regimens had a statistically significant difference in the shedding curves compared to the naïve, unvaccinated mice (by either Log-rank (Mantel-Cox) test or Gehan-Breslow-Wilcoxon test). Though not significant, only 40% of DDD vaccinated BALB/c mice were shedding at day 7 post challenge while 80% of unvaccinated mice were still shedding.

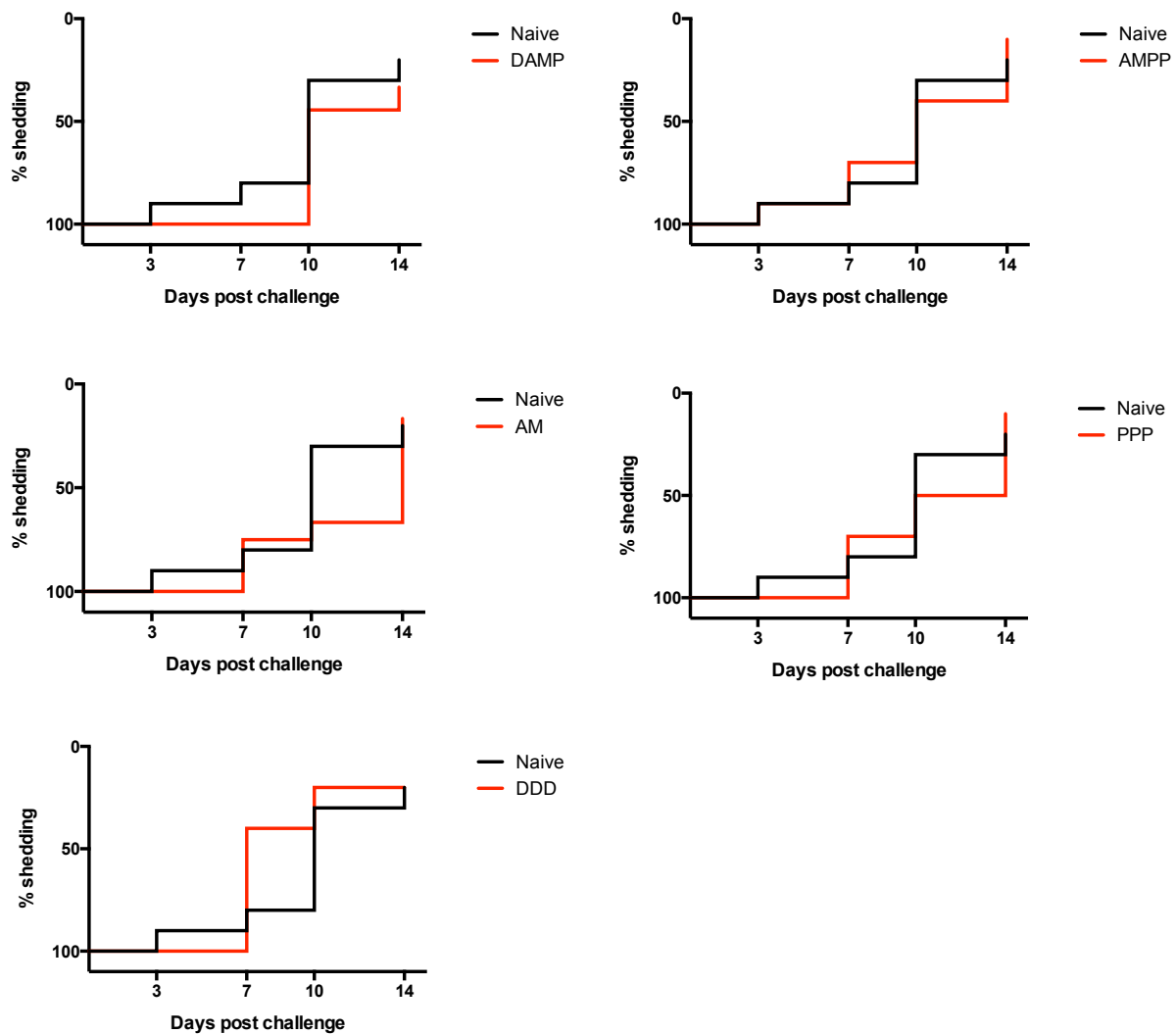


Figure 5.7. Percentage of BALB/c mice shedding *C. trachomatis* following vaccination with prime-boost regimens.

Six weeks after the final vaccination and 1 week after 2 mg/mouse subcutaneous Depo-Provera treatment, BALB/c mice (n = 5 to 8) were infected intravaginally with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx. The vaginal vault of each mouse was sampled using individual swabs at 3, 7, 10 and 14 days post challenge, and vaginal Chlamydial loads quantified by infection assay and immunoflorescent microscopy. Mice are designated non-shedding when below the limit of detection (6 IFU/swab). Shedding curve differences were analysed by both the Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test.

5.2.8 A common protective vaccination regimen in both B6C3F1 and BALB/c mice.

From the five investigated prime-boost regimens, only the DAMP regimen was consistently protective, significantly reducing chlamydial IFU per swab at day 3 post challenge compared to unvaccinated controls in both BALB/c (* $p = 0.0359$, 2-tailed Mann-Whitney test) and B6C3F1 (* $p = 0.0303$, 2-tailed Mann-Whitney test) (Figure 5.8). The regimens AM and DDD were consistently non-protective in both mouse strains, while PPP was protective in B6C3F1 and AMPP protective in BALB/c though with moderate p-values of 0.0451 and 0.0447 respectively (2-tailed Mann-Whitney test).

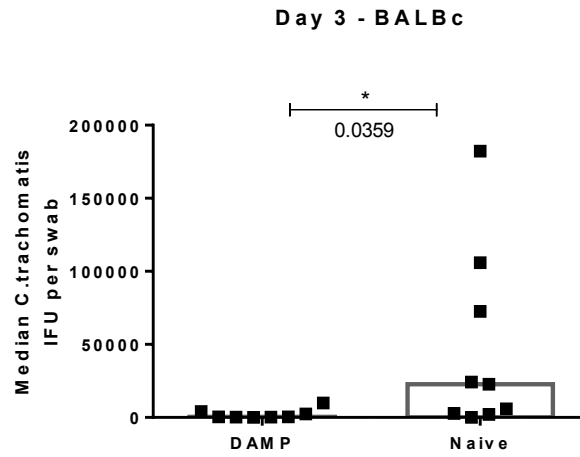
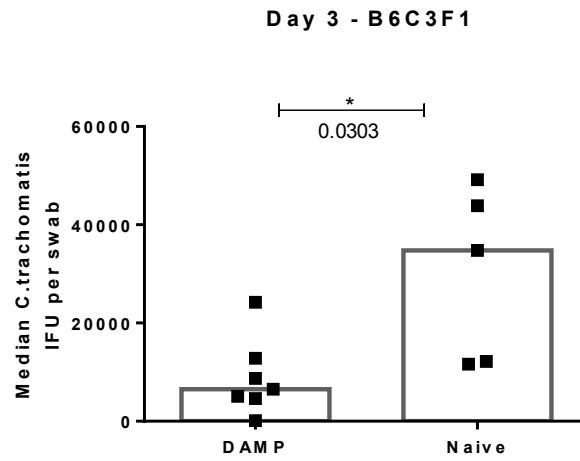


Figure 5.8. A common *C. trachomatis* protective vaccine regimen.

Vaginal Chlamydial loads quantified by infection assay and immunoflorescent microscopy at day 3 post challenge. Groups include DAMP vaccination (n = 7) and naïve controls (n = 5) for B6C3F1 mice and DAMP vaccination (n = 8) and naïve controls (n = 9) for BALB/c mice. Median values are represented as clear bar charts. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, 2-tailed Mann-Whitney U test.

5.2.9 CD4+ T cells contribute to the DAMP regimen vaccine-induced protective response.

In an effort to elucidate the role of CD4+ T cells in the vaccine-induced protective response, monoclonal antibodies were used to deplete CD4+ cells in DAMP vaccinated and unvaccinated control B6C3F1 mice (Figure 5.9). The CD4+ T cell depletion is detailed in Chapter 2.4.11. The significant reduction in *C. trachomatis* shedding in undepleted B6C3F1 (* $p = 0.0303$, 2-tailed Mann-Whitney test) was lost when CD4+ T cells were depleted. Following CD4+ T cell depletion, DAMP vaccinated B6C3F1 mice had median chlamydial loads of 46888 IFU/swab while unvaccinated depleted B6C3F1 had median loads of 15675 (N.S. $p = 0.1638$, 2-tailed Mann-Whitney test). Thus, CD4+ T cells contribute significantly to the vaccine-induced protective response. Confirmation of the CD4+ depletion in antibody-treated mice by FACS analysis is provided in the Appendix (Figure 9.3).

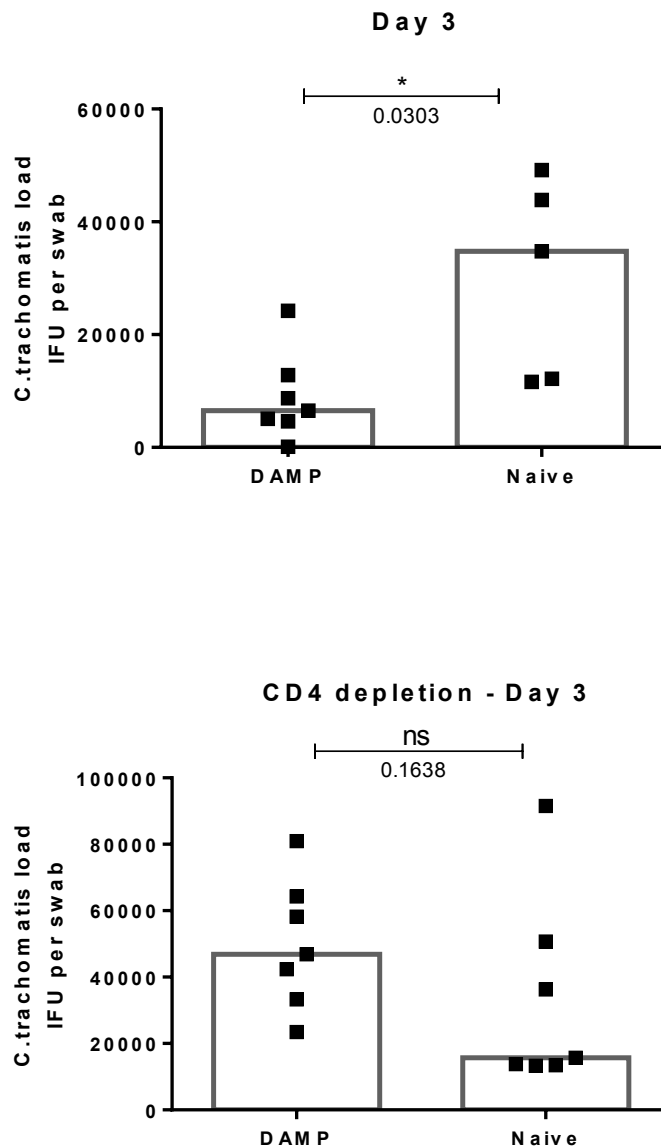


Figure 5.9. Importance of CD4+ T cell immunity in DAMP vaccine regimen-mediated protection.

B6C3F1 mice were depleted of CD4+ T cells by i.p. injections of 500 µg/mouse of anti-mouse CD4 monoclonal antibody (clone GK1.5) on days -1 and +1 with respect to day of challenge day 0. Day 3 swab samples were analysed as only day 3 provided significant reduction in chlamydial load in undepleted controls. Undepleted DAMP vaccinated mice (n = 7) and undepleted naïve controls (n = 5), with CD4 depleted DAMP vaccinated mice (n = 7) and depleted naïve controls (n = 7). Median values are represented as clear bar charts. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, 2-tailed Mann-Whitney U test.

5.2.10 Serum antibody neutralisation ability does not contribute to the DAMP regimen vaccine-induced protective response

One immunological mechanism that could potentially contribute to Chlamydia immunity is the elicitation of antibodies that neutralise EB cell entry. The ability of anti-MOMP antibodies from the heterologous prime-boost regimens to prevent EB infection of McCoy cells *in vitro* was therefore investigated, with the neutralisation assay fully detailed in Chapter 2.5.7. The DAMP, AMPP and PPP regimens have similar neutralisation profiles with mean neutralisation abilities of 50-60% at a 1/10 serum dilution, dropping to 40-45% at 1/20 and ~30% at 1/40 serum dilutions (Figure 5.10). The regimens without the protein boosts, AM and DDD had mean neutralisation abilities of around 20% at 1/20 and only 10% at 1/40 serum dilutions, perhaps reflective of the lower quantity of antigen-specific anti-MOMP per unit serum. Interestingly, the AM and DDD regimen differed greatly in their mean neutralisation ability at a 1/10 serum dilution, with AM exhibiting similar neutralisation abilities as DAMP, AMPP and PPP (43%), but DDD only having a mean neutralisation ability of 16%. The comparable neutralisation profiles of the protective DAMP regimen and the non-protective regimens AMPP, PPP and AM indicate that the observed protection is unlikely dependent on the induction of neutralising antibodies.

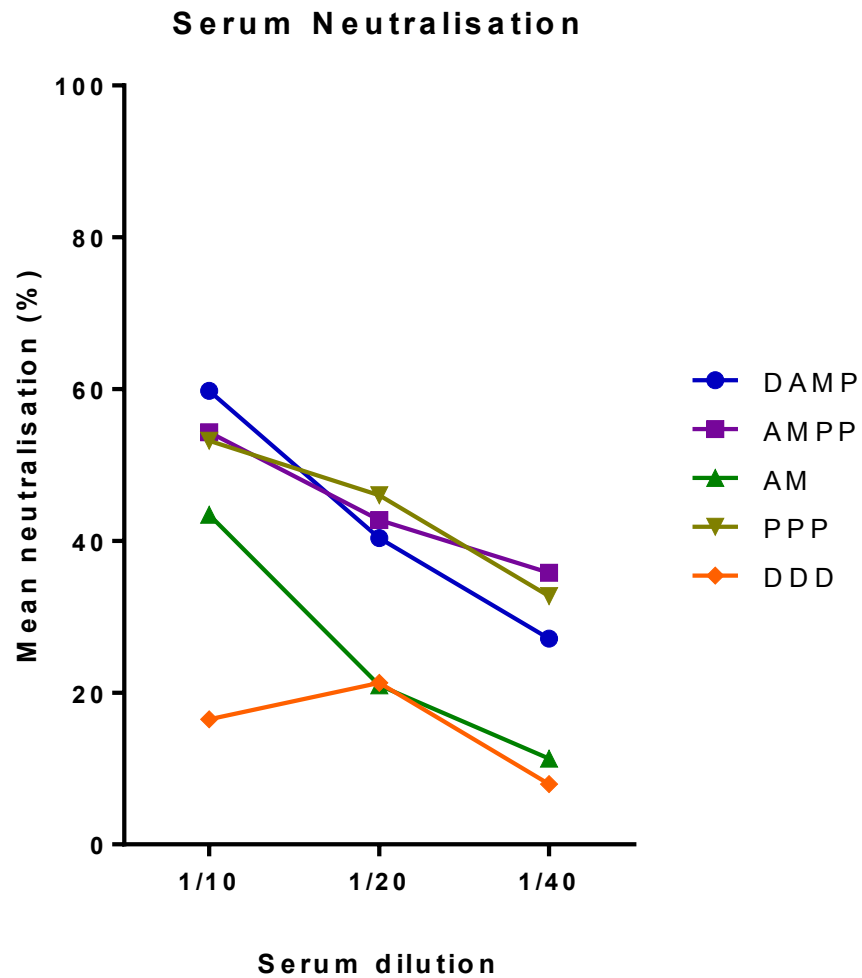


Figure 5.10. Comparable serum neutralisation ability between protective and non-protective vaccine regimens.

Serum antibodies from the differing prime-boost regimens were pre-incubated at varying dilutions with infectious *C. trachomatis* elementary bodies (EBs) and used to infect McCoy cell monolayers and compared with EBs treated with corresponding dilutions of naïve sera. The antiserum neutralisation activity was determined by measuring the reduction of the number of inclusions generated by antibody-opsonised EBs after correction to the background inhibition observed with naïve sera controls.

5.2.11 Serum antibodies do not contribute to the DAMP regimen vaccine-induced protective response

To assess the role of vaccine induced antibodies via the DAMP and PPP regimens, serum from these vaccinated mice were passively transferred into naïve C57BL/6 mice (Figure 5.11). 15 µg of anti-MOMP serum IgG was intravenously injected into each mouse, 4 hr prior to *C. trachomatis* vaginal challenge. Naïve serum was also intravenously injected for the naïve control. There was no statistically significant reduction in *C. trachomatis* vaginal load at day 3, 6 or 10 post challenge in C57BL/6 mice for any of the serum transfused groups (Kruskal-Wallis test with Dunn's multiple comparison post-test). To assess whether the vaccine induced antibodies via the DAMP and PPP regimens may have a greater effect in an IFN- γ deficient environment, serum from these vaccinated mice were also passively transferred into naïve IFN- γ knock-out C57BL/6 mice. C57BL/6 mice were genotyped to confirm their IFN- γ KO status as described in Chapter 2.2.12. Again, there was no statistically significant reduction in *C. trachomatis* vaginal load at day 3, 6 or 10 post challenge in C57BL/6 for any of the serum transfusion groups (Kruskal-Wallis test with Dunn's multiple comparison post-test). Moreover, none of the IFN- γ knock-out C57BL/6 mice could control the chlamydia vaginal loads, with median loads of 9040 IFU/swab, 20418 IFU/swab 19662 IFU/swab for DAMP, PPP and Naïve transfusion respectively at 10 days post challenge.

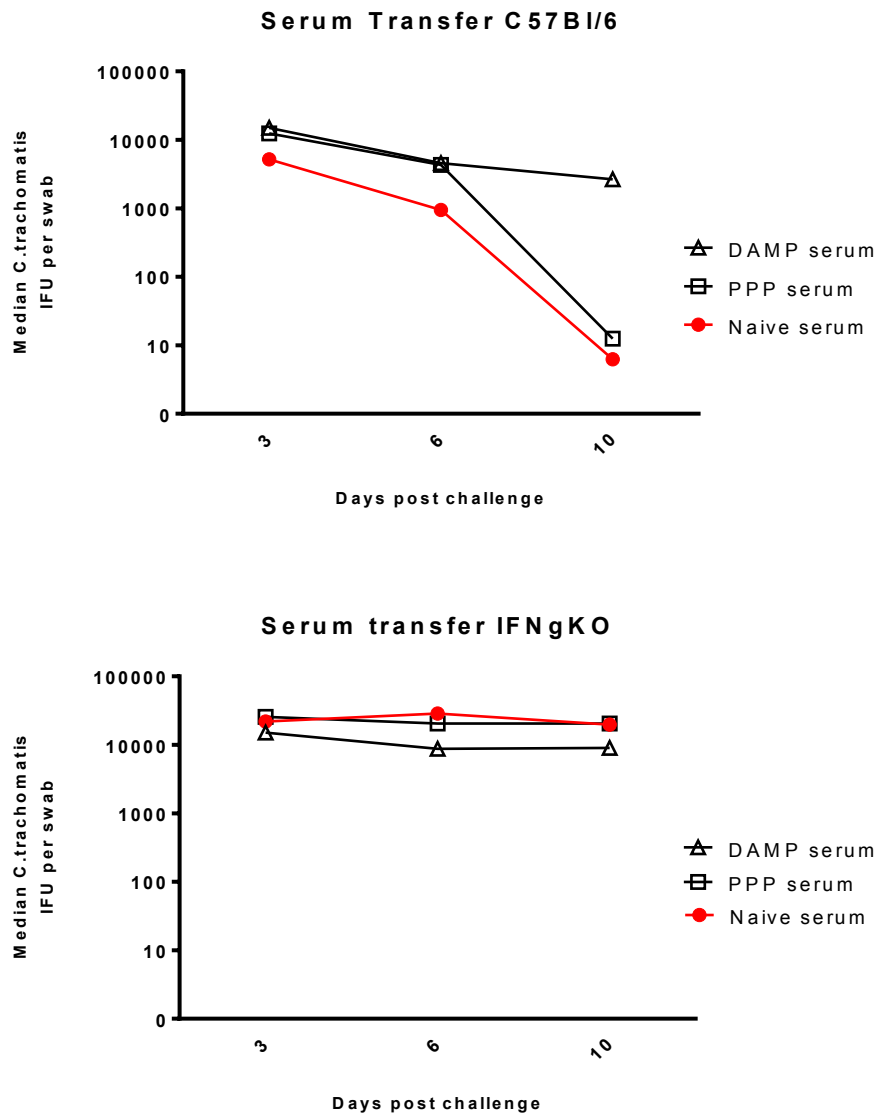


Figure 5.11. No role for serum anti-MOMP antibodies in DAMP vaccine regimen-mediated protection, in both IFN- γ present and IFN- γ deficient environments.

Anti-MOMP sera from either DAMP vaccinated mice, PPP vaccinated mice or naïve unvaccinated mice was evaluated by ELISA prior to passive transfer. 15 μ g of anti-MOMP sera was intravenously infused via the tail vein into C57BL/6 mice (n = 7 per group) (A) and into IFN- γ K.O. C57BL/6 mice (n = 7 per group) (B) 4 hours prior to intravaginal infection with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx. The vaginal vault of each mouse was sampled using individual swabs at 3, 7 and 10 days post challenge, and vaginal Chlamydial loads quantified by infection assay and immunofluorescent microscopy. Median values are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, 2-tailed Mann-Whitney U test.

5.2.12 Intranasal immunisation with MOMP +MPLA or MPLA alone does not protect against *C. trachomatis* vaginal challenge

The intranasal PPPP regimen, adjuvanted with MPLA, was the only regimen investigated capable of inducing MOMP-specific vaginal IgA. To assess whether these MOMP-specific vaginal IgA antibodies could confer a protective role, mice immunised with the intranasal PPPP regimen adjuvanted with MPLA, were subsequently challenged vaginally with *C. trachomatis*.

The intranasal PPPP regimen, adjuvanted with MPLA, did not significantly reduce the Chlamydial bacterial load at 3, 7, 10 or 14 days post challenge below that of unvaccinated control BALB/c mice (Figure 5.12). As a control, four intranasal immunisations with MPLA alone and no MOMP antigen were carried out. This immunisation regimen also did not significantly reduce the Chlamydial bacterial load at 3, 7 or 10 days post challenge below that of untreated control BALB/c mice.

The intranasal PPPP + MPLA regimen was the only regimen to induce MOMP-specific vaginal IgA concentrations, and the absence of protection suggests a lack of a role for the MOMP-specific vaginal IgA concentrations induced. This may be due to the concentrations of IgA induced being too low, or the IgA antibodies being not specific enough to the infectious Chlamydia encountered. The lack of protection may also be due to a minimal or unnecessary role for anti-MOMP IgA antibodies in this murine model of *C. trachomatis* vaginal infection.

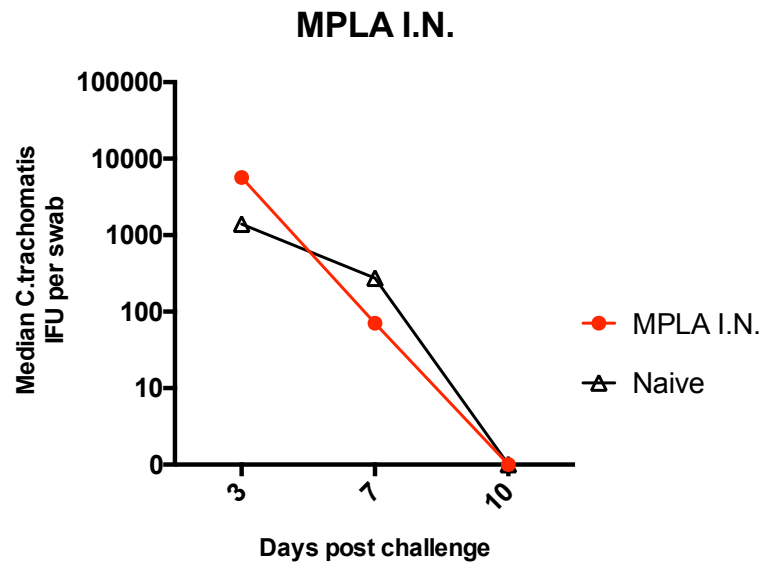
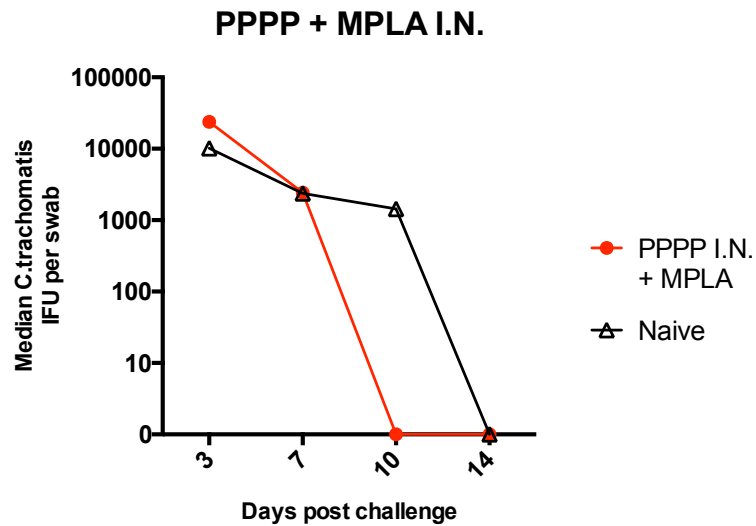


Figure 5.12. PPPP + MPLA intranasal immunisation's protective ability against *C. trachomatis* in BALB/c mice.

Six weeks after the final vaccination and 1 week after 2mg subcutaneous Depo-Provera treatment, BALB/c mice (n = 8) were infected intravaginally with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx. The vaginal vault of each mouse was sampled using individual swabs at 3, 7, 10 and 14 days post challenge, and vaginal Chlamydial loads quantified by infection assay and immunofluorescent microscopy. Median values are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, 2-tailed Mann-Whitney U test.

5.2.13 Intranasal and intravaginal immunisation with Adenoviral prime-MVA boost does not protect against *C. trachomatis* vaginal challenge

Neither the intranasal administration of the AM regimen, nor the intravaginal administration of the AM regimen resulted in a significant reduction of the vaginal chlamydial bacterial load at 3, 7, 10 or 14 days post challenge (Figure 5.13).

The intranasal and the intravaginal administration of the AM regimen did not elicit significantly greater humoral or cellular responses than naïve controls, so the lack of protection afforded is not surprising. If there were any significant reduction in chlamydial load following these regimens it would have suggested an immune mechanism that we did not screen for such as NK cell killing.

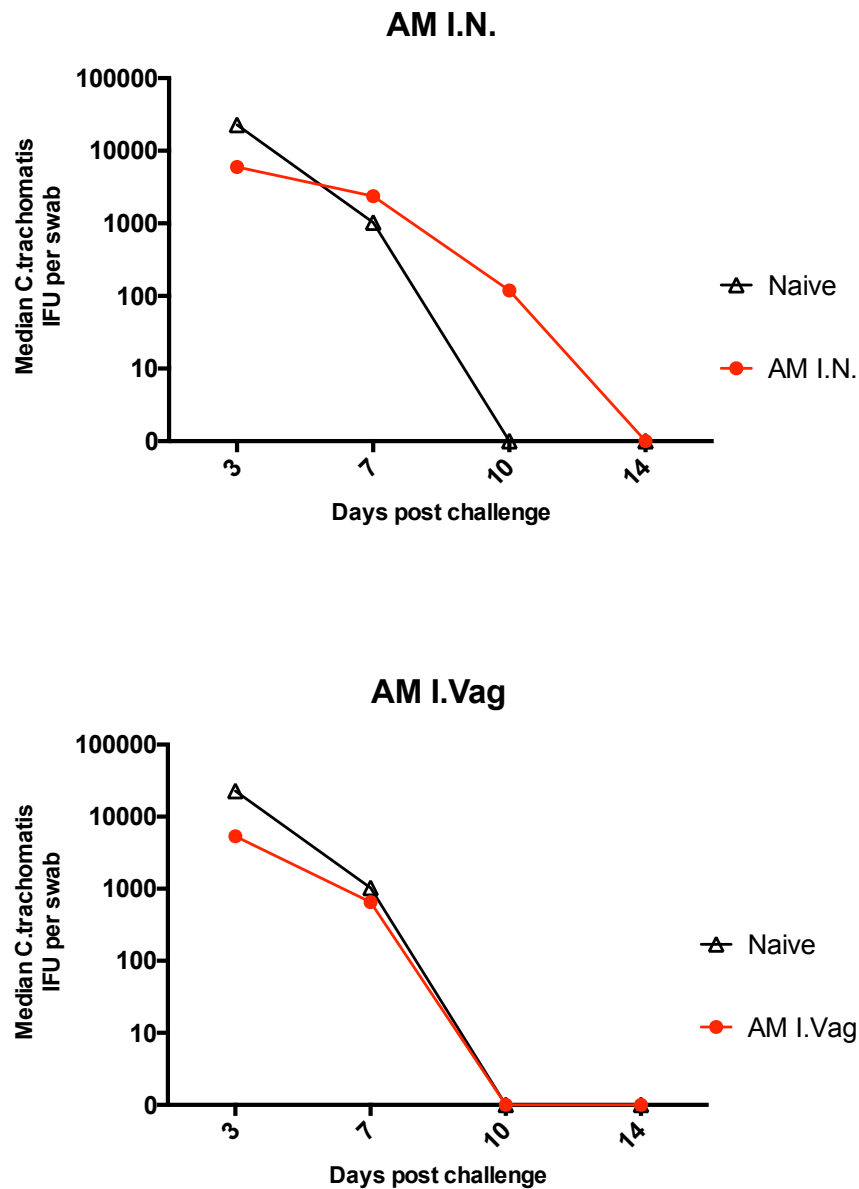


Figure 5.13. AM regimen by intranasal or intravaginal immunisation and its protective ability against *C. trachomatis* in BALB/c mice.

Six weeks after the final vaccination and 1 week after 2 mg/mouse subcutaneous Depo-Provera treatment, BALB/c mice (n = 10) were infected intravaginally with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx. The vaginal vault of each mouse was sampled using individual swabs at 3, 7, 10 and 14 days post challenge, and vaginal Chlamydial loads quantified by infection assay and immunofluorescent microscopy. Median values are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, 2-tailed Mann-Whitney U test.

5.3 Discussion

We employed two differing mouse strains for our challenge studies: BALB/c and B6C3F1 (female C57BL/6 and male C3H/HeN cross). Genital tract infection of mice with *C. trachomatis* has been previously shown to be dependent on the mouse strain used, with a genital *C. trachomatis* serovar E infection having a significantly longer duration of infection in C3H/HeN mice than in C57BL/6 [273]. Furthermore, *C. trachomatis* serovar D genital infections in BALB/c mice are of significantly shorter duration than infection of both C57BL/6 and CF-1 [108]. We observed no statistical differences in the *C. trachomatis* serovar D chlamydial loads between our BALB/c and B6C3F1 at any sampling point within the first 15 days post challenge, but there were statistically different immune responses in the differing mice strains to certain regimens. The DAMP regimen induced significantly greater MOMP-specific serum IgG concentrations in B6C3F1 mice than in BALB/c mice, and the T cell responses induced by the DAMP, DDD, AMPP and AM regimens were significantly greater in B6C3F1 mice than in BALB/c mice. The DAMP regimen was the only regimen to significantly reduce *C. trachomatis* vaginal shedding in both BALB/c and B6C3F1 mice, and this protection was subsequently lost following CD4⁺ T cell depletion. There appears to be a lack of a role for antibodies in the protection afforded by the DAMP regimen, as shown by passive serum transfer experiments and *C. trachomatis* EB serum neutralisation assays.

Our DAMP vaccination regimen resulted in a median reduction in genital *C. trachomatis* serovar D bacterial load by $\sim 1 \log_{10}$ in B6C3F1 and by $\sim 2 \log_{10}$ in BALB/c at day three post infection. Both are significant reductions compared to unvaccinated naïve mice. Previous chlamydia vaccine studies have resulted in differing degrees of protection. In BALB/c mice, compared to the AS01B adjuvant alone, there were 1 \log_{10} reductions in genital *C. trachomatis* serovar K bacterial loads at day 7 post infection in groups vaccinated with either PmpDpd, CT622, MOMP-F, or CT875 [276]. Following a challenge with 10^5 IFU of *C. trachomatis* MoPn in the left ovarian bursa, the number of IFU recovered was on average 1 \log_{10} lower in mice immunised with glutaraldehyde-fixed MoPn MOMP than in those inoculated with ovalbumin [148]. Pal *et al.* investigated the protection afforded by MOMP formulated with adjuvants developed for human vaccines. In BALB/c mice, MOMP + MF59 resulted in a genital bacterial load of 10 IFU after 7 days, however the OVA + MF59 control also had a low bacterial load of only 30 IFU. Furthermore, the percentage shedding between the two groups were similar with 69% to 78% respectively [279]. We could only obtain significant reductions in chlamydial shedding compared to unvaccinated naïve controls at day 3 post challenge, as by day 7 the unvaccinated naïve mice appear to control the infection, with a dramatic reduction in shedding in both vaccinated and unvaccinated BALB/c and B6C3F1 mice. The use of *Chlamydia muridarum* as the challenge species may have provided a longer sampling window to investigate differences in shedding loads between vaccinated and unvaccinated mice but would have involved redesigning and remaking all vaccines against this murine-specific Chlamydia.

It is important to note that the DAMP regimen was not compared against the other investigated regimens directly, so it has not been proven to be superior to say PPP or AMPP. The DAMP regimen has been shown, however, to afford protection that is significantly better than no intervention (the unvaccinated naïve control mice) in both BALB/c and B6C3F1 mouse strains.

We decided to screen the protective efficacy of our vaccine regimens in two different mouse strains: BALB/c and the cross strain B6C3F1. These are two currently used models, and have distinct Th1/Th2 profiles. Few chlamydia vaccine studies examine vaccine efficacy in multiple mouse strains, one of note being the screening of human adjuvants alongside MOMP in both BALB/c and C3H/HeN mice. None of the three human adjuvants tested in that study was convincingly protective in either mouse strain [279]. Another study investigated live intranasal immunisation with the elementary bodies of *C. trachomatis* MoPn in C3H/HeN, BALB/c and C57BL/6 mouse strains. Eight weeks following the intranasal immunisations the mice were challenged in the genital tract and all three strains of mice were significantly protected, with no shedding at any time point post challenge in C3H/HeN and C57BL/6, but with low bacterial shedding in the BALB/c mice [280]. These subtle infection differences in the differing mouse strains even following live chlamydial immunisation indicate the importance of screening novel vaccines and the vaccination regimens in multiple mouse strains.

The distinct characteristic of the DAMP vaccination regimen was the significantly

high IgG2a:IgG1 ratio it induced. There are examples of chlamydial-specific IgG2a antibody concentrations correlating with protection in animal studies. Anti-CPAF IgG2a levels were significantly elevated in both the serum and vaginal fluids after CPAF + IL-12 vaccination in comparison to animals that received CPAF alone, and it was the CPAF + IL-12 vaccinated mice which displayed significantly reduced bacterial shedding upon challenge [151]. MOMP with Freund's adjuvant, formulated by vortexing, elicited higher IgG2a than IgG1, while formulation by sonication elicited the reverse. It was then only the mice immunised with vortexed MOMP that were significantly protected against *C. muridarum* challenge in terms of number of IFUs and length of shedding time, compared to sonicated MOMP vaccinated mice [148]. Intramuscular immunisation with recombinant MIP (macrophage infectivity potentiator – an immunodominant *C. muridarum* antigen) induced more antigen specific IgG2a than IgG1 as well as elevated IFN- γ , and these immunised mice had significantly reduced vaginal bacterial shedding by day 12 after intravaginal chlamydial challenge and less severe hydrosalpinx compared to GST-immunised controls [281]. An intramuscular immunisation with a *C. trachomatis* serovar D MOMP formulation that included a PorB DNA plasmid and a *Vibrio cholera* ghost preparation elicited high concentrations of IgG2a and IgA, and significantly reduced vaginal *C. trachomatis* shedding both in terms of duration and intensity, with complete resolution by two weeks post challenge [282]. There are potential mechanisms by which these high IgG2a and low IgG1 chlamydial-specific antibodies may be operating. EB opsonisation by IgG isotypes has been shown to affect the uptake of chlamydia into cells. The coating of EBs with the mouse isotype IgG2b,

resulted in the increased infection of cells through the interaction with the mouse FcγR_{II} [283]. More recently, EB opsonisation with a high IgG1 and low IgG2a anti-MOMP polyclonal sera was shown to enhance infection of a cell line through the FcRn mediated uptake of these IgG-coated EBs, suggesting a negative role for MOMP-specific IgG1 antibodies [284]. The IgG2a isotype can mediate effector functions such as antibody-dependent cellular cytotoxicity (ADCC) through its Fc region binding to FcγR [285] [286]. Specific anti-chlamydial antibodies have previously been shown to augment the macrophage killing of infected epithelial cells via ADCC suggesting this effector function may facilitate the early clearance of a chlamydial infection [287]. Collectively, these suggest that the anti-MOMP IgG isotype may have a causative role in the protection observed from the DAMP regimen, though this would need to be investigated further.

The protection afforded by the DAMP regimen was lost when CD4⁺ T cells were depleted prior to genital challenge, revealing the protection is CD4-dependent. Previous chlamydia vaccine investigations have also revealed a role for CD4⁺ T cells in their protection. Olsen *et al.*, showed that the genital protection induced by subcutaneous immunisation with recombinant CTH1 adjuvanted with CAF01 in mice was solely CD4⁺ T cell mediated [105]. The same research group also revealed that the genital protection provided to C57BL/6 mice by the liposomal delivery of a *C. muridarum* MOMP antigen was dependent on CD4⁺ T cells and not dependent on the conformation of MOMP in this instance [262]. Farris *et al.*, vaccinated female

mice with *C. muridarum* MOMP and the adjuvants CpG-1826 and Montanide ISA 720 resulting in 2-3 log₁₀ fewer IFU than naïve controls. The depletion of CD4+ T cells, but not the depletion of CD8+ T cells, diminished the vaccine-induced protection, with CD4-depleted mice shedding 2-4 log₁₀ more IFU than CD8-depleted or nondepleted mice [288]. The role of CD4+ T cells in protection were clearly confirmed following their depletion after CPAF + IL-12 vaccination, but moreover, anti-chlamydial immunity could be adoptively transferred to naïve recipients using CPAF-specific CD4+ T cells. Therefore this CPAF mediated anti-chlamydial immunity is highly dependent upon antigen-specific CD4+ T cells [289]. Our observation of the inability of IFN-γ KO mice to control chlamydial infection further supports the role of CD4+ T cell responses in Chlamydia control.

Following our serum passive infusion experiments, in which the transfer of DAMP serum did not significantly reduce the genital bacterial loads compared to naïve serum controls, we concluded a lack of a role for antibody below the transfused amount of 15 µg/ml MOMP-specific IgG. Of course, serum antibody may have a significant protective effect at concentrations higher than the amount we could transfuse. There are, however, a few chlamydial vaccine studies where the role of antibody is debatable. The contribution of antibodies to the vaccine-induced protection of a *C. muridarum* MOMP based vaccine was demonstrated by the absence of protection in vaccinated B-cell-deficient mice, and by a 2-3 log₁₀ decrease in bacterial shedding by mice passively administered an anti-MOMP serum [288]. In contrast, B-cell-deficient (micromT) mice were used to evaluate the role of antibody in vaccination with CPAF + CpG, and there was in fact comparable chlamydial

clearance in both micromT and wild-type mice after vaccination. In addition, both vaccinated micromT and vaccinated WT mice exhibited similar reductions in pathology, revealing the antibodies playing a largely dispensable role in CPAF+ CpG induced chlamydial clearance [290]. While antibodies may have had a protective role in the liposomal delivered *C. muridarum* MOMP adjuvanted with CAF01 vaccine, the equal protection yielded by the native and recombinant MOMP antigens suggests the protective mechanism isn't reliant on conformationally dependent antibodies [262].

The serum neutralisation abilities did not significantly differ between the protective and non-protective regimens, implying a minimal role for neutralising antibodies in the DAMP protection. The role of neutralising antibodies in previous chlamydia vaccine studies is chequered. An alum-adsorbed MOMP-derived oligopeptide which when immunised subcutaneously elicited high levels of anti-chlamydial serum IgG neutralising antibodies did not in fact protect against intravaginal or intrauterine *C. trachomatis* serovar D challenge. There were no significant differences between immunised or control mice in either colonisation, shedding, or duration of infection, leading the authors to suggest the serum neutralising antibodies are ineffective in preventing chlamydial genital tract infection [291]. In another study native MOMP proteins were stabilised with two differing detergents, with the amphipol A8-35 providing higher neutralising titres (titre = 1250) than sera from mice immunised with nMOMP/Z3-14 (titre = 250). The nMOMP/A8-35 did elicit more robust protection against *C. muridarum*, but it was not investigated whether it was due to the neutralising ability of the induced antibodies. The authors used cell depletions

and demonstrated that protection was CD4+ but not CD8+ T cell mediated [292]. Novartis Vaccines expressed the chlamydia antigen HtrA in outer membrane vesicles (OMVs) to try and conserve native conformation, and following immunisation in mice they analysed the neutralising ability of the elicited serum. They observed 60% neutralisation at the serum dilution of 1/30, and 40% neutralisation at 1/90, more effective sera than that elicited with our various heterologous prime boost regimens. However, none of these vaccinated mice were challenged, so there is no evidence that these neutralising anti-HtrA antibodies are protective *in vivo* [293].

Our vaccine regimen involving four intranasal protein immunisations adjuvanted with MPLA was the only regimen to elicit vaginal MOMP-specific IgA antibodies, yet this group was still not statistically protected from vaginal *C. trachomatis* challenge. In Brunham *et al.*, from 95 women naturally infected with *C. trachomatis*, the secretory IgA specific to *C. trachomatis* in cervical secretions demonstrated a striking inverse correlation with recovery of the bacteria from the cervix, suggesting IgA may regulate chlamydial shedding in humans [294]. However, the resolution of primary and secondary *C. muridarum* genital infections in IgA-deficient (IgA^{-/-}) mice was not different from that in IgA^{+/+} mice. Furthermore, the depletion of CD4+ T cells prior to the reinfection of IgA^{+/+} or ^{-/-} mice had limited impact on immunity to reinfection indicating that IgA antibodies are not an absolute requirement of the protective response [295]. However, IgA may play a different role in humans compared to mice.

Our vaginal challenge in IFN- γ receptor ^{-/-} mice revealed an inability of these mice to

control and reduce bacterial load levels for 14 days post infection. This confirmed the importance of IFN- γ *in vivo* to chlamydia control and that our intravaginal *C. trachomatis* infection procedure is correct and capable of establishing a vaginal infection. The importance of IFN- γ *in vivo* to chlamydia control has been demonstrated previously in IFN- γ $-/-$ mice, and mice treated with anti-IFN- γ antibodies [114] [296] [297]. The indoleamine 2,3-dioxygenase (IDO) pathway which converts L-tryptophan to N-formylkynurenine, and thus limits tryptophan availability, is implicated in the IFN- γ mediated inhibition of chlamydial replication [298]. The mechanism of IFN- γ depleting tryptophan availability is supported by the observation that *C. trachomatis* trachoma serovars whose growth is inhibited by IFN- γ lack tryptophan biosynthesis genes [299]. IFN- γ mediated iron deprivation has been suggested to control chlamydial infections *in vitro* [300] [301] [302], and IFN- γ may have a regulatory role through its ability to alter the Th1/Th2 balance, or by enhancing the expression of chemokines or adhesion molecules [303] [304], though the relative roles of these mechanisms remains to be studied. NK cells participate in the resistance against a number of bacterial infections through their ability to secrete IFN- γ , and dendritic cells and macrophages have been shown to produce IFN- γ in response to stimulation with bacteria, IL-12 and IFN- γ itself [305]. Furthermore, both CD4⁺ and CD8⁺ T cells produce IFN- γ in response to infection, and are probably complementary in providing protective levels of this cytokine [306]. Our IFN- γ R $-/-$ mice did eventually resolve the genital chlamydial infection, suggesting the presence of IFN- γ -independent mechanisms of chlamydial load reduction.

In this chapter we aimed to assess the protective capacity of MOMP-based

heterologous prime-boost regimens against vaginal *C. trachomatis* infection in two differing mouse models and to investigate the mechanism of the vaccine induced *C. trachomatis* protection. The major finding from this chapter is that the DAMP vaccination regimen significantly reduced the *C. trachomatis* vaginal load at day 3 post-infection in both BALB/c and B6C3F1 mouse strains. This vaccine-induced reduction in chlamydial load was lost following the depletion of CD4⁺ T cells from vaccinated mice indicating the protection is CD4⁺ mediated. Protective and non-protective vaccination regimens yielded similar *C. trachomatis* EB serum neutralisation profiles suggesting a minimal role for neutralising antibodies in this vaccine-induced protection, and the passive transfer of DAMP serum into naïve C57BL/6 mice did not significantly reduce *C. trachomatis* shedding, further indicating a lesser role for antibodies in this vaccine-induced protection. Antibodies may play a role in the protection as suggested by the DAMP regimen's significantly skewed IgG2a:IgG1 ratio, but they would be required at higher concentrations than what we could passively transfer in these experiments.

Chapter 6

Inducing Chlamydia-specific ocular antibodies

6 Inducing Chlamydia-specific ocular antibodies

6.1 Introduction

Trachoma is a chronic conjunctivitis caused by the infection of epithelial cells with *Chlamydia trachomatis* and is the world's leading cause of preventable blindness, with approximately 146 million people affected globally according to WHO estimates. 540 million people live in trachoma endemic areas and are at risk from the disease and trachoma is currently the target of a WHO campaign to eradicate the disease by 2020. Trachoma is caused by the eye-to-eye transmission of infection with *C. trachomatis*, and in many areas eye-seeking (synanthropic) flies are the major factor in bacterial spread [307]. Another significant route of trachoma transmission is the close contact with infected ocular secretions within the family [308] [309].

Active trachoma, which involves the intermittent shedding of viable chlamydiae, has five clinical gradings. Follicular trachoma (TF grade) is mild asymptomatic inflammation with immune cells visible on the upper tarsal conjunctiva; intense inflammatory response (TI grade) has most of the tarsal plate obscured by capillary congestion; scarring trachoma grade (TS) is the scarring of the conjunctiva from repeated ocular infections; trichiasis grade (TT) is the distortion of the eye-lids (entropion) and deviation of the eyelashes to cause abrasion of the orb of the eye, ultimately resulting in the most severe clinical outcome, corneal opacity and

blindness (grade CO).

Surgery and antibiotic therapy dominate the trachoma prevention programs. Surgery has a sustained effect in preventing an individual going blind but has no effect on trachoma transmission, while prophylactic antibiotics reduce the transmission of infection but unless frequently repeated has no long-lasting effect on disease eradication [310]. Sustainable reductions in transmission are likely through environmental improvement and improved hygiene. Having said this, the most effective treatment in terms of practicality and cost would be a vaccine against trachoma. Empirical attempts in the 1960s and 1970s to vaccinate with crude whole organism vaccines were unsuccessful, with some subjects protected, but a suggestion that other vaccinees in fact developed enhanced disease [311]. These adverse hypersensitivity responses were not related to serovar, and they persisted longer than the protective immunity [312]. Chlamydial heat shock proteins have been notably implicated in these exaggerated responses, with higher antibody levels to chlamydial HSP60 found in subjects with trachomatous scarring compared to non-disease controls [313]. Interestingly, the antibody response to chlamydial HSP was found to be associated with specific HLA class II alleles indicating that there are also host genotype determined differences in susceptibility to severe scarring disease [314].

Research into the protective immune responses required against trachoma is currently on going, using both nonhuman primate models alongside human sampling. Kari *et al.* recently investigated potential contributing factors to the protracted

clearance of a primary *C. trachomatis* ocular infection in nonhuman primates [315]. It was found that a slow maturation of the humoral immune response specific to some chlamydial antigens correlated strongly with the reduction in early infectious burden, and other chlamydial antigens correlated with the complete eradication of infection.

Kari *et al.* used a serovar A trachoma clinical isolate (A2497), a strain with enhanced virulence characteristics for macaques, and ocular infection with A2497 resulted in a long duration primary infection that did not spontaneously resolve until ~9-15 weeks post-challenge. This A2497 ocular infection had a phenotype of intermittent disease, changing from culture negative to culture positive periods over the course of a primary infection and was accompanied by moderate to severe clinical disease. Thus A2497 infection of NHPs closely mimics trachoma in humans and the authors re-challenged A2497 infected macaques after spontaneous clearance to evaluate protective immunity. They employed radioimmunoprecipitation (RIP) as opposed to Western blotting to study the humoral immune response to the A2497 infection, an assay that utilises radiolabeled chlamydiae and is methodologically important due to the ability of this assay to detect all chlamydial antigens associated with both the EB and RB forms, in addition to any chlamydial secreted antigens. This provided a larger diversity of antigenic targets extracted under non-denaturing native conditions, an important difference from Western blotting which usually involves denatured, non-native antigen conformations. Antibody recognition from the NHP sera was restricted to the antigenically variable MOMP and a few antigenically conserved antigens. Recognition of MOMP occurred early (2-4 weeks) post-infection, and

correlated with the reduction in infectious ocular burdens, but not with infection eradication. However, antibody recognition against the conserved antigens PmpD, Hsp60, CPAF and Pgp3 appeared late (9-14 weeks post infection) and correlated with infection eradication.

Though in the previous chapter we revealed CD4⁺ T cells to be important in conferring protection against the vaginal infection with *C. trachomatis*, with a minimal role (if any) for antibodies, it is entirely feasible for a differing mechanism to be at play in the ocular environment. Ocular strains of *C. trachomatis* lack tryptophan synthase function, a function thought to have arisen or maintained due to IFN- γ pressure, suggesting a lack of a sole role for IFN- γ in the clearance of trachoma [316]. Any role for IFN- γ dependent Th1 cells in human ocular infection has yet to be confirmed and the importance of humoral immunity to trachoma is currently unclear [317].

MOMP has already been extensively discussed within this thesis. PmpD is a polymorphic outer membrane protein found on the surface of chlamydial EBs and has previously been shown to generate neutralising antibodies [205]. It is highly conserved and involved in the chlamydial attachment to host cells [318]. Pgp3 is transcribed and expressed from a highly conserved gene contained within an episomal cryptic plasmid of *C. trachomatis* and is secreted into the inclusion lumen and the host cell cytosol. Purified Pgp3 can stimulate macrophages to release inflammatory cytokines suggesting a role in chlamydial pathogenesis [319].

In this chapter we aim to assess whether heterologous prime-boost vaccine

regimens utilising the third generation vaccines constructed within this thesis are capable of inducing Chlamydial specific antibody responses on the murine ocular mucosa. As trachoma is an ocular disease and the MOMP-, PmpD-, and Pgp3-specific antibodies correlated with reduction and eradication, we wanted to investigate whether a vaccination regimen could induce antibodies specific to these potentially important chlamydial antigens on the murine eye. We screened a set of prime-boost regimens using DNA, HuAd5, MVA and recombinant protein vaccines expressing MOMP to test which were capable of inducing the highest concentrations of anti-MOMP antibodies on the murine eye. This regimen was then used with the additional recombinant protein antigens PmpD and Pgp3 to investigate whether as vaccine cocktails all antigen specific antibodies could be raised and whether there was any ability of these elicited immune responses to protect from infection. Here, we used the vaginal mucosal infection model to gauge the protective capacity of the immunity, as we do not have an ocular challenge model.

Aim

- To investigate whether DNA, HuAd5, MVA and/or recombinant protein-based vaccines can elicit antigen-specific antibody responses on the ocular mucosa of mice.

Objectives

- To screen a set of prime-boost regimens using DNA, HuAd5, MVA and recombinant protein vaccines expressing MOMP to investigate which are capable of inducing the highest concentrations of anti-MOMP antibodies on

the murine eye by ELISA.

- To take this regimen and utilise it with additional chlamydial antigens which have been implicated in having a protective role in trachoma.

6.2 Results

6.2.1 Intramuscular heterologous prime-boost regimens induce MOMP-specific ocular IgG responses

The *C. trachomatis* antigen MOMP was assessed in BALB/c mice immunised in heterologous prime-boost regimens with DNA, HuAd5, MVA and protein (+ MF59) based vaccines. Immunisations were administered three weeks apart and the ocular mucosa sampled two weeks post-final immunisation (Figure 6.1A). The ocular mucosa sampling is described in detail in Chapter 2.4.12. PPP induced significantly higher MOMP-specific ocular IgG concentrations than DDPP (** $p \leq 0.005$) and naïve unvaccinated controls (** $p \leq 0.005$, one-way ANOVA with Bonferroni's multiple comparison post-test). PPP + MF59 induced the highest mean MOMP-specific ocular IgG concentrations of 38.28 ng/ml, with the DDPP regimen inducing the lowest mean MOMP-specific ocular IgG concentrations with 4.13 ng/ml.

Total IgG concentrations of the ocular samples were measured by ELISA and the MOMP-specific ocular IgG concentrations were normalised to 1 μg total IgG revealing the proportion of total ocular IgG which is specific to the antigen in each heterologous prime-boost vaccination regimens (Figure 6.1B). The PPP regimen induced MOMP-specific ocular IgG antibodies that accounted for more than half of the total IgG on the ocular mucosa (mean = 511 ng specific/ μg total). The AMPP, APP and PP regimens induced MOMP-specific ocular IgG antibodies accounting for around a third of the total IgG on the ocular mucosa, with DAMP and DDPP inducing less than 10% of the total IgG to be MOMP-specific.

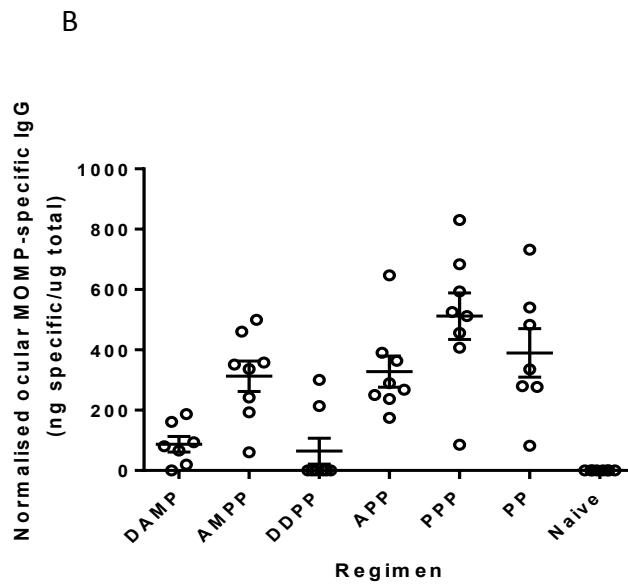
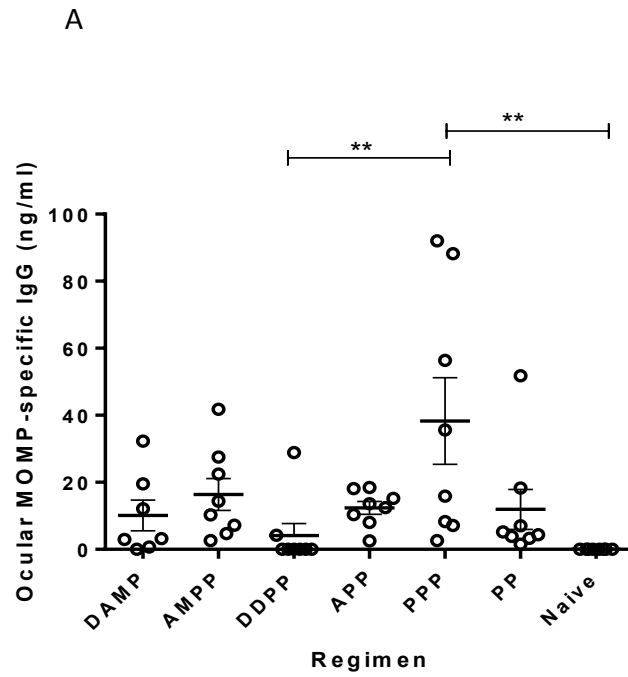


Figure 6.1. Ocular antibody responses following prime-boost intramuscular vaccination.

MOMP-specific ocular IgG (A) and normalised specific to total ocular IgG (B) from BALB/c ocular mucosa were assessed ($n = 8$ per group) two weeks after the final immunisation. Antibody results are expressed as group means (+SEM). * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ and **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni's multiple comparison post-test.

6.2.2 PPP regimen induces chlamydial-specific ocular IgG antibodies.

Three selected chlamydial antigens, MOMP, PmpD, and Pgp3, were used in three protein immunisations adjuvanted with MF59. Hsp60 was not chosen as a vaccine antigen due to its potential immunopathogenic effect. Exposure to the chlamydial hsp60 is thought to generate an immune response that also recognises regions of human hsp60 due to their close homology and this production of cross-reactive antibodies and cell-mediated immunity to human hsp60 is implicated in increase susceptibility to atherosclerosis and autoimmune disorders [320]. With MOMP being implicated in the chlamydial load reduction and PmpD and Pgp3 implicated in the chlamydial eradication, the antigens were thus investigated in cocktail formulations as they would likely be in an actual vaccine formulation. Mice were vaccinated in groups (n = 8) with individual or combinations of recombinant proteins; PmpD alone, Pgp3 alone, MOMP + Pgp3 cocktail, and MOMP + Pgp3 + PmpD cocktail (Figure 6.2A). PmpD, whether administered alone, or in combination, consistently had lower PmpD-specific ocular IgG concentrations (PmpD alone, mean = 7.5 ng/ml; PmpD with MOMP and Pgp3, mean = 1.06 ng/ml) than MOMP- or Pgp3-specific ocular IgG concentrations. Pgp3-specific ocular IgG concentrations were consistent whether immunised with Pgp3 alone (mean = 24.2 ng/ml) or immunised in combination with MOMP (mean = 18.4 ng/ml) or with MOMP and PmpD (mean = 14.2 ng/ml). MOMP-specific ocular IgG concentrations were mean = 31.1 ng/ml when co-administered with Pgp3, and mean = 15.6 ng/ml when co-administered with Pgp3 and PmpD.

The vaccine cocktail of MOMP + Pgp3 + PmpD elicited statistically different antigen-

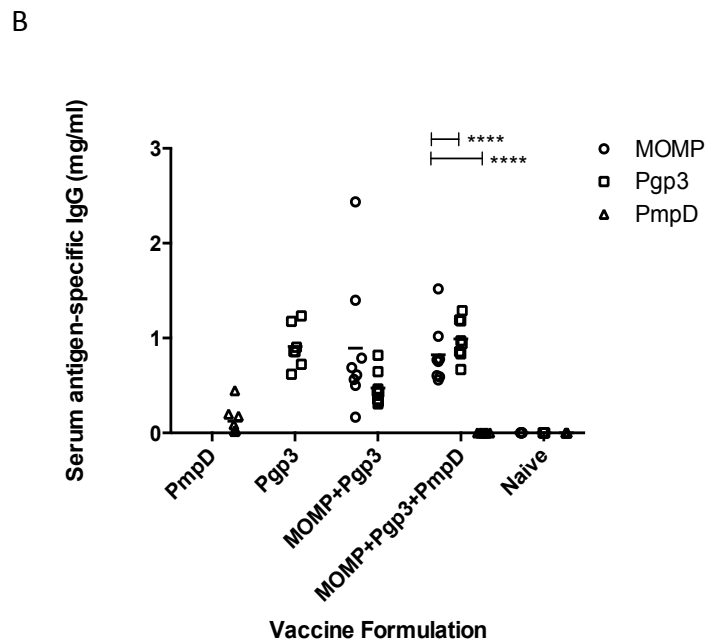
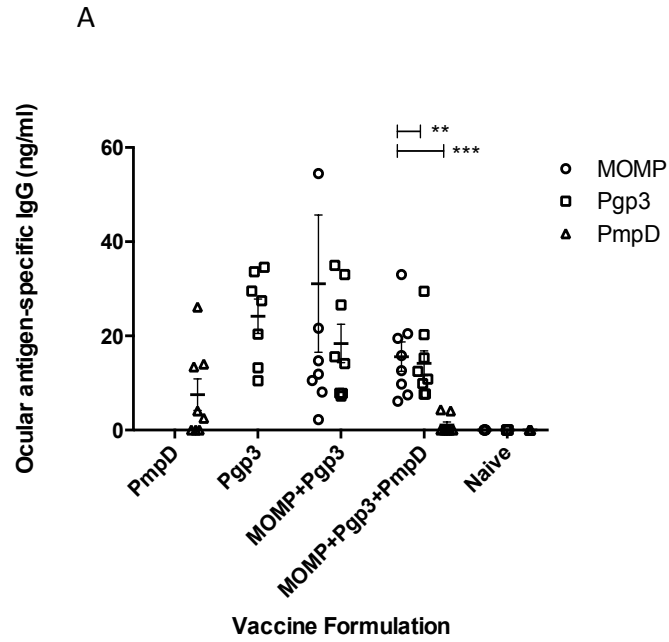


Figure 6.2. Ocular and serum IgG antibody responses following PPP + MF59 vaccination.

Antigen-specific IgG from ocular mucosa (A) and serum (B) were assessed ($n = 8$ per group) two weeks after the final immunisation. Antibody results are expressed as group means (\pm SEM) (ng/ml or mg/ml). * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$ and **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni's multiple comparison post-test.

specific ocular IgG concentrations. There were significantly higher MOMP-specific ocular IgG concentrations (***) $p \leq 0.0005$) and significantly higher Pgp3-specific ocular IgG concentrations (** $p \leq 0.005$) than PmpD-specific ocular IgG (one-way ANOVA with Bonferroni's multiple comparison post-test). There was no statistical difference between the antigen-specific ocular IgG concentrations in the MOMP + Pgp3 vaccine cocktail.

The same trend was observable in the serum, with the lowest serum IgG concentrations being specific to PmpD (PmpD alone mean = 124 $\mu\text{g/ml}$, PmpD with MOMP + Pgp3 mean = 0.3 $\mu\text{g/ml}$), and comparable specific serum IgG concentrations for the MOMP and Pgp3 antigens whether alone or in combination. The ocular IgG concentrations were compared against the antigen-specific serum IgG concentrations for all the antigens (Figure 6.3). A significant correlation was observed between the antigen-specific serum IgG concentrations and the antigen-specific ocular IgG concentrations (* $p = 0.0131$) with a Pearson R squared of 0.1107. This significant correlation in IgG concentrations between the serum and ocular samples suggests the ocular antibodies are likely to be serum exudates.

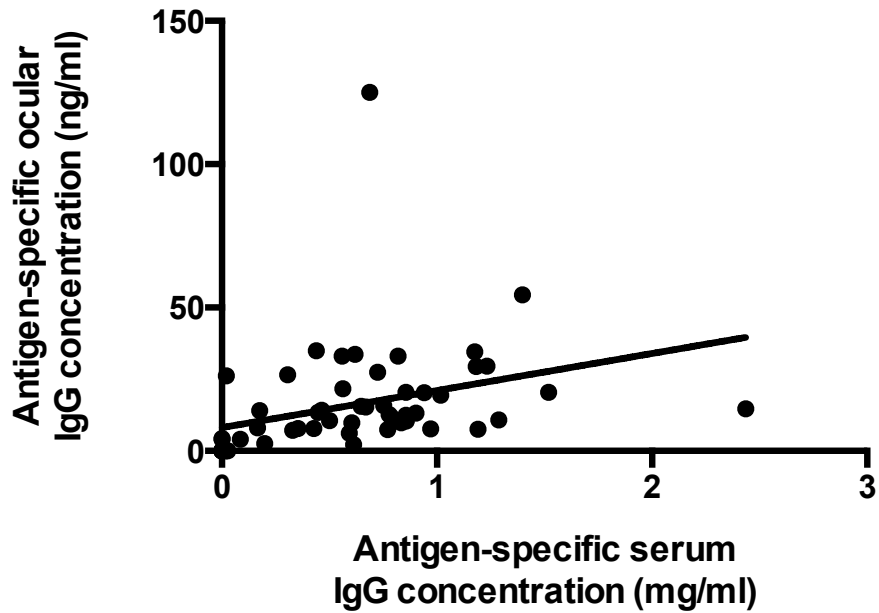


Figure 6.3. Correlation between antigen-specific serum IgG and ocular IgG concentrations

Antigen-specific IgG concentrations from the serum and ocular sampling were compared. There is a significant correlation (*, $p = 0.0131$) between the serum and ocular concentrations, with a Pearson R squared of 0.1107. The Pearson product-moment correlation test was performed on 55 sample pairs from PmpD, Pgp3, MOMP + Pgp3, and MOMP + Pgp3 + PmpD immunisation regimens.

6.2.3 PPP regimen does not induce chlamydial-specific ocular IgA antibodies

The chlamydial antigens MOMP, Pgp3, and PmpD did not induce antigen-specific ocular IgA antibodies in detectable concentrations (Figure 6.4). This is interesting because there is a high concentration of total IgA on the ocular mucosa as evident from total IgA ELISAs on the ocular samples, with naïve unvaccinated BALB/c ocular samples having a mean total IgA concentration of 494 ng/ml. The mean concentration of total IgA differed following the heterologous prime-boost regimens, though not significantly from the naïve. The PPP regimen had mean total ocular IgA concentrations of 1.01 µg/ml, and the DAMP regimen of 0.98 µg/ml. This is in comparison to total IgG concentrations of mean 67 ng/ml for PPP and 127 ng/ml total IgG for DAMP. The proportions of IgG to IgA differ on the ocular surface in comparison to the serum suggesting that if a vaccination regimen could induce IgA antibodies, the active transport of IgA should allow for appreciable and perhaps effective antigen-specific IgA on the murine ocular mucosa. Either the three antigens used here are poor at inducing IgA antibodies, or the use of the adjuvant MF59 or the intramuscular route of vaccination all contributed to this lack of antigen-specific IgA antibody.

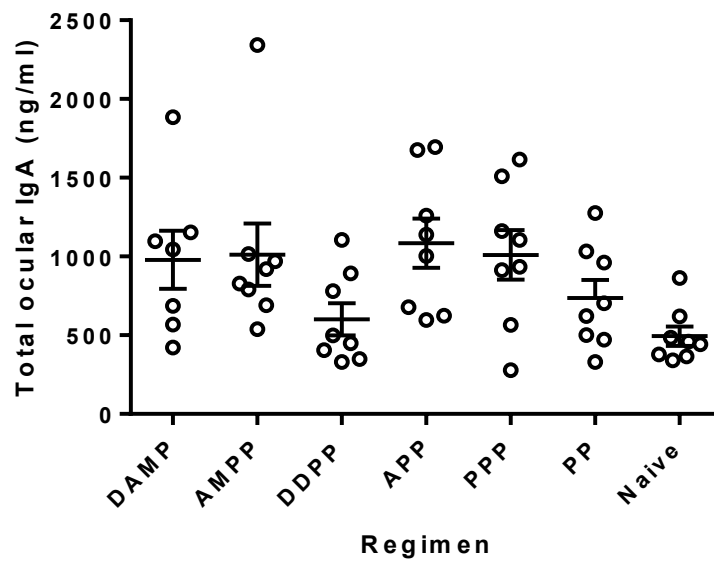
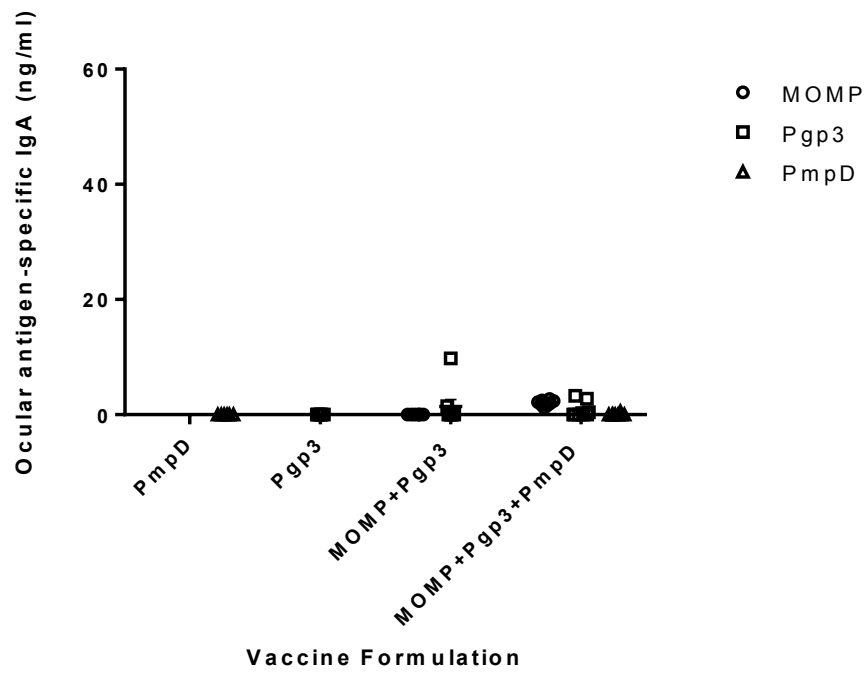


Figure 6.4. Ocular IgA antibody responses following prime-boost vaccination.

Antigen-specific IgA from ocular mucosa (A) and total IgA from the ocular mucosa (B) were assessed (n = 8 per group) two weeks after the final immunisation. Antibody results are expressed as group means (+SEM) (ng/ml).

6.2.4 Pgp3, PmpD and MOMP protein vaccine cocktails in a PPP regimen are not protective against vaginal *C. trachomatis* challenge

The mouse model for trachoma is rarely used, with the establishment of a chlamydia infection technically difficult on the small size of the mouse eye. Therefore, as a potential correlate, the immunised BALB/c mice were infected vaginally with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx as in previous challenge experiments throughout this work. None of the vaccines (PmpD, Pgp3, MOMP+Pgp3, nor MOMP+Pgp3+PmpD) in the PPP regimen (adjuvanted with MF59) significantly reduced chlamydial loads compared to unvaccinated controls (Figure 6.5). Though different antigens, this data corroborates with the lack of protection seen for MOMP alone + MF59 in the PPP regimen in BALB/c (chapter 5.2.6). It would have been of interest to investigate the partially protective DAMP regimen utilising the additional antigens PmpD and Pgp3, to assess whether the chlamydial load reduction was greater and the chlamydial clearance was quicker with respect to MOMP alone. Unfortunately this would have required the production of six extra vaccine constructs and wasn't feasible within the scope of this thesis.

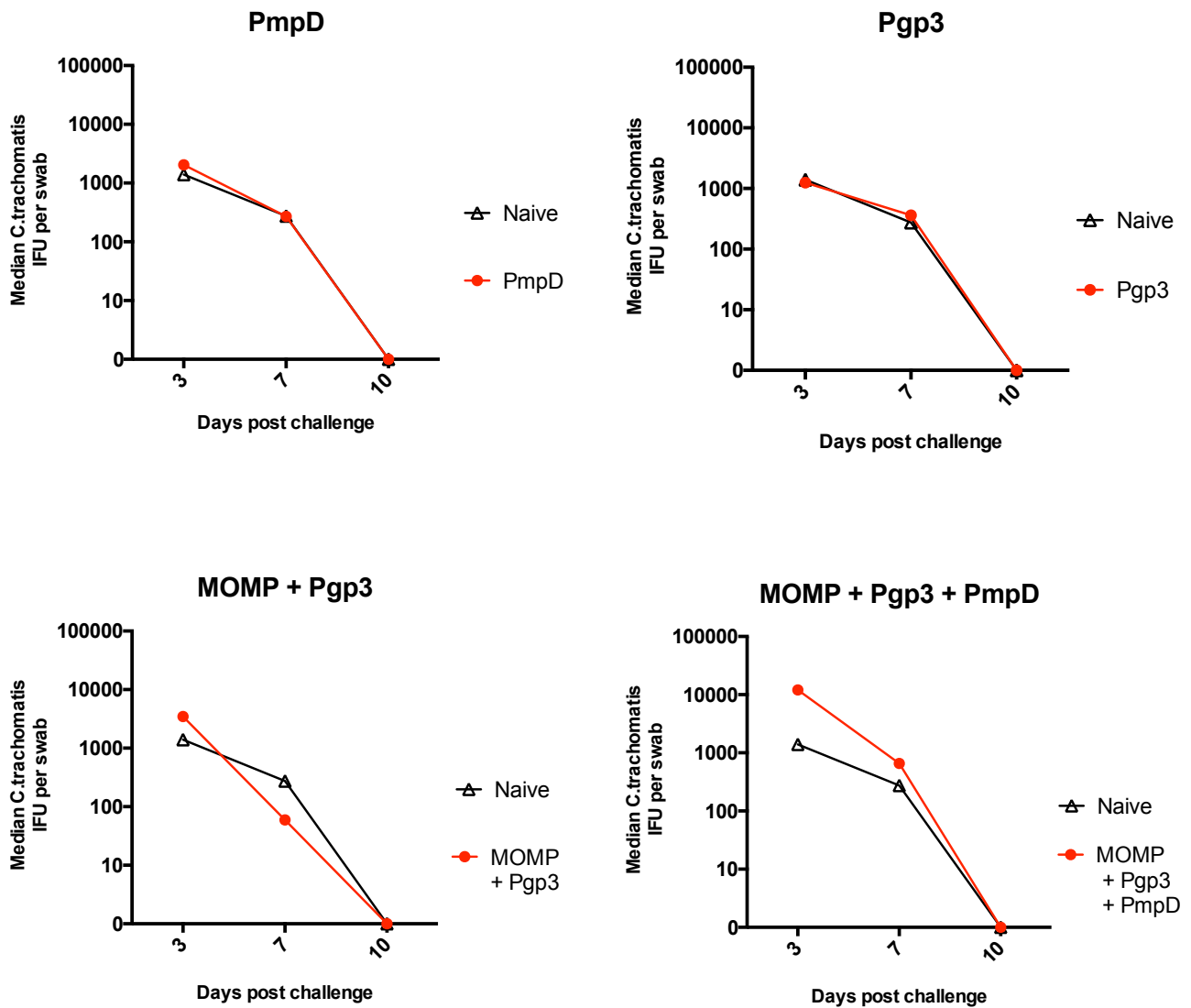


Figure 6.5. PPP+MF59 vaccine regimens *C. trachomatis* protective capacity in BALB/c mice.

Six weeks after the final vaccination and 1 week after 2 mg/mouse subcutaneous Depo-Provera treatment, BALB/c mice ($n = 8$) were infected intravaginally with 4×10^5 IFU of *C. trachomatis* D/UW-3/Cx. The vaginal vault of each mouse was sampled using individual swabs at 3, 7, and 10 days post challenge, and vaginal Chlamydial loads quantified by infection assay and immunofluorescent microscopy. Median values are represented. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, 2-tailed Mann-Whitney U test.

6.3 Discussion

To the best of our knowledge, our sampling of chlamydia-specific antibodies on the murine eye following vaccination is unique. From literature searches, ocular antibodies are normally sampled from non-human primates (NHPs) [321] [322] or humans [323] [324].

Kari *et al.* showed that specific serum antibody signatures correlated with ocular infection reduction and differing signatures correlated with ocular infection eradication in macaques [315]. If the antigen-specific antibodies have in fact a direct mechanistic role, we have developed an optimal vaccination regimen to induce them on the murine eye allowing the future prospect of screening the specific antibody induction potential of new chlamydia vaccine antigens. Our data demonstrated that not all antigens are capable of inducing equal concentrations of antigen-specific IgG, with for example the PmpD inducing significantly less than either Pgp3 or MOMP antigens. This may be because PmpD is a less immunogenic antigen, or due to the slight dose difference during vaccinations. Our model for the induction of chlamydial specific antibodies also lends itself to the screening of antigens that may induce neutralising antibodies, with the antibodies sampled from the eye being used in neutralisation assays. This is certainly an avenue that needs to be explored in the future development of this model.

The chlamydial-specific ocular IgG concentrations significantly correlated with the chlamydial-specific serum IgG concentrations (Figure 6.3), strongly suggesting that the antibodies detected on the ocular mucosa are serum exudate. It would be of interest in future studies to investigate whether this holds true outside of the mouse model. Other experimental data have shown that establishing a high-level serum response can protect mucosally through the antibody exudation mechanism. HPV-specific neutralising serum antibodies that are important for reliable protection against HPV infection are able to transudate or exudate from the serum to the cervical mucosa and may protect against new HPV infection, prevent a current infection from becoming established at other sites and/or reduce viral load after shedding of viral particles [325] [326] [327] [328].

The PPP regimen adjuvanted with MF59 induced the highest MOMP-specific ocular IgG concentrations from the regimen screening. It would be of interest to investigate whether other adjuvants used in the PPP regimen could elicit equally high IgG concentrations but of differing isotypes, as MF59 skews the IgG type to IgG1 predominance [329] [330]. With the same antigen, differing adjuvants have been shown to influence the antibody isotype in mice. The IgG3 isotype was produced mainly after immunisation with lipopolysaccharide (LPS) or Lithium salt, IgG1 was produced with incomplete Freund's adjuvant (IFA), complete Freund's adjuvant (CFA), alum and poly I:C. Poly A:U favoured the production of IgG2b, and CFA, alum and poly I:C favoured the IgG2a isotype besides the main isotype [331]. Similar adjuvant inducing antibody isotype skewing has been observed against *Mycoplasma*

agalactiae in mice, with poly I:C again shifting towards IgG2a/IgG3 production [332].

There was a high concentration of measurable total IgA on the murine ocular mucosa, however neither MOMP, PmpD, nor Pgp3 by the PPP + MF59 regimen induced any specific IgA. This maybe an antigen issue, or an effect of the adjuvantation or due to the intramuscular route of immunisation. Concerning adjuvantation, the TLR-independent molecule Cholera Toxin subunit B (CTB) is widely used as a mucosal adjuvant to induce IgA [333] [334], with CTB enhanced IgA responses against inhaled allergens in the mouse model [335]. The route of immunisation has been shown to alter IgA induction, with the intranasal immunisation of the influenza X:31 antigen inducing significantly higher IgA titres in all mucosal secretions (lungs, nasal and vaginal) compared to the subcutaneous, oral and intrarectal routes [336]. Moreover, oral administration of a GBS type III CPS conjugated vaccine with the recombinant cholera toxin B subunit induced high specific IgA levels in the lungs and rectum of mice [337]. In Chapter 4 we showed that four intranasal MOMP immunisations adjuvanted with MPLA were capable of inducing MOMP-specific IgA in the vaginal washes, yet the IgA concentrations were low suggesting there may be issues in inducing sizable MOMP-specific IgA concentrations with this antigen or adjuvant combination.

There is a school of thought in which antibodies do not have a role in controlling trachoma. This may be the case in natural infection, but vaccine induced protection

can act through different mechanisms to that of naturally acquired immunity. Bailey *et al.*, found that the presence of antichlamydial IgG antibodies in ocular secretions of disease-free individuals was in fact associated with an increased incidence of trachoma. IgA antibody showed an opposite trend, but was not statistically significant. This led the authors to conclude that antichlamydial IgG antibodies could possibly enhance the infectivity of *C. trachomatis* for the human eye [338]. The IgG isotype might be of importance in this case as well as the chlamydial protein the IgG antibodies are raised against, as isotype differences and antigen-specificities have shown differential uptake and translocation of chlamydia into cells *in vitro* [284].

There is a murine model of ocular infection by a human biovar of *C. trachomatis*. Unfortunately this could not be explored within the scope of this work due to the serovar specificity of the MOMP sequences in our vaccines, due to the lack of availability of high titre *C. trachomatis* ocular serovar stocks, due to our serovar specific chlamydia detection reagents, and due to the local animal facility guidelines. However, future work would be to assess the different antigens in varying prime-boost regimens within this murine model of ocular infection. The murine model of ocular infection has been established in BALB/c and C3H/HeN mice involving the topical infection of the conjunctiva with *C. trachomatis* serovar C at a dose of 5000 IFU. The conjunctival surfaces of both eyes are then swabbed with Dacron urethral swabs, with these strains of mice exhibiting microbiologic, histopathologic and immunologic evidence of ocular infection, and a peak of infection at 10 to 14 days

post infection [339]. Though there is this murine model of ocular infection, there is no apparent evidence of trachoma vaccine efficacy testing or screening in this mouse model. The vast majority of trachoma vaccine testing has instead been undertaken in NHPs [340].

We carried out vaginal chlamydial challenges as a surrogate, to investigate any protective efficacy of the three antigens, either individually or in combination. However none of the vaccinated groups significantly reduced chlamydial shedding below that of unvaccinated naïves. Potentially relevant to PmpD and MOMP is the likely requirement to induce humoral immunity to conformationally dependent epitopes, such as epitopes capable of inducing neutralising antibodies. This is supported by the findings that native MOMP is a superior immunogen for eliciting protective immunity in both the mouse and NHP [163] [341]. More recently Lu *et al.* used GST-tagged antigens expressed in a heterologous system and described the recognition of multiple potentially important chlamydial proteins in trachoma patients. However, their experiments failed to recognise PmpD and MOMP, the two most immunogenic proteins of Chlamydia, likely because recognition of these antigens in humans is dependent on native conformation [342]. Another potential reason why none of the antigens (either individually or in combination) conferred protection may be due to the swift clearance of chlamydia in our mouse model. From the Kari *et al.* paper, the antibodies specific for MOMP appeared to function early in infection thus reducing early chlamydial burdens, whereas antibodies specific to Pgp3 and PmpD appeared later and were suggested to be required to eradicate infection at later time points [315]. In a separate study, the use of Pgp3 as

an immunogen in BALB/c mice revealed protection against *C. muridarum* was only limited to later time points [343]. An issue with the murine genital *C. trachomatis* challenge model is that the mice do not have long enough periods of infection to assess the role of late acting antibodies. Due to the clearance of *C. trachomatis* in naïve mice, there are only three days post challenge where one can confidently observe any significant chlamydial load reduction via vaccination. The use of *C. muridarum* may avoid this issue. A final reason for the lack of significant protection afforded by the antigens and a major caveat of the research from Kari *et al.*, is that one cannot conclude that antibodies are the primary protective mechanism. The antibody signatures observed in the NHPs may simply be surrogate markers of protective T cell immune responses. However, if antibodies (whether neutralising, opsonising, ADCC) do prove to be of protective importance from future trachoma research, we have developed a model for the sampling and analysis of ocular antibodies and have revealed the vaccination regimen required to induce significantly greater concentrations on the ocular mucosa.

This chapter aimed to assess whether heterologous prime-boost vaccine regimens utilising the third generation vaccines constructed in this thesis were capable of inducing Chlamydial specific antibody responses on the murine ocular mucosa. All the heterologous prime-boost vaccine regimens tested using the DNA, HuAd5, MVA and recombinant MOMP based vaccines were capable of inducing MOMP-specific IgG antibodies on the murine ocular mucosa, however the PPP regimen induced significantly greater anti-MOMP ocular antibody concentrations. This regimen was employed with the additional chlamydial antigens PmpD and Pgp3, of which all

antigens induced antigen-specific ocular IgG antibodies but no antigen-specific ocular IgA antibodies. Antigen-specific serum IgG concentrations directly correlated with the antigen-specific ocular IgG concentrations suggesting serum exudation. We believe this is the first study to investigate whether different prime-boost regimens are capable of inducing chlamydial specific antibodies on the murine ocular mucosa. As such the work presented in this chapter provides a potential new model for the screening of future potential trachoma vaccines.

Chapter 7

Discussion and Future Directions

7 Discussion and Future Directions

It is widely held that a chlamydia vaccine is of urgent need. *Chlamydia trachomatis* is the most commonly reported communicable disease in North America and Europe [344]. It is the most prevalent sexually transmitted bacterial infection; indeed the highest clinically reported bacterial infection in the United States and in many other developed countries [345]. Chlamydia is as important in developing countries with the WHO estimating that 92 million sexually transmitted infections occur annually and the majority of infections occur in the most impoverished areas of the world where control programs are effectively absent [94]. Due to untreated infections in women that result in long-term reproductive problems such as infertility and ectopic pregnancy, Chlamydia has been the focus of public health control programs for nearly two decades. In 2002 estimates of the tangible costs of *C. trachomatis* illness in the United States exceeded \$2.6 billion, with the costs uncalculated and perhaps incalculable on a global scale [346]. National screening programs for *C. trachomatis* have been in place for nearly 20 years in many countries, and despite the wide scale roll out of such control programs in the United States, Canada and Scandinavia, the reported case counts have not exhibited sustained declines [347]. In 2000, in the United States nearly 710,000 cases of Chlamydia were reported, rising to over 1.2 million reported infections in 2009, and 2.8 million infections estimated to have occurred that year [345]. Over the course of the British Columbia (Canada) control program, Chlamydia cases initially declined but since 1998 have risen to levels ~50%

above what was seen prior to the introduction of the program, with reinfection rates significantly increasing at ~5% per year in particular among young women [348]. Brunham & Rappuoli (2013) propose that due to these important public health issues, an accelerated *C. trachomatis* vaccine effort is required with a *C. trachomatis* vaccine as a global public health priority [344].

The significance of this work and a major accomplishment of this research is the discovery of a protective vaccination regimen, which furthermore was protective in two mouse models that had differing immune bias potentials. The vaccination regimen was protective in that it significantly reduced the vaginal chlamydial load compared to unvaccinated controls and though not sterilising in its protection, a protective regimen nonetheless. It is important to note that the DAMP regimen was not compared against the other investigated regimens directly, so it has not been proven to be superior to say PPP or AMPP. The DAMP regimen has been shown, however, to afford protection that is significantly better than no intervention (the unvaccinated naïve control mice) in both BALB/c and B6C3F1 mouse strains. This DAMP regimen has a distinct characteristic and thus provides a potential correlate of protection – the significantly greater IgG2a:IgG1 ratio than any of the other tested regimens. This IgG2a:IgG1 ratio is unlikely a mechanistic correlate, but a surrogate of a good CD4⁺ T cell response. The transcription factor T-bet directly regulates Th1 commitment by CD4⁺ T cells but also regulates IgG class switching, especially to IgG2a in response to IFN- γ . T-bet serves as a mediator to activate the classical IFN- γ related Ig isotype IgG2a yet inhibit the classical Th2-related isotype IgG1 [349]. Thus

CD4+ Th1 commitment can be inferred from the IgG2a:IgG1 ratio.

This is the first use of DNA, HuAd5, MVA and recombinant protein in sequential immunisations against Chlamydia, and the first ever use of the MVA viral vector in chlamydia vaccine research. Another first is the use of a bioinformatically designed MOMP antigen to encompass the variability of *C. trachomatis* serovars D and E, and which has been shown here to provide protection against a challenge with *C. trachomatis* serovar D/UW-3/Cx.

A vaccine conferring even partial protection, such as this DAMP regimen in the mouse models, may still have significant population effects if it confers partial protection in humans. Gray *et al.*, mathematically modeled the impact of potential vaccines on the epidemics of *C. trachomatis*, and showed that a fully protective vaccine could eradicate epidemics of chlamydia infection within 20 years. If lifelong sterilising immunity cannot be attained, then their models reveal that a chlamydia vaccine would need to be effective for at least 10 years for population-level eradication to take place. However, partially protective vaccines could still be beneficial at the population-level as long as they increased the infectious threshold required for transmission to $\sim 1-2 \log_{10}$ from the peak load. Similarly, vaccines that reduce the peak load or the duration of infection in infected individuals can have substantial population-level effects [350]. As it is still unknown what the development of PID is most dependent on, both a vaccine that reduces the peak load and reduces the duration of infection will likely have an effect on pathology.

It is of importance to place this work in the context of the most recent approaches to

Chlamydia vaccination. Plasmid-free, attenuated strains of Chlamydia have shown promising results in both mouse and NHP models. A plasmid-free, attenuated strain of *C. muridarum* was revealed to be attenuated during murine genital tract infections and thus not result in upper genital tract disease [351]. This plasmid-deficient strain also functioned as a live attenuated vaccine in mice, with vaccination with the plasmid-deficient strain protecting against oviduct disease following subsequent genital infections [352]. A plasmid-deficient *C. trachomatis* strain has also been recently utilised in the macaque model to investigate protection against virulent ocular *C. trachomatis* challenge. This vaccine delivered ocularly elicited solid and partial protective immunity in macaques [353], with mechanistic investigations later revealing an important and unexpected role for CD8⁺ T cells in the solid protection observed [354]. Plasmid-deficient Chlamydial strains are another vaccine approach to induce both B and T cell responses, but with the added potential of inducing responses against an array of antigens. However, these vaccines have no safety record to date, with genetically manipulated *Chlamydia* being a relatively new advancement in the field. The full molecular mechanisms behind the action of these plasmid-deficient, future “vaccine” strains will need to be elucidated before they are a viable vaccine platform [355].

7.1 Conclusions

An initial aim of this thesis was to computationally assess whether the amino acid variability within the Chlamydial antigen MOMP could be encompassed within a consensus or a mosaic based vaccine. We bioinformatically attempted to encompass the variability of the chlamydial antigen MOMP, with the consensus approach providing greater epitope coverage and native structure than a mosaic antigen, and DNA, HuAd5 and MVA based vaccines were successfully constructed expressing a consensus MOMP. A recombinant consensus MOMP was poorly expressed from *E. coli*, so a closely related MOMP serovar D antigen was adopted as the recombinant protein. This toolbox of MOMP-based vaccines allowed us to investigate which vaccine-induced immune responses may be protective against *C. trachomatis* genital infections in the murine model.

We subsequently set out to comparatively assess the induction of humoral and cellular immunity against the MOMP antigen following heterologous prime-boost vaccinations with the newly constructed DNA, HuAd5, MVA and recombinant protein based vaccines. This revealed a capability to induce an array of MOMP-specific immune responses, both cellular and humoral, using the four differing MOMP-based vaccines in heterologous prime-boost regimens. Protein boosting significantly increased both serum and vaginal antibody concentrations, while DNA and viral vector alone regimens induced the greatest cellular responses as analysed by IFN- γ ELISpot. Protein boosts increased the avidity of the induced specific-serum antibodies, while CD4+ T cell polyfunctionality was broadest following regimens with

DNA or HuAd5 prime. Regimens were grouped on the distinct MOMP-specific immune environments they elicited, and taken through into *C. trachomatis* vaginal challenge studies to shed light on the relative contribution of each environment to protective immunity.

We then aimed to assess the protective capacity of these MOMP-based heterologous prime-boost regimens against vaginal *C. trachomatis* infection in two differing mouse models and to investigate the mechanism of the vaccine induced *C. trachomatis* protection. The major finding was that the DAMP vaccination regimen significantly reduced the *C. trachomatis* vaginal load at day 3 post-infection in both BALB/c and B6C3F1 mouse strains. This vaccine-induced reduction in chlamydial load was lost following the depletion of CD4⁺ T cells from vaccinated mice indicating CD4⁺ mediated protection. Protective and non-protective vaccination regimens yielded similar *C. trachomatis* EB serum neutralisation profiles suggesting a minimal role for neutralising antibodies in this vaccine-induced protection, and the passive transfer of DAMP serum into naïve C57BL/6 mice did not significantly reduce *C. trachomatis* shedding, further indicating a lesser role for antibodies in this vaccine-induced protection.

The final aim of this thesis was to assess whether heterologous prime-boost vaccine regimens utilising the third generation vaccines constructed in this thesis were capable of inducing Chlamydial specific antibody responses on the murine ocular mucosa. Recently, Kari *et al.* revealed serum anti-MOMP antibodies correlated with the reduction in chlamydial ocular burden in non-human primates, while anti-PmpD

and anti-Pgp3 serum antibodies correlated with chlamydial eradication, and thus we sought to investigate whether such antibodies could be induced ocularly. All the heterologous prime-boost vaccine regimens tested using the DNA, HuAd5, MVA and recombinant MOMP based vaccines were capable of inducing MOMP-specific IgG antibodies on the murine ocular mucosa, however the PPP regimen induced significantly greater anti-MOMP ocular antibody concentrations. This regimen was therefore employed with the additional chlamydial antigens PmpD and Pgp3, and all antigens induced antigen-specific ocular IgG antibodies. We believe this is the first study to have evaluated the effects of vaccination on the induction of chlamydial specific antibodies on the murine ocular mucosa. This provides a potential new model for the screening of future potential trachoma vaccines.

7.2 Future Directions

One criticism of the protective DAMP regimen is the requirement of four separate immunisations. It is not unusual to have three immunisations in a vaccination regimen, yet the addition of another intervention significantly increases the costs and reduces patient adherence. A potential way to circumvent this issue is the option of mixed vector immunisation. Reyes-Sandoval *et al.*, have recently revealed that the administration of mixed HuAd5 and MVA delivered as part of the same formulation in fact elicited optimal immunogenicity and conferred protection to mice against a Plasmodium challenge. This vector mixture was dose dependent with higher doses of vector having detrimental effects on immunogenicity [219]. This

mixed vector immunisation could be tested with our MOMP-based vectors to establish whether it can still elicit such IgG2a:IgG1 ratios for this antigen, and is a potential avenue for future work.

There are other potential vaccine delivery platforms that warrant investigation in *C. trachomatis* vaccine research. Vaccine antigens expressed in attenuated strains of *Salmonella enterica* (Vaxonella®) have been proven safe in clinical trials. The bacteria is ingested, passes through the stomach and then traverses the lining of the small intestine via M cells into lymphatic nodules. Here they are phagocytosed by APCs such as macrophages and express the antigen genes to stimulate both humoral and cellular immune responses [356]. Salmonella is an intracellular Gram-negative bacteria like Chlamydia, so the presentation of antigens in this similar way may be of importance in stimulating a protective immune response. Another recent delivery platform are outer membrane vesicles (OMVs). OMVs are closed spheroid particles released from all Gram-negative bacteria studied so far and are generated through a budding out of the bacterial outer membrane [357]. OMVs purified from *Neisseria*, *Salmonella*, *Pseudomonas*, *Vibrio* and *E. coli* have all induced potent protective immune responses against the pathogens they are derived from [358] [359] [360] [361], and anti-*Neisseria* OMV-based vaccines are already licensed [362]. The protection may not be provided only by the presence of conformationally correct immunogenic antigens but also that OMVs carry many PAMPs which play a role in stimulating innate immunity and promoting adaptive immune responses [363] [364] [365]. OMVs would appear to be a promising delivery platform for the Chlamydial MOMP antigen, as MOMP is membrane bound and requires the native conformation

to induce the most robust immune responses [163].

The antibody responses elicited by our vaccines were poorly neutralising. If neutralising antibodies were found to have a positive mechanistic role in reducing chlamydial loads, approaches to induce such neutralising antibodies would need to be explored. The neutralising epitopes of MOMP or other chlamydial surface exposed proteins would need to be defined using a panel of neutralising mAbs [366], following which they could be presented on scaffold proteins to ensure they were conformationally correct. This scaffold approach has recently been used to present conformational HIV Env epitopes in an attempt to elicit broad neutralising antibodies [367], and these heterologous recombinant protein-based vaccines could be used to attempt to induce highly neutralising antibodies against *C. trachomatis*.

One issue with the murine genital *C. trachomatis* challenge model that we encountered was that the mice did not have long enough infection periods to assess any late acting immune responses. With naïve mice clearing the *C. trachomatis* genital infection, it leaves only three days post chlamydial infection where one can confidently observe any significant chlamydial load reduction due to vaccination. The use of *C. muridarum* as the challenge species could avoid this issue. The intravaginal inoculation of *C. muridarum* in mice results in a genital tract infection that closely resembles acute genital *C. trachomatis* infections in women, with mice generally resolving the genital tract infection within 4 weeks [120]. The use of *C. muridarum* as

the challenge species may provide a longer sampling window to investigate differences in shedding loads between vaccinated and unvaccinated mice but would require redesigning and remaking all the vaccines against this murine-specific Chlamydia.

The four vaccine constructs created as part of this research are to be carried through into immunogenicity and intravaginal *C. trachomatis* challenge studies in cynomolgus macaques (*Macaca fascicularis*) by the ADITEC Consortium. Several species of nonhuman primates (marmoset [368], grivet [369], baboon [370]) have previously been used as models for genital *C. trachomatis* infections, with the most frequently used model being the pig-tailed macaque (*Macaca nemestrina*) developed by Patton *et al.* [371, 372]. Macaques have a similar anatomy and physiology to the female reproductive tract of humans, with a similar 28-30-day menstrual cycle and similar vaginal microflora [135]. They are also naturally susceptible to genital tract infection with human biovars of *C. trachomatis* and do not need hormonal pretreatment to influence the infection [136]. In the *in situ* model, macaques are infected with *C. trachomatis* by cervical inoculation, producing cervicitis and salpingitis [373] [374], with repeated salpingeal infections causing tubal scarring and distal tubal obstruction – similar to the development of pelvic inflammatory disease (PID) in women [137]. Macaques are a very good model for human chlamydial disease, but there are practical disadvantages such as high costs, adequate facilities and expertise.

Short-term future work should involve further characterisation of the DAMP regimen. Though the antibodies induced following the DAMP regimen have low neutralisation ability, and there appeared to be no role for antibodies following our passive serum transfer experiments, it should be considered whether high enough concentrations of MOMP-specific antibodies were transfused. This would be tested by transfusing higher concentrations of MOMP-specific DAMP sera from concentrating the MOMP-specific antibodies from multiple DAMP vaccinated mice. The antibodies induced by the DAMP regimen could be further characterised in functional experiments including transwell assays to assess antibody-mediated phagocytosis or chromium-51 release assays to assess ADCC potential.

As well as these, further optimisation of the murine ocular sampling model should be carried out. We have used the ocular sampling method to assess antibody type and their concentrations, yet this same procedure could be used to assess cytokine type and cytokine concentration, either by ELISA or Luminex® assays. This would provide indicators of cellular responses on the ocular mucosa following vaccinations, alongside the humoral responses. The logical conclusion to this model would be to set up an ocular infection model with *C. trachomatis*, in a similar vein to Whittum-Hudson *et al.* [339], to assess the protective efficacy of future potential trachoma vaccines.

7.3 Final Remarks

An efficacious vaccine for *Chlamydia trachomatis* would have an enormous public health impact. Infection-induced immunity in both animal models and humans suggests a strong role for CD4+ Th1-biased immune responses. Work in this thesis described the utility of DNA, HuAd5, MVA and recombinant protein vaccines in heterologous prime-boost vaccine regimens to elicit an array of MOMP-specific immune responses, following which the protection of genital *C. trachomatis* infection was assessed. The DAMP regimen, which induced a significantly greater MOMP-specific IgG2a:IgG1 ratio than any other investigated regimen, was the only regimen to significantly reduce genital *C. trachomatis* loads below that of unvaccinated control mice. This protection was shown to be CD4+ T cell mediated. The vaccines constructed within this thesis and their use in the DAMP regimen are soon to be assessed in macaque experiments to reveal whether their immunogenicity and their protective capacity translates out of the mouse model.

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643-50.

9 Appendix

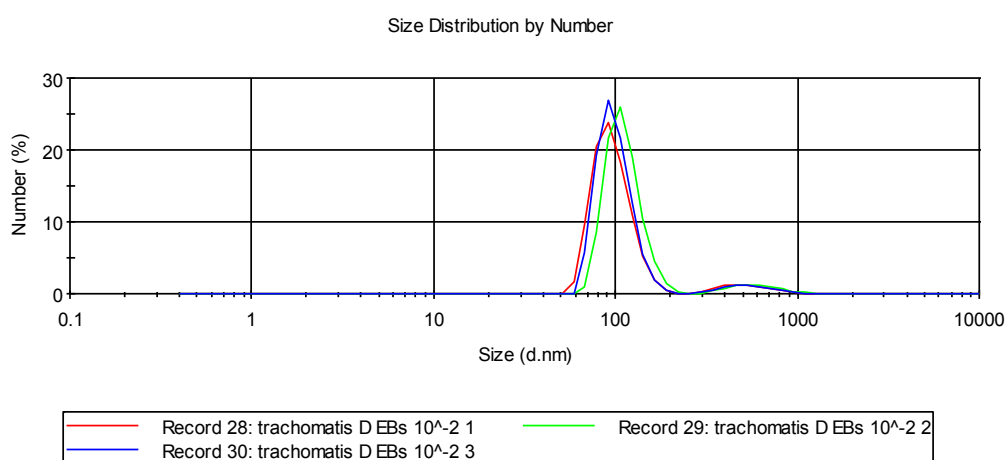


Figure 9.1. *C. trachomatis* elementary body (EB) sizing by dynamic light scattering (DLS).

Analysis of the size distribution of the purified *C. trachomatis* EB stocks using a Malvern Zetasizer revealed a consistent purity of particles of 100 nm diameter.

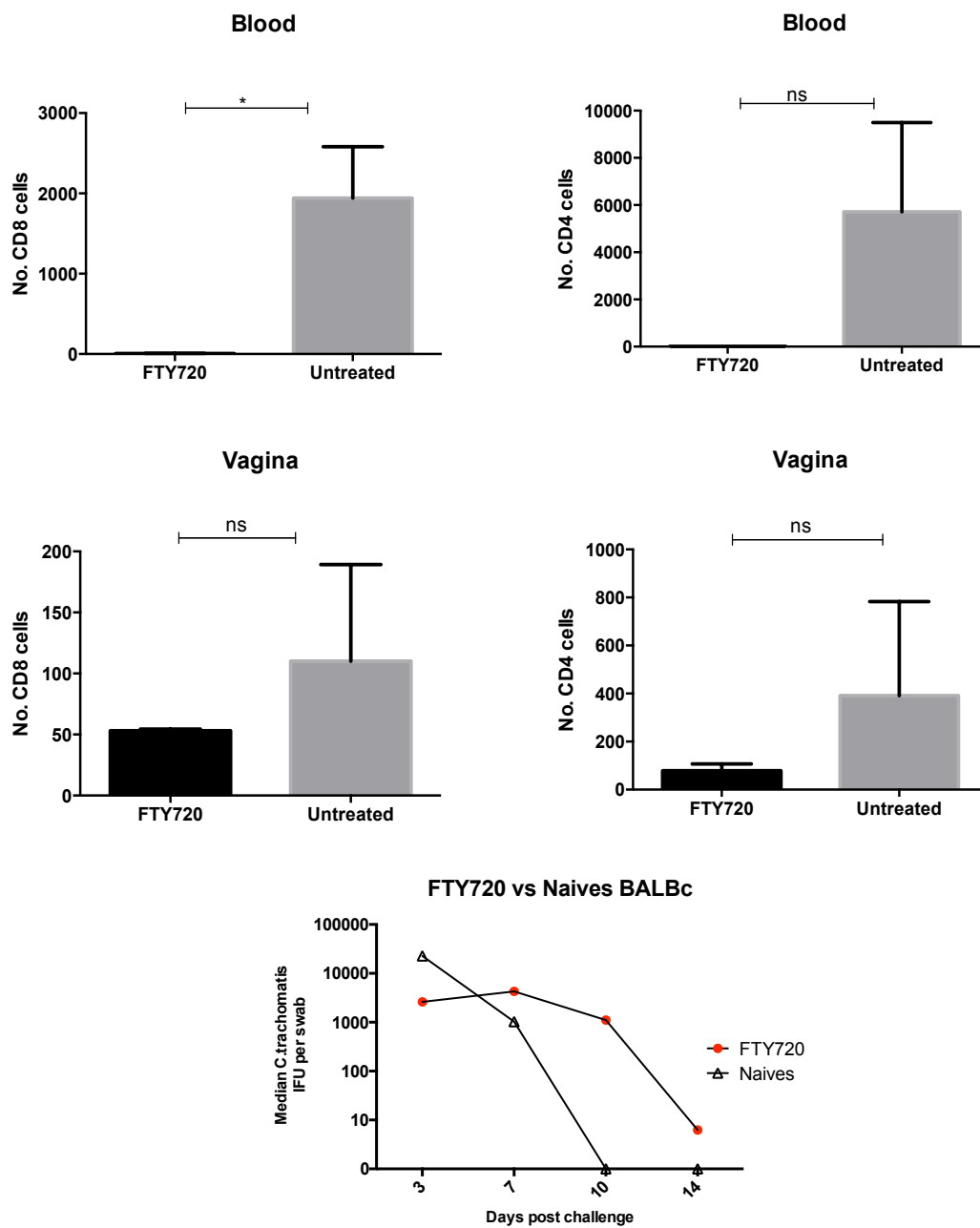


Figure 9.2. FTY720 treatment.

Bar charts representing the number of CD8 and CD4 cells from FACS analysis in the blood and vaginal tissue of BALB/c mice either under FTY720 treatment or untreated. Unpaired two tailed T test used to compare T cell numbers. Shedding data following *C. trachomatis* genital challenge in FTY720 treated and naïve BALB/c mice (N=5). Median values are represented with a 2-tailed Mann-Whitney U test.

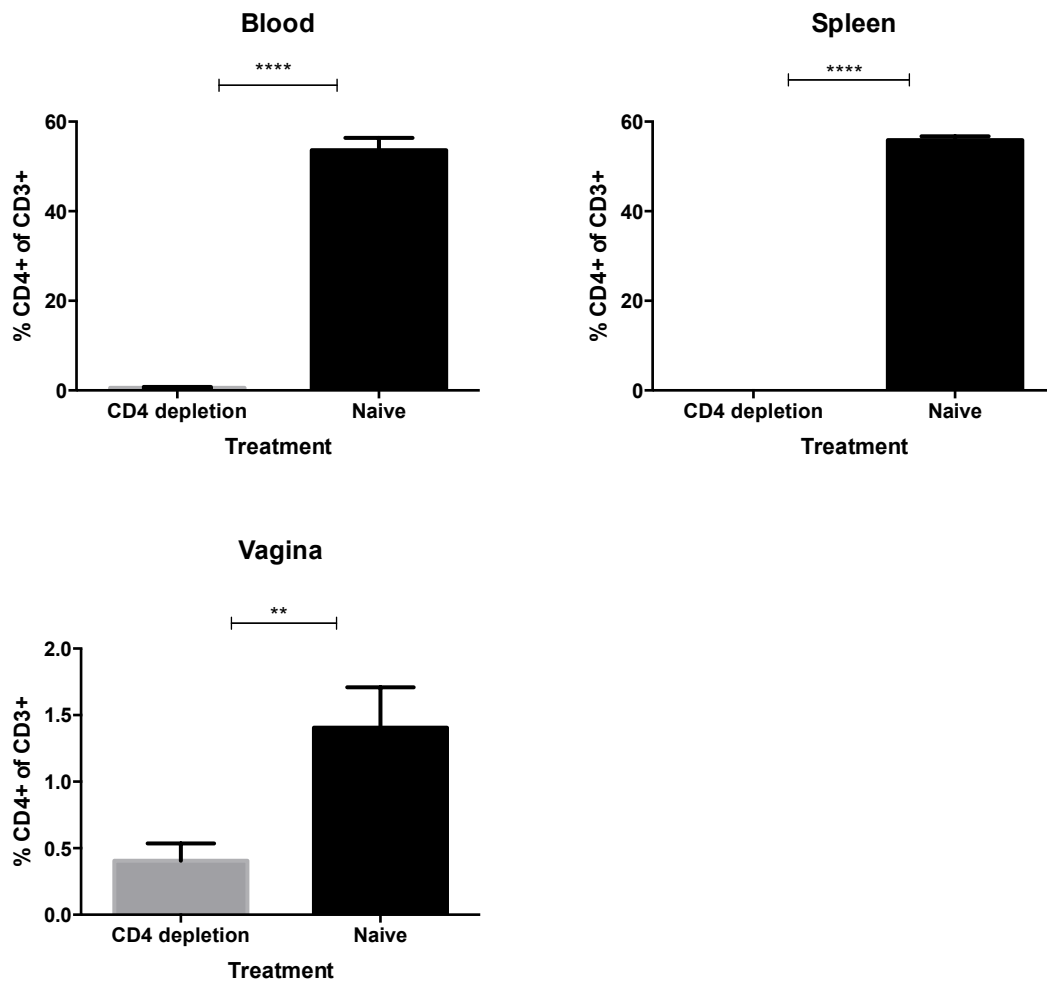


Figure 9.3. mCD4 depletion.

Bar charts representing the percentage of CD4+ T cells of all CD3+ T cells following FACS analysis of blood, splenocytes and vaginal tissue of BALB/c mice either following mCD4+ antibody depletion or naive controls. Percentages analysed by an unpaired two tailed T test, * P<0.05, ** P<0.01; *** P<0.001.

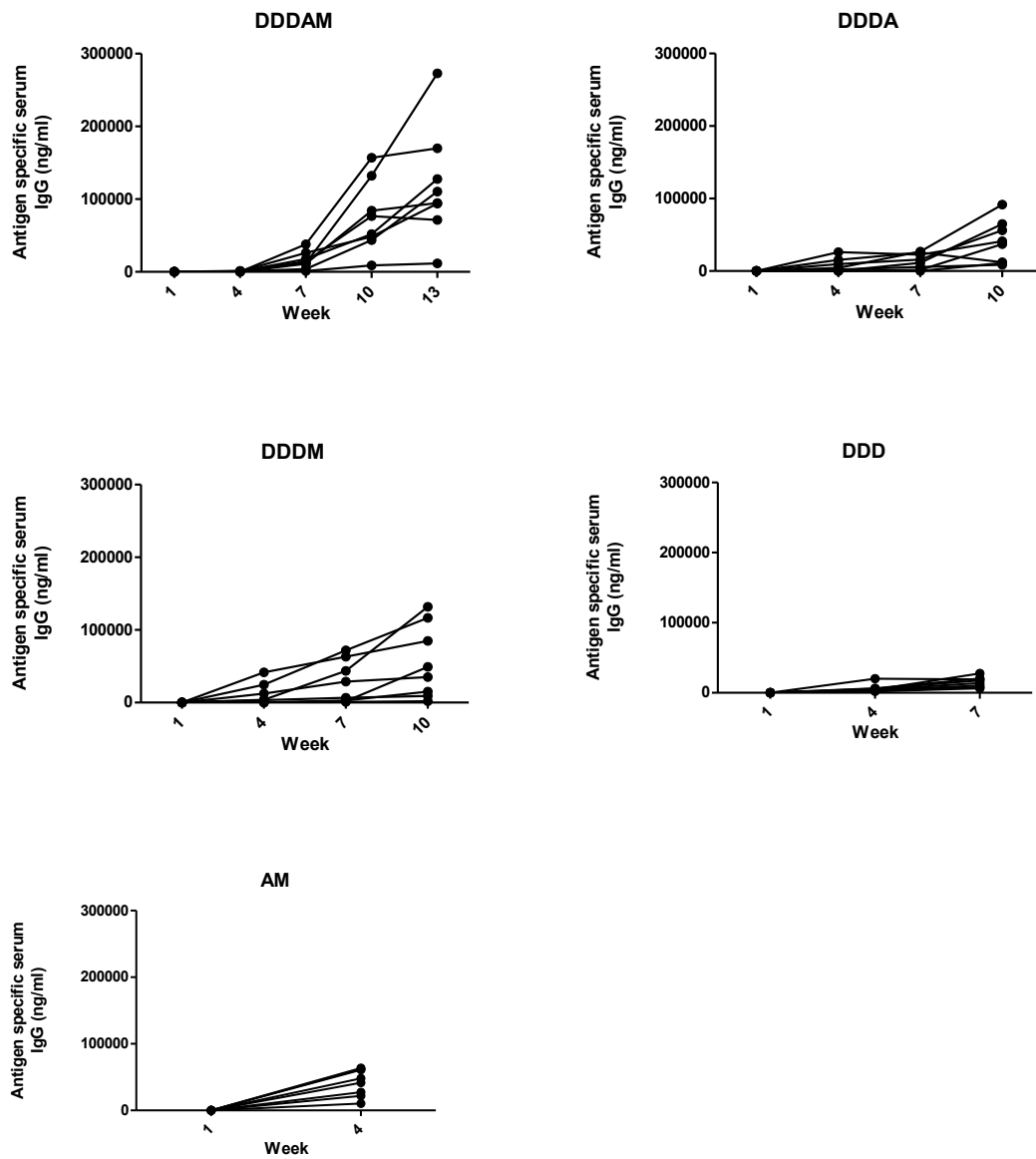


Figure 9.4 Antibody kinetics of protein-free vaccination regimens.

MOMP-specific serum IgG concentrations following individual immunisations within heterologous prime-boost regimens.

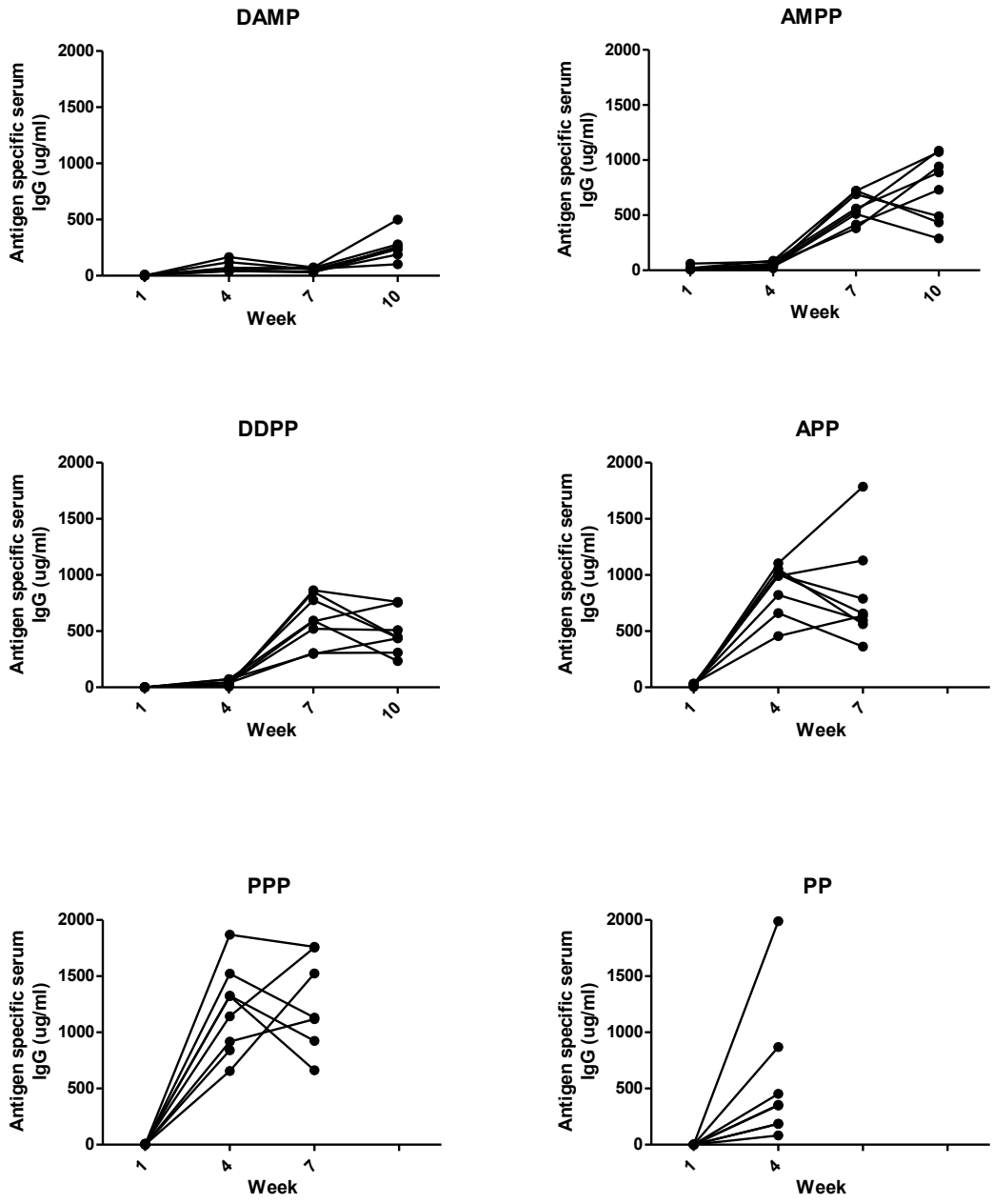


Figure 9.5 Antibody kinetics of protein-boost vaccination regimens.

MOMP-specific serum IgG concentrations following individual immunisations within heterologous prime- protein boost regimens.

ATGAAGAAGCTGCTGAAGAGCGTGCTGGTGTTCGCCGCCCTGAGCAGCGCCAGCAGCCTG
CAGGCCCTGCCCCTGGGCAACCCCGCCGAGCCAGCCTGATGATCGACGGCATCCTGTGGG
AGGGCTTCGCGGGCGACCCCTGCGACCCCTGCACCACCTGGTGGCAGCCATCAGCATGCG
CATGGGCTACTACGGCGACTTCGTGTTCGACCGCGTGCTGAAGACCGACGTGAACAAGGA
GTTCCAGATGGGCGACAAGCCCACCAGCACCACCGGCAACGCCACCGCCCCACCACCCTG
ACCGCCCGGAGAACCCCGCCTACGGCCGCCACATGCAGGACGCCGAGATGTTACCAACG
CCGCCTGCATGGCCCTGAACATCTGGGACCGCTTCGACGTGTTCTGCACCCTGGGCGCCAG
CAGCGGCTACCTGAAGGGCAACAGCGCCAGCTTCAACCTGGTGGGCCTGTTTCGCGACAAC
GAGAACCAGAGCACCGTGAAGACCAACAGCGTGCCCAACATGAGCCTGGACCAGAGCGTG
GTGGAGCTGTACACCGACACCGCCTTCAGCTGGAGCGTGGGCGCCCGCGCCGCCCTGTGG
GAGTGCGGCTGCGCCACCCTGGGCGCCAGCTTCCAGTACGCCAGAGCAAGCCCAAGGTG
GAGGAGCTGAACGTGCTGTGCAACGCCCGGAGTTCCACCATCAACAAGCCCAAGGGCTAC
GTGGGCCAGGAGTTCCTCCCTGGCCCTGATCGCCGGCACCGACGCCGCCACCGGCACCAAG
GACGCCAGCATCGACTACCACGAGTGGCAGGCCAGCCTGGCCCTGAGCTACCGCCTGAACA
TGTTACCCCTACATCGGCGTGAAGTGGAGCCGCGCCAGCTTCGACGCCGACACCATCCG
CATCGCCAGCCCAAGAGCGCCACCGCCATCTTCGACACCACCACCCTGAACCCACCATCG
CCGGCGCCGCGGACGTGAAGGCCAGCGCCGAGGGCCAGCTGGGCGACACCATGCAGATC
GTGAGCCTGCAGCTGAACAAGATGAAGAGCCGCAAGAGCTGCGGCATCGCCGTGGGCACC
ACCATCGTGGACGCCGACAAGTACGCCGTGACCGTGGAGACCCGCCTGATCGACGAGCGC
GCCGCCACGTGAACGCCAGTTCCGCTTCTAATAA

Figure 9.6 Human codon optimised nucleotide sequence of consensus MOMP.

MKLLKSVLVFAALSSASSLQALPVGNPAPESLMIDGILWEGFGDPCDPCTTWCDALSMRMG
YYGDFVFDRLKTDVNKEFQMGGDKPTSTTGNATAPTTLTARENPAYGRHMQDAEMFTNAAC
MALNIWDRFDVFCGLGASSGYLKGNSASFNLVGLFGDNENQSTVKTNSVPMNSLDQSVVELYT
DTAFSWSVGARAALWECGCATLGASFQYAQSKPVEELNVLNAAEFTINKPKGYVQGFEPLA
LIAGTDAATGTKDASIDYHEWQASLALSYRLNMFTPYIGVKWSRASFDADTIRIAQPKSATAIFD
TTTTLNPTIAGAGDVKASAEGQLGDTMQIVSLQLNKMKSRSKSGIAVGTIVDADKYAVTVETRLI
DERAAHVNAQFRF**

Figure 9.7 Amino acid sequence of consensus MOMP.