



Of Mice and MenB:
Assessing the Immune Mechanisms Underlying
Meningococcal Capsular Group B Vaccines in Mice

Thesis submitted for the degree of Doctor of Philosophy

(Michaelmas Term 2019)

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St. Cross College

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Abstract

Neisseria meningitidis is the causative agent of invasive meningococcal disease (IMD) which claims between 50,000 and 135,000 lives worldwide annually. Disease caused by the capsular group B meningococcus (MenB) is the leading cause of infectious death in UK infants. A four component vaccine, 4CMenB, has been developed to prevent IMD caused by this capsular group, but is associated with a high incidence of fever in infants and requires multiple doses to induce adequate protection. A novel adenovirus-based vaccine encoding the MenB factor H binding protein (fHbp) with an N-terminal signal sequence (Ad-fHbp) has been developed at the Oxford Vaccine Group and was found to induce high titres of protective antibody after a single dose in mice. The central aims of the work presented in this thesis were to elucidate the immune mechanisms underlying the reactogenicity of 4CMenB and the immunogenicity of Ad-fHbp. ‘Systems vaccinology’ has provided many insights into vaccine-induced responses by combining high-throughput sequencing with conventional immunogenicity assays to characterise the gene signatures and immunological pathways underlying them. However, the RNA-sequencing (RNA-seq) component of such studies can be compromised by the impact of computational choices on the final differential gene expression (DGE) call. A novel DGE pipeline was developed in the present study to mitigate the impact of this computational ‘noise’. A ‘systems’ approach was then taken to comparatively assess 4CMenB vaccine combinations and components in order to determine the cause of fever in a mouse immunisation model. Using a combination of RNA-seq, temperature, and inflammation markers, the reactogenicity was ultimately attributed to the membrane-bound endotoxin contained in the outer membrane vesicle component of the vaccine. To explore the phenomenon of the early, sustained protective immune response induced by Ad-fHbp, a panel of N-terminal signal sequence insertion/deletion variants of the antigen were comparatively assessed for *in vitro* expression from mammalian cells and for immunogenicity in mice. The full-length sequence demonstrated superior early expression and induction of bactericidal antibody and was also found to significantly boost antigen-specific T cell responses when incorporated ahead of other adenovirus-encoded bacterial antigens, highlighting its potential as an immune-enhancing sequence element for other viral vectored vaccine transgenes. Finally, antigen-specific immunological pathways were defined for the fHbp transgene product by comparative transcriptomics. The work presented in this thesis contributes to our understanding of what constitutes a well-tolerated and immunogenic meningococcal vaccine.

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Abbreviations

WHO	World Health Organization
VPD	Vaccine preventable disease
DTP	Diphtheria, tetanus, pertussis
UNICEF	United Nations Children's Emergency Fund
MMR	Measles, mumps, rubella
4CMenB	Four component meningococcal capsular group B
IPV	Inactivated polio vaccine
HBV	Hepatitis B virus
PCV	Pneumococcal conjugate vaccine
Hib	<i>Haemophilus influenzae</i> type B
Alum	Aluminium hydroxide
HIV	Human immunodeficiency virus
TB	Tuberculosis
PRR	Pattern recognition receptor
PAMP	Pathogen-associated molecular patterns
DC	Dendritic cell
TLR	Toll-like receptor
Nod	Nucleotide oligomerisation domain
NLR	Nucleotide oligomerisation domain-like receptor
IL	Interleukin
RIG	Retinoic acid inducible gene
RLR	Retinoic acid inducible gene-I-like receptor
TNF	Tumour necrosis factor

NK	Natural killer
mDC	Myeloid dendritic cell
pDC	Plasmacytoid dendritic cell
LAIV	Live-attenuated influenza virus
NF- κ B	Nuclear factor-kappa B
MHC	Major histocompatibility complex
ER	Endoplasmic reticulum
APC	Antigen-presenting cell
T _H	T helper
BCR	B cell receptor
T _{FH}	T follicular helper
PGE ₂	Prostaglandin E ₂
BEC	Brain endothelial cell
mPGES-1	Microsomal prostaglandin E synthase-1
IL-1R1	Interleukin-1 type 1 receptor
COX-2	Cyclooxygenase-2
LPS	Lipopolysaccharide
BBB	Blood-brain-barrier
CVO	Circumventricular organs
AEFI	Adverse events following immunisation
<i>N. meningitidis</i>	<i>Neisseria meningitidis</i>
IMD	Invasive meningococcal disease
CNS	Central nervous system
MenA	Meningococcal capsular group A
MenW	Meningococcal capsular group W
MenC	Meningococcal capsular group C

MenB	Meningococcal capsular group B
Tfp	Type IV pilus
OMP	Outer membrane protein
Opa	Opacity-associated
CECAM	Carcinoembryonic antigen cell adhesion molecules
NadA	Neisserial adhesin A
Ig	Immunoglobulin
Fc	Fragment crystallisable
MBL	Mannose-binding lectin
MASP	Mannose-binding lectin-associated serine proteases
MAC	Membrane-attack complex
SBA	Serum bactericidal antibody
LOS	Lipooligosaccharide
C4BP	C4 binding protein
PorA	Porin A
fH	Factor H
OMV	Outer membrane vesicle
β2AR	β2-adrenoreceptor
IU	Infectious units
dOMV	Detergent-extracted outer membrane vesicle
nOMV	Native outer membrane vesicle
fHbp	Factor H binding protein
ORF	Open reading frame
<i>E. coli</i>	<i>Escherichia coli</i>
FDA	Food and Drug Administration
EMA	European Medicines Agency

NHBA	Neisserial heparin binding antigen
JCVI	Joint Committee on Vaccination and Immunisation
IFN	Interferon
HCV	Hepatitis C virus
CAR	Coxsackie and adenovirus receptor
RGD	Arginine-glycine-aspartic acid
AdHu5	Human adenovirus serotype 5
ChAd	Chimpanzee adenovirus
dLN	Draining lymph node
eGFP	Enhanced green fluorescent protein
AdC68	Chimpanzee adenovirus serotype 68
CTL	Cytotoxic T lymphocyte
BCG	Bacillus Calmette-Guérin
MVA	Modified vaccinia Ankara
EBOV	Ebola virus
EVD	Ebola virus disease
NHP	Non-human primate
GP	Glycoprotein
ChAd3	Chimpanzee adenovirus serotype 3
rVSV	Recombinant vesicular stomatitis virus
OVG	Oxford Vaccine Group
<i>H. sapiens</i>	<i>Homo sapiens</i>
<i>M. musculus</i>	<i>Must musculus</i>
GTF	Gene transfer format
DAPI	4',6-diamidino-2-phenylindole,dihydrochloride
BSA	Bovine serum albumin

Carb-bicarb	Carbonate-bicarbonate
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
HBSS	Hank's Balanced Salt Solution
Isoflo	Isofluorane
L/G	L-glutamine
LB	Lysogeny broth
P/S	Penicillin-streptomycin
PGN	Peptidoglycan
PBS	Phosphate-buffered saline
RBC	Red blood cell
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TE	Tris-ethylenediaminetetraacetic acid
TMB	Tetramethylbenzidine
PBST	Phosphate-buffered saline-Tween
RNA-seq	RNA-sequencing
WCHG	Wellcome Centre for Human Genetics
scRNA-seq	Single cell-RNA-sequencing
GEM	Gel bead-in-emulsion
UMI	Unique molecular identifier
RT-qPCR	Real-time quantitative polymerase chain reaction
Δ CT	Normalised threshold cycle
SAM	Sequence alignment map

BAM	Binary alignment map
STAR	Spliced transcript alignment to reference
PatMaN	Pattern matching in nucleotide databases
p2pPCC	Point-to-point Pearson correlation coefficient
.csv	Comma-separated variable
.tsv	Tab-separated variable
CPM	Counts per million
TMM	Trimmed mean of m-values
DGE	Differential gene expression
eBayes	Empirical Bayes
LFC	Log ₂ fold change
DEG	Differentially expressed gene
FDR	False-discovery rate
CCA	Canonical correlation analysis
CMV	Cytomegalovirus
bGH	Bovine growth hormone
VVCF	Viral vector core facility
ELISA	Enzyme-linked immunosorbent assay
PHE	Public Health England
OD	Optical density
CFU	Colony-forming units
EUCLIDS	European childhood life-threatening infectious disease study
DE	Differentially expressed
NICE	National Institute for Health and Care Excellence
MHRA	Medicines and Healthcare Products Regulatory Agency
IM	Intramuscularly

MDS	Multidimensional scaling
ORA	Over-representation analysis
INOH	Integrating network objects with hierarchies
JAK	Janus kinase
STAT	Signal transducer and activator of transcription protein
Mal	MyD88-adaptor-like
TREM1	Triggering receptor expressed on myeloid cells
PGLYRP1	Peptidoglycan recognition protein 1
ImmGen	Immunological Genome Project
tSNE	t-distributed stochastic neighbour embedding
PC	Principal component
PCA	Principal component analysis
CYP	Cytochrome P450
GM-CSF	Granulocyte-macrophage colony-stimulating factor
PBMC	Peripheral blood mononuclear cell
tPA	Tissue plasminogen activator
FL	Full-length
SP	Signal peptide
KO	Knockout
MOI	Multiplicity of infection
SFU	Spot-forming units
JEV	Japanese encephalitis virus
HPV	Human papillomavirus
T3SS	Type III secretion system
preF	Prefusion F
IRF	Interferon regulatory factor

IFIT	Interferon-induced transcript
OAS	Oligoadenylate synthase
TFBS	Transcription factor binding site

Papers Published from this Thesis

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“Issues in Vaccinology: Present Challenges and Future Directions.”

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“Gene expression profiling reveals insights into infant immunological and febrile responses to capsular group B meningococcal vaccine.”

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Chapter 1

Introduction

1.1 Vaccines

1.1.1 The importance of vaccine uptake

According to World Health Organization (WHO) estimates, between two and three million deaths are averted worldwide on an annual basis as a result of childhood vaccination ¹. There is no doubt that vaccines as a medical intervention have had a profound effect on controlling disease and improving the status of global health. In the two centuries since Edward Jenner's pioneering work on the smallpox vaccine, smallpox has successfully been eradicated as a result of improvements made to the vaccine over time and effective immunisation programmes. The WHO currently lists 26 vaccine preventable diseases (VPDs [at the time of writing, June 2019]), a repertoire that is constantly expanding with a further 24 pipeline vaccines listed by the WHO's Product Development for Vaccines Advisory Committee ². Improvements are also being made on existing vaccines to enhance tolerability, the acellular preparations of pertussis vaccines ³ and the rabies vaccine produced from improved cell culture methods ⁴ being good examples, and increase the duration of protection, as was demonstrated by the development of glycoconjugate vaccines ⁵. Such improvements are important to

ensure positive public perception of vaccines and the continued success of immunisation campaigns.

The ongoing decline in infant mortality over the past century can be partly attributed to the control of infectious disease through public health measures including immunisation. Fig. 1.1 illustrates the impact that immunisation programmes have had on reducing disease over the course of the past century in the US.

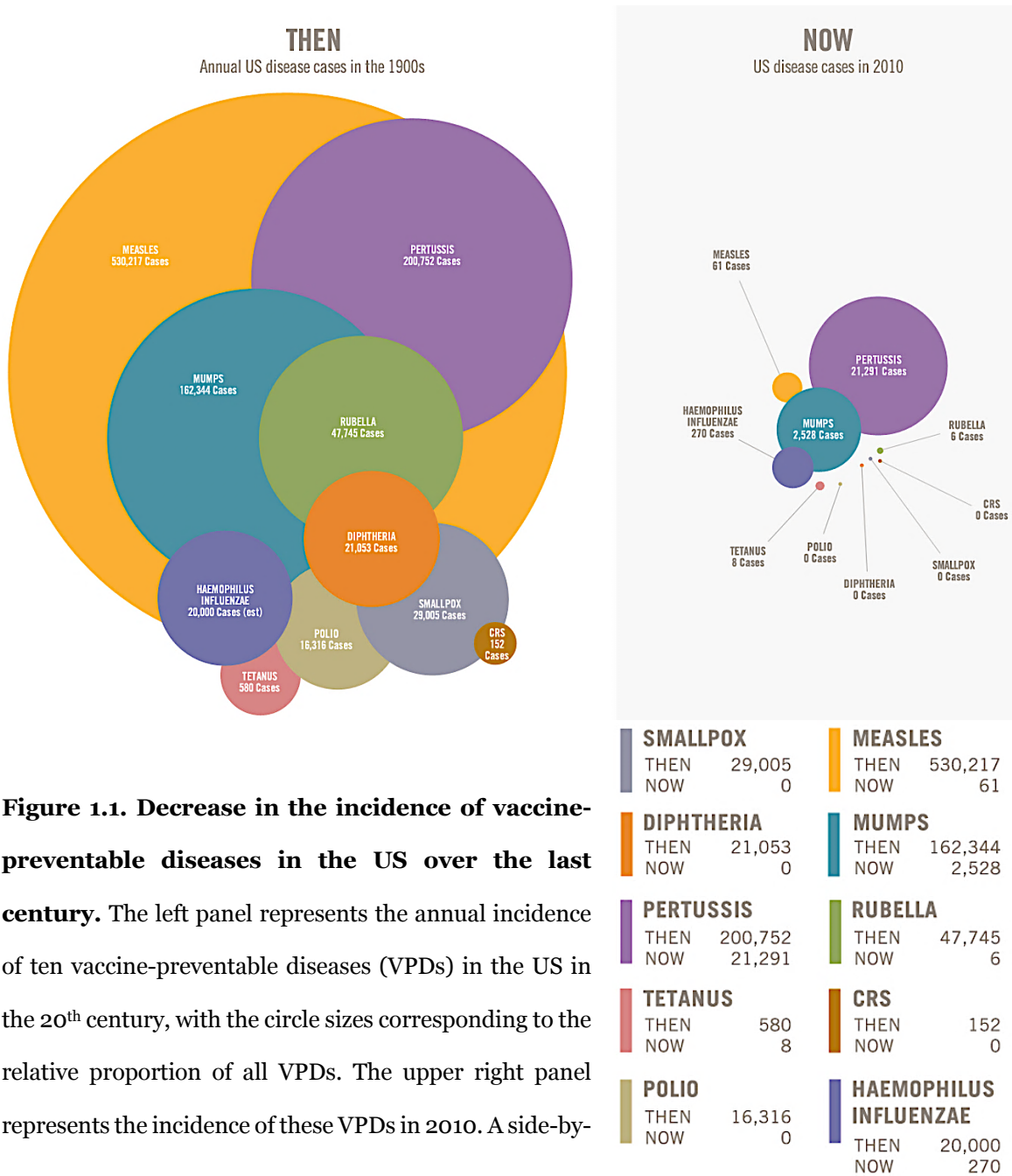


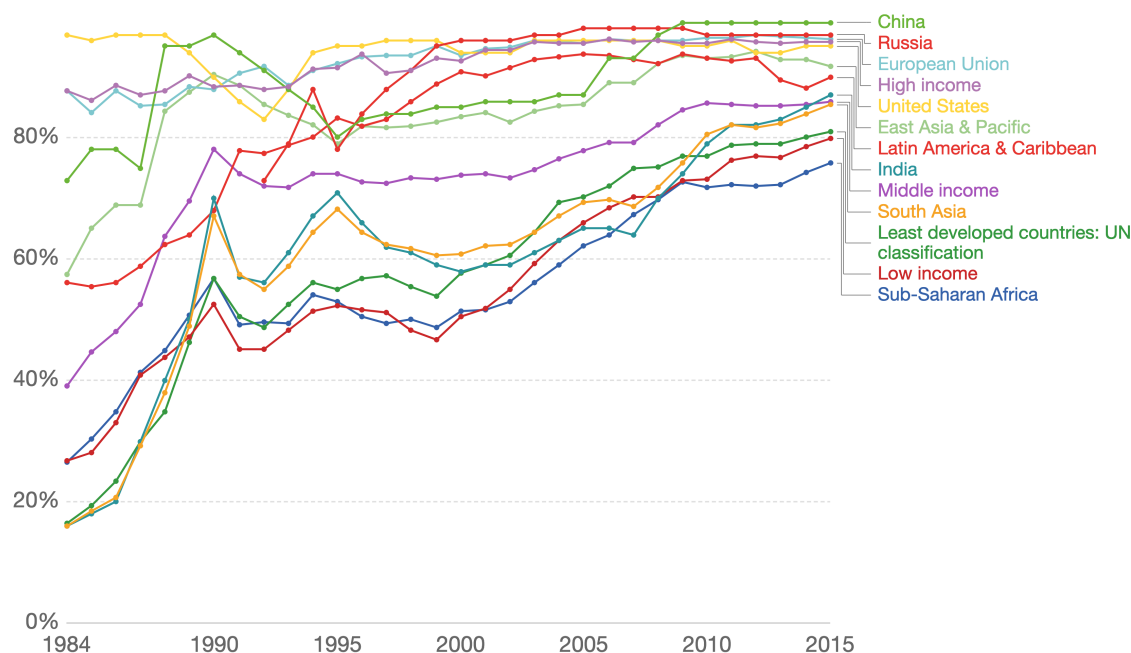
Figure 1.1. Decrease in the incidence of vaccine-preventable diseases in the US over the last century. The left panel represents the annual incidence of ten vaccine-preventable diseases (VPDs) in the US in the 20th century, with the circle sizes corresponding to the relative proportion of all VPDs. The upper right panel represents the incidence of these VPDs in 2010. A side-by-

side numerical comparison of the incidence of these diseases is shown in the lower right panel. Figure adapted from Centers for Disease Control and Prevention data ^{6,7}.

However, the burden of VPD and infant mortality remains substantial in low- and middle-income countries. Recent figures show that diphtheria, tetanus, and pertussis (DTP) vaccine three dose series coverage has reached 86% worldwide (Fig. 1.2), but it is estimated that, globally, as many as 19.4 million children do not have access to essential vaccines – according to WHO and United Nations Children's Emergency Fund (UNICEF) estimates, close to one in ten infants (12.9 million) were not immunised with DTP in 2016 ⁸ – and up to 1.5 million more deaths could be avoided annually by improving vaccine coverage ¹. Access to vaccines is clearly the greatest problem to be overcome in reducing infant mortality worldwide.

Share of childrean immunised against diphtheria, tetanus and pertussis (DTP)

Vaccination coverage of 12 to 23 month old children.



Source: Immunization against DPT – World Bank (WDI) (2016)

OurWorldInData.org/vaccination/ • CC BY

Figure 1.2. Diphtheria, tetanus, pertussis vaccine coverage rates across major geographic regions. Percentage of diphtheria, tetanus, pertussis (DTP) vaccine coverage from 1984–2015 across major geographic regions, countries, and socio-economic classifications. Lowest rates of coverage are associated with low- and middle-income countries, particularly those of sub-Saharan Africa. The highest rates of coverage are associated with China, Russia, the European Union, and the United States; generally speaking, high-income countries. Generated from OurWorldInData.org ⁹ using data published with the World Bank and sourced from the World Health Organization and the United Nations Children’s Emergency Fund ¹⁰.

However, despite widespread access to basic and novel vaccines, there is sub-optimal uptake of vaccines in high-income countries with full immunisation programmes in place. The association between unvaccinated individuals and VPD epidemics has been highlighted by a retrospective study of recent measles and pertussis outbreaks in the US which found that a significant proportion of cases in each outbreak affected those who had not received the relevant vaccine ¹¹. There

are concerns relating to the safety and necessity of vaccination among a small percentage of the population. The factors contributing to this are many, but controversies have had a substantial impact on attitudes towards vaccination. One recent example is the now refuted claim over a proposed link between the measles, mumps, rubella (MMR) vaccine and autism which was set in motion by a paper published in a medical journal in the UK and, as a result of media influence and advocacy groups, diffused across the globe ¹². It took many years before public opinion reverted and MMR vaccine uptake was restored ¹³, though many parents still cite fear of autism as a concern ¹². We have seen a global decrease of greater than 80% in the number of deaths related to measles infection since the new millennium began but there has been a startling resurgence in the incidence of the disease over the past year, with rates increasing by 300% in Europe and 700% in Africa ¹⁴. The WHO have cited vaccine hesitancy among parents as a key driver of this resurgence and have included this phenomenon among its ten greatest threats to global health this year ¹⁵. Identifying, understanding the aetiology of, and managing rare adverse events following immunisation is essential to ensure that such occurrences do not taint the reputation of effective vaccines. Section 1.1.3B will describe how the whole cell pertussis immunisation campaign was compromised by fears of neurological side effects. One of the central aims of this thesis was to improve our understanding of the determinants of fever induced by the four component meningococcal capsular group B (4CMenB) vaccine with a view to informing the design of better tolerated meningococcal vaccines. An explanation of the immune mechanisms of vaccines is warranted at this point, before proceeding to put these responses in the context of fever and to discuss how vaccine adverse events have contributed to public perception in the past.

1.1.2 The immunology of immunisation

Vaccines can be broadly categorised as live-attenuated, inactivated, toxoid-based, or subunit-based vaccines. Vaccines in the former category maintain elements of the infectious cycle of a pathogen while removing the capacity to cause severe infection and include the MMR, smallpox, and yellow fever vaccines ¹⁶. The principle underlying these vaccines is that the immune response to a natural infection confers the host with protection against reinfection and so a mild infection with an attenuated pathogen can induce protection while minimising disease symptoms. Live-attenuated vaccines provide continuous stimulation of the immune system, allowing time for cell-mediated and memory immune responses to form. An alternative to live-attenuated vaccines are inactivated vaccines that contain chemically- or heat-killed killed microorganisms, such as the whole cell pertussis vaccine or the inactivated polio vaccine (IPV). While these vaccines have a better safety profile than the live-attenuated vaccines, as they do not contain live microorganisms, they do not induce as strong of an immune response and may require multiple doses to achieve lasting protection. Certain bacteria, such as *Clostridium tetani* (tetanus) and *Corynebacterium diphtheriae* (diphtheria), produce toxins that can be chemically- or heat-inactivated. Subunit-based vaccines contain purified immunogenic fragments of a pathogen referred to as antigens, such as the hepatitis B virus (HBV) subunit-based vaccine, and some can be conjugated to toxoids to improve memory responses – the pneumococcal conjugate vaccine (PCV) or *Haemophilus influenzae* type B (Hib) and meningococcal conjugate vaccines consist of bacterial polysaccharide conjugated to tetanus or diphtheria toxoid ¹⁶. Inactivated, toxoid, and subunit-based vaccines

are usually combined with substances that enhance the immune response to them, known as adjuvants, with aluminium hydroxide (alum) being used most commonly. The vaccines mentioned thus far represent some of the most effective to date and their development constitutes the era of empirical vaccine design. However, the development of vaccines against difficult diseases, including human immunodeficiency virus (HIV), malaria, tuberculosis (TB), and others for which conventional vaccine development strategies have failed, has proved challenging, largely because of lack of knowledge of what constitutes a protective response against these pathogens and, where this is known, how to induce this response through immunisation ¹⁷. Increasing our understanding of the immune mechanisms of immunisation and how we can tailor these to promote specific protective immune responses is central to the development of the next generation of rationally designed vaccines. The following two sections detail our current understanding of the human innate and adaptive immune responses in the context of immunisation.

1.1.2A Innate immune response

The primary response to an antigenic stimulus is mediated by cells of the innate immune system whose primary function is to restrict the replication of pathogens while signalling their presence to other cells. This occurs through the stimulation of pattern recognition receptors (PRRs) by cognate pathogen-associated molecular patterns (PAMPs), that act as ‘danger’ signals ¹⁸, present in vaccine formulations. Live-attenuated or heat-killed whole microorganism vaccines tend to provide more of these signals than subunit-based vaccines which must usually be administered with an adjuvant to enhance this innate immune

activation. PRRs are germline-encoded and tasked with distinguishing self from non-self in order to elicit this response only when appropriate. They are mainly expressed on innate immune cells that engage in the uptake, degradation, and presentation of exogenous molecules through the process of phagocytosis, and include neutrophils, macrophages, and dendritic cells (DCs) ¹⁹. Toll-like receptors (TLRs), of which there are six surface-exposed and four intracellular located in humans, are abundantly expressed PRRs found in all cell types and recognise a variety of stimuli; TLRs 1, 2, 4, 5, 6, and 10 (absent in mice) are outer membrane-bound and recognise the lipids, proteins, and lipoproteins of microbes; TLRs 3, 7, 8, and 9 (11 absent in humans) are located on vesicles within cells and respond to exogenous nuclear material ²⁰. The best studied of the TLRs is TLR4, which plays a key role in the response to endotoxin, and therefore the recognition of Gram-negative bacteria. TLR4 forms a complex with surface-located MD2 to bind the lipid chains of these molecules ²¹. In addition to TLRs, there are several families of PRRs located in the cytosol, such as the nucleotide oligomerisation domain (Nod)-like receptors (NLRs) that activate a multiprotein complex called the inflammasome leading to the release of the fever-inducing or pyrogenic cytokine interleukin (IL)-1 β ²² and retinoic acid inducible gene (RIG)-I-like receptors (RLRs) that respond to viral RNA ²³. The subsequent cascade of intracellular events culminate in the production of pro-inflammatory signalling proteins, termed cytokines, or immune cell attractants, termed chemokines ¹⁹. The cytokines produced following PRR engagement belong mostly to the IL family of molecules that promote cell differentiation and effector function in an autocrine or paracrine manner ¹⁹. Tissue-resident macrophages have a high capacity to phagocytose, process, and present the vaccine components at the initial site of immunisation while releasing cytokines such as IL-6, tumour necrosis factor (TNF) α , and IL-1 β

to signal an inflammatory response to other cells. Neutrophils can also process and present antigen to prime adaptive immune responses ^{24,25} and natural killer (NK) cells play an important role in mediating between and enhancing the innate and adaptive arms of the immune response to vaccines ²⁶. DCs are the major players in the early immune response to immunisation owing to their capacity for antigen processing and presentation as well as their role in bridging the innate and adaptive arms of the immune response: they possess a wide array of PRRs that are selectively activated in specific DC subsets depending on the administered vaccine. The archetypical live-attenuated vaccine against yellow fever, YF-17D, is known to engage TLRs 2, 7, 8, and 9 as well as RIG-I on both conventional myeloid and plasmacytoid DCs (mDCs and pDCs, respectively) ²⁷, while the live-attenuated influenza virus (LAIV) stimulates RIG-I on mDCs and TLR7 on pDCs ²⁸. The specific PRRs that are stimulated have a downstream influence on the nature of the adaptive response induced ²⁹. Receptor signalling by PAMPs or pro-inflammatory cytokines has the effect of upregulating the nuclear factor-kappa B (NF- κ B) in DCs leading to their activation and maturation which enhances their ability to phagocytose, migrate, and express co-stimulatory molecules ^{30,31}. The processing and presentation of antigen can occur in all nucleated cells via proteasomal degradation and loading of ~eight–ten amino acid peptide fragments onto major histocompatibility complex (MHC) class I molecules that then traffic from the endoplasmic reticulum (ER) to the cell surface (Fig. 1.2) ³². However, exogenous antigens delivered by vaccines are predominantly presented by class II MHC molecules, the expression of which is mostly limited to professional antigen-presenting cells (APCs) ³³. Acidification of phagolysosomal compartments results in degradation of antigen into smaller peptides which can then be loaded onto MHC class II molecules for surface presentation (Fig. 1.3) ³². A mature DC

presenting antigen-derived peptide will then traffic to secondary lymphoid organs where it is capable of stimulating T cells and influencing the subsequent immune responses ³⁴. This interaction delimits the innate and adaptive immune responses.

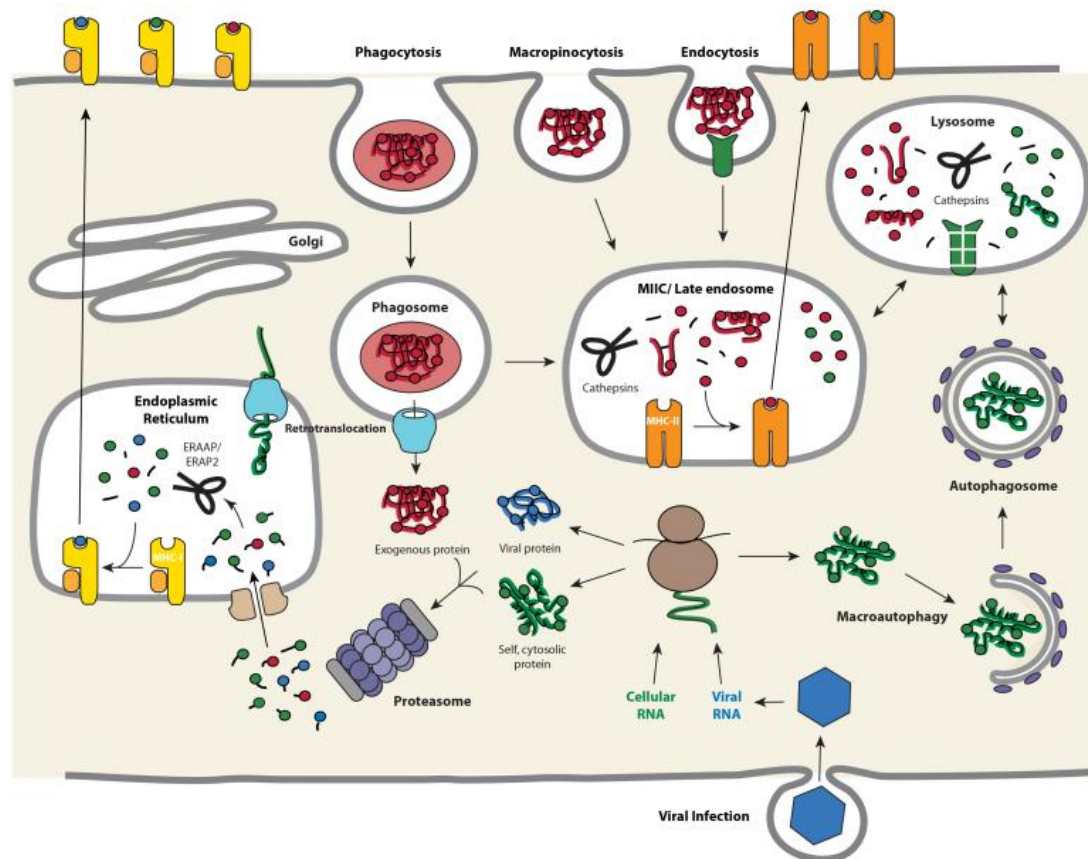
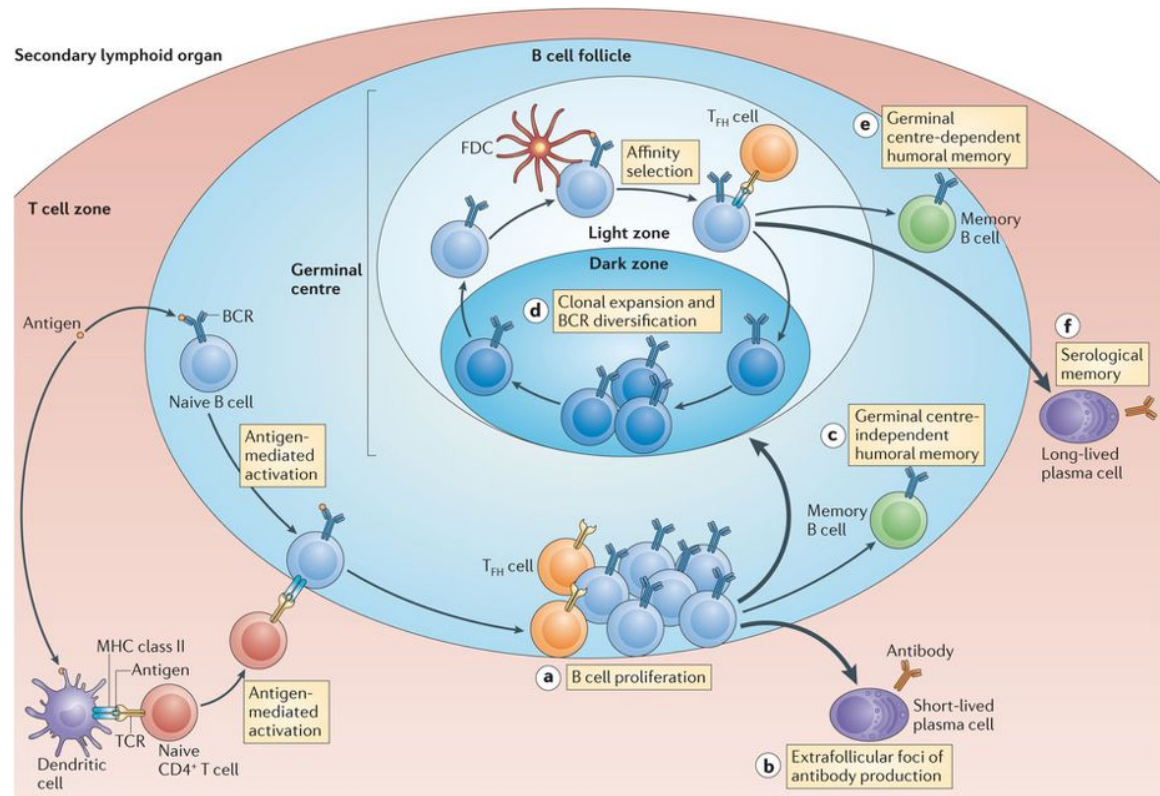


Figure 1.3. Antigen processing and presentation via major histocompatibility complex molecules. Antigen processing and presentation is initiated by cellular uptake of antigenic material through phagocytosis, micropinocytosis, or endocytosis. Exogeneous protein is typically processed via proteasomal degradation followed by loading of small fragments onto major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum. These MHC class I-associated peptides are then transported to the cell surface for presentation. MHC class II presentation occurs through increased acidification of lysosomal or late endosomal compartments leading to disintegration of antigen and loading of peptide onto MHC class II molecules. The late endosome then traffics to the cell membrane for surface exposure of the class II-associated peptide. From Blum *et al.*, 2013 ³².

1.1.2B Adaptive immune response

The primary aim of most vaccines, particularly those that have been effective at preventing disease to date, is the induction of antigen-specific antibodies capable of neutralising a pathogen ³⁵. These responses are generated by B cells in the lymphatics that mature into circulating antibody-secreting plasma cells. Unlike the cells of the innate immune system that express germline-encoded receptors for antigen sensing, the cells of the adaptive immune system express clonally selected receptors that afford greater specificity to their interactions and allow them to recognise MHC-restricted peptides. B cells must undergo multiple rounds of affinity selection and clonal amplification in order to generate this specificity. Activation of naïve B cells occurs predominantly within specialised compartments of lymph nodes known as B cell follicles (Fig. 1.4). T cell-dependent activation of B cells occurs at the interface between this compartment and the surrounding T cell zone where B cells encounter recently primed CD4⁺ T helper (T_H) cells; however, B cells can also be activated by certain antigens, such as endotoxin or polysaccharide, in a T cell-independent manner due to the inherent capacity of B cell receptors (BCRs) to recognise those particular antigens ³⁶. The role of follicular helper T cells (T_{FH}) in the establishment, maintenance, and function of germinal centres in which B cells undergo clonal selection has gained attention in recent years for its functional significance in vaccine-induced responses ³⁷. Activated B cells undergo antibody isotype switching and proliferation with the support of T_{FH} cells and cytokine signals, leading to the formation of transient antibody-secreting plasma cells that depart from the follicle, switching to a memory phenotype, or migration to specialised germinal centre compartments within the B cell follicle ³⁸. Germinal centres consist mainly of B

cells, T_{FH} cells, and follicular DCs. It is here that B cells undergo the iterative process of binding antigen within the light zone, presenting it via MHC to T_{FH} cells to receive survival signals that trigger relocation to the dark zone where they clonally expand and point mutations in the variable regions of their immunoglobulin genes, referred to as somatic hypermutation, occur to produce a repertoire of BCRs ³⁹. Upon returning to the light zone, T_{FH} cells select for the BCRs with the highest affinity and initiate further mutation and selection in a process of affinity selection ³⁹. The result is B cells with highly antigen-specific BCRs capable of leaving the germinal centres to form memory B cells in the follicle or long-lived plasma cells in the serum ³⁸. The formation of immunological memory to specific antigens is key to mounting a strong anamnestic response when it is reencountered in the event of natural infection and is the cornerstone of vaccine-induced protection ⁴⁰.



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Figure 1.4. Activation of B cells within germinal centres. B cells can be activated either directly by certain antigens in a T cell-independent manner, or in a T cell-dependent manner when they engage with antigen-presenting dendritic cell (DC)-activated CD4⁺ T cells at the border between the T cell zone and B cell follicle in secondary lymphoid organs. **A.** Activated B cells then proliferate with help from follicular helper T cells (T_{FH}) and can either **B.** exit the B cell follicle as short-lived antibody-secreting plasma cells in the serum, **C.** switch to a memory B cell phenotype in a germinal centre-independent manner, or **D.** enter the germinal centre to undergo affinity maturation. Within the germinal centre, B cells can undergo iterative rounds of T_{FH} or follicular DC stimulation in the light zone, migration to the dark zone where somatic hypermutation leads to B cell receptor (BCR) diversification, and return to the light zone for selection of BCRs with high affinity for antigen. Clonally expanded and affinity matured B cells can then **E.** exit the germinal centre to switch phenotype to that of a germinal centre-dependent memory B cell or **F.** exit the follicle to become a long-lived high affinity antibody-secreting plasma cell in the serum. From Kurosaki *et al.*, 2015³⁸.

Humoral immune responses are not sufficient to induce protection against several difficult pathogens that require the activation of potent antigen-specific T cell responses ⁴¹⁻⁴³. For this reason, the induction of CD4⁺ and CD8⁺ T cell responses has become the focus of many current vaccine development strategies. The various subsets of CD4⁺ T_H cells are specialised at dealing with particular types of infection – generally speaking, T_H1 cells promote antiviral and antibacterial responses, T_H2 cells defend against extracellular infections such as those caused by parasites, and T_H17 cells are important players in the anti-fungal response and defend against intracellular and extracellular bacterial infections ^{44,45} – and do so by releasing lineage-specific cytokines that activate other immune cells and influence antibody class-switching in B cells ⁴⁶. Further subsets of CD4⁺ T cells have been discovered in recent years, but from the point of view of vaccinology, we are concerned mostly with the aforementioned subsets. T-cell-inducing vaccine strategies aim to induce memory T cell responses directed against the vaccine antigen that can generate a rapid cellular response upon infection. The differentiation choices made by naïve T cells are determined to a large degree by MHC-dependent DC co-stimulation. CD4⁺ T cells receive stimulation from antigen presented via MHC class II molecules interacting with the T cell receptor (TCR) and CD4, cognate receptor binding of co-stimulatory molecules expressed on DCs, and exposure to polarising cytokines will bring about signal transduction and transcriptional changes that determine T cell commitment to a particular T_H lineage ⁴⁷. The transient connections formed between these cell types is referred to as the immunological synapse. CD4⁺ T cells can also be activated through MHC class I-presented peptide through a phenomenon known as cross-presentation ⁴⁸. A cross-primed T cell can then licence a DC to stimulate, via MHC class I, the differentiation of CD8-expressing cytotoxic T cells capable of eliminating infected

cells ⁴⁹. The cellular decisions that influence CD8⁺ effector or memory cell formation in the context of immunisation are the subject of section 1.3.3.

Before discussing novel methods that have been employed to further resolve the mechanisms of vaccine-induced immune responses, the side effects of some of these responses will be discussed in the context of vaccine reactogenicity.

1.1.3 Vaccine-induced fever

The febrile response is a complex process coordinated by the hypothalamus, a part of the brain which facilitates communication between the nervous and endocrine systems. It can be instigated by a range of etiologic and inflammatory agents, but its mechanisms are generalisable and occur independently of the stimulus. The downstream mechanisms of temperature regulation that increase body temperature during fever are the same as those which ensure temperature homeostasis. However, the upstream mechanisms of inflammation-induced fever are important for the understanding of how vaccines elicit fever, the primary objective of the work presented in chapter 4, and will be discussed in the context of immune-physiology and considerations for public perception in the following sections.

1.1.3A Neural mechanisms of fever

The role of the brain in the initiation of fever has been known since the early 1980s when it was demonstrated that animals with severed spinal connection to the brain did not succumb to fever after receiving exogenous pyrogenic stimuli.

However, the exact mechanisms by which certain stimuli promote fever remain to be fully elucidated. Several models have been proposed to explain the pro-inflammatory responses that lead to the initiation of fever in the part of the brain that coordinates thermogenesis, the hypothalamus (Fig. 1.5). The model of inflammation-induced fever that is supported by the most conclusive evidence is that of prostaglandin E₂ (PGE₂) production by brain endothelial cells (BECs) in response to inflammatory signals in the circulation ⁵⁰. It was determined that the expression of microsomal prostaglandin E synthase-1 (mPGES-1) in the brain vasculature is localised to cells possessing the markers of endothelial cells that express the IL-1 type 1 receptor (IL-1R1), cyclooxygenase-2 (COX-2), a key PGE₂-synthesising enzyme and the target of non-steroidal anti-inflammatory drugs; when the genes encoding mPGES-1 and COX-2 are selectively deleted in mouse BECs the mice display greatly reduced fever following intraperitoneal administration of lipopolysaccharide (LPS) despite the generation of a strong inflammatory response ⁵¹. Another study specifically deleted these genes from surrounding nerve and myeloid cells but did not see a similar reduction in fever ⁵². Specific deletion of *Il1r1* on mouse BECs delays the onset of LPS-induced fever indicating that this receptor plays an important role but does not act alone in the transduction of inflammatory signal to the brain ⁵³. This immune stimulus also induces substantial IL-6 signalling and deletion of its cognate receptor also abrogates fever in mice ⁵⁴. TNF α is another endogenous pyrogen capable of eliciting fever, but its mechanism is thought to be more reliant on triggering the production of other cytokines ⁵³. Together, these data help us to build a picture of how immune stimuli release signals that act on the brain to generate a febrile response. These signals play an important role in vaccine-induced fever and will be explored in depth in chapter 4.

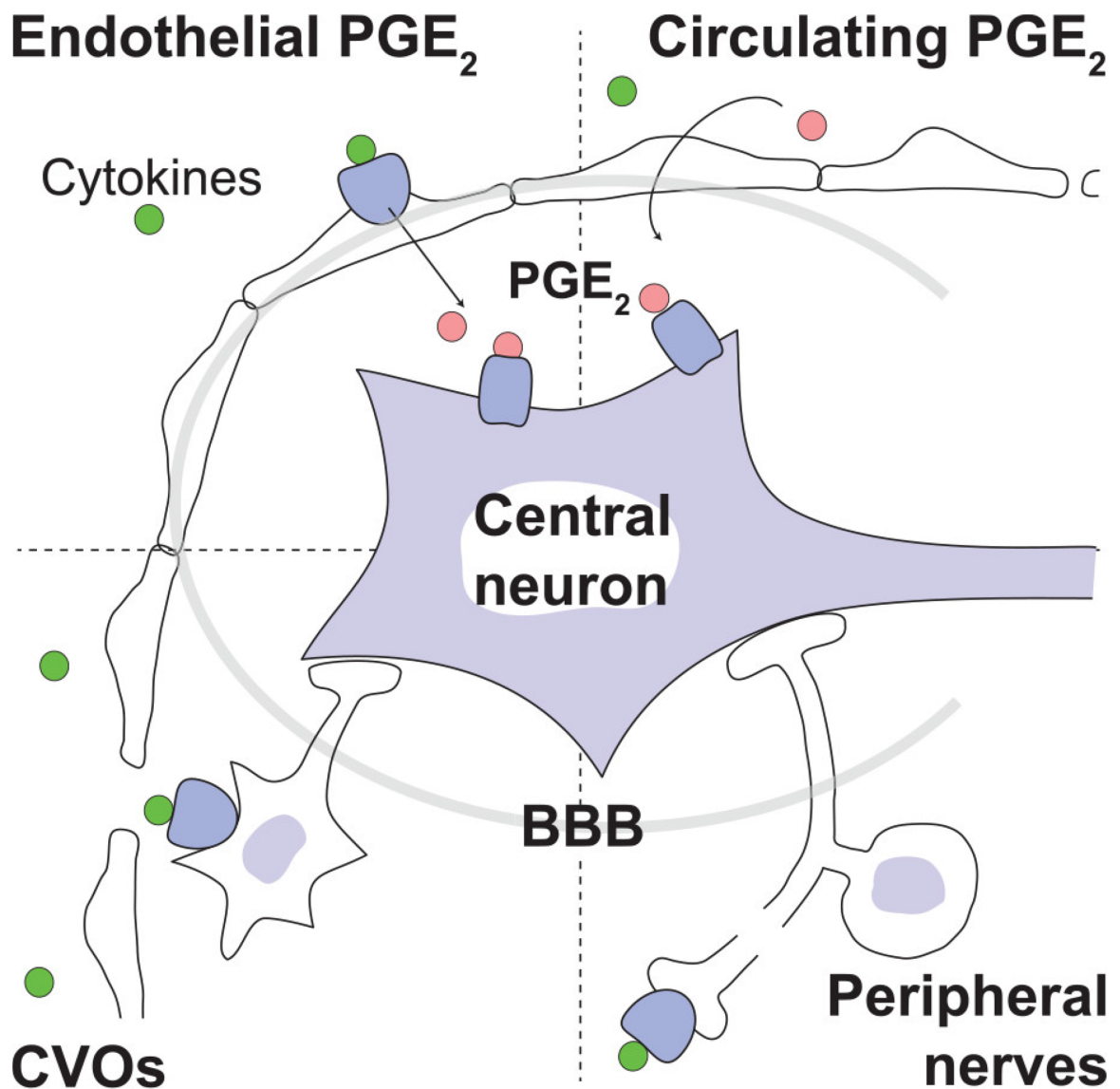


Figure 1.5. Proposed models for transduction of systemic signals on the brain. The hypothesis with the most evidence provided by experimental data involves the release of pro-inflammatory cytokines by immune cells into the circulation which then act on brain endothelial cells at the blood-brain-barrier (BBB) to elicit prostaglandin E_2 (PGE_2) synthesis (top left). PGE_2 then acts on the central neurons to alter the thermoregulatory set point in the brain. Other hypotheses include the transmigration of circulating PGE_2 across the BBB (top right), or the transduction of pro-inflammatory cytokine signals via the circumventricular organs (CVOs) or the peripheral nerves. From Blomqvist and Engblom, 2018 ⁵⁰.

1.1.3B Adverse events following immunisation: a cautionary tale

Immunisation is commonly associated with a range of adverse events that are, more often than not, transient and benign occurrences in the case of licensed vaccines. Candidate vaccines undergo rigorous testing through successive rounds of clinical trials during which reactogenicity is monitored. However, ongoing post-licensure monitoring is essential to ensure that rare events do not emerge when the vaccine administered to large populations. Management of adverse events is also key to managing public perceptions of vaccines. Vaccines have, in the past, been discontinued or replaced in certain countries due to concerns relating to adverse events following immunisation (AEFI). A prime example was the highly efficacious whole cell pertussis preparation based on chemically-inactivated *Bordetella pertussis* bacilli, the causative agent of whooping cough, that was incorporated into a trivalent DTP vaccine for several decades leading to a dramatic decline in disease incidence ⁵⁵. However, concerns began to arise in the 1970s and 1980s about the adverse reactions experienced after its administration with high rates of fever in infants, 47% presenting with a fever > 38 °C, and local inflammation at the site of injection ⁵⁶. Occasionally, severe systemic reactions such as febrile seizures and swelling of entire limbs were observed but investigation of these AEFI found there to be no associated long-term morbidity ⁵⁷. As the prevalence of the disease fell, the fear of vaccine side effects rose among concerned parents. The main reason for the decline in vaccine uptake in the UK was the accusation that the vaccine caused neurological side effects. Uptake of the vaccine also declined considerably in several other European countries due to these reservations, while safety concerns prompted Sweden and Japan to terminate their pertussis immunisation

programmes and several high-profile lawsuits led US firms to cease manufacture of the vaccine ³. An analysis of disease incidence across countries found evidence of causality between pertussis-related anti-vaccine movements and epidemics of the disease ⁵⁸. Complications related to vaccines do occur but are exceedingly rare; however, the conflation of other rare, unrelated maladies with these events can lead to erroneous claims about vaccine safety. These concerns tend to persist in spite of evidence provided against them by subsequent investigation, leading to a loss of confidence among the public and an accompanying resurgence in disease. An alternative DTP vaccine incorporating an acellular pertussis vaccine, containing an inactivated pertussis toxin among other bacterial components, was developed in response to reactogenicity concerns and adopted by several countries, but studies have demonstrated the acellular vaccine to be less effective than the whole cell counterpart ⁵⁹. It is worth noting that the whole-cell vaccine is still the most widely-used pertussis vaccine worldwide and there are no major safety concerns about its use. Nonetheless, it is important to understand the causes, mechanisms, and possible solutions to AEFI as early as possible in the development pipeline to ensure continual uptake of vaccines even as they fulfil their remit. Therefore, there is merit to investigating these phenomena and learning from their occurrence to ensure that vaccine effectiveness needn't be compromised in favour of minimising reactions in future endeavours.

1.1.4 Systems vaccinology

The exciting new discipline of “systems vaccinology” (Fig. 1.6), a term coined by Bali Pulendran in 2008 ⁶⁰, that often combines high-throughput sequencing with conventional immunological assays and other novel techniques to determine genes and pathways responsible for and predictive of vaccine-induced responses ⁶¹. The complex mechanisms that coordinate vaccine responses through the interplay of innate and adaptive immune systems cannot be entirely understood by studying components of these responses in isolation using reductionist methodologies – an integrative, holistic approach is preferable ^{62–64}. By sampling whole blood, we can obtain a snapshot of the immune response to a particular intervention at the level of gene expression that can be correlated with readouts such as antibody or T cell responses. The pioneering work on applying a systems biology approach to vaccinology involved separate studies of the early transcriptional response in peripheral blood to immunisation with the live-attenuated YF-17D vaccine, chosen for its ability to induce strong and persistent protection after a single shot ⁶⁵, in order to identify how the effective cytotoxic CD8⁺ T cell and humoral responses are coordinated and can be reliably predicted at this stage ^{66,67}. This approach has since been used to characterise host responses to candidate vaccines for diseases such as malaria ⁶⁸, HIV infection ⁶⁹, and Ebola ⁷⁰. The identification of early gene signatures that can act as robust correlates of protection would provide many benefits to vaccine clinical trials, particularly those at early stages of the development pipeline ⁷¹. The use of functional genomics to explore innate immune responses to vaccines may also identify the cause of AEFI and inform the design of better tolerated vaccines and adjuvants ⁷². This

underexplored strategy will be applied to the analyses of vaccine-induced fever presented in chapter 4. The integrated analyses of data generated from new -omics technologies promises to deliver unprecedented insight into the interactions between gene regulation, molecular signals, and immune cell populations ⁷³ and will no doubt prove useful in the field of systems vaccinology. However, it is important that the era of “big data” in vaccinology does not fulfil the cautionary prophesising of the late Nobel prize-winning geneticist, Sydney Brenner:

“We are drowning in a sea of data and thirsting for knowledge. Most biology today is low input, high throughput, no output biology...The idea that we'll dissect [cellular] complexity by making lots of measurements is bound to fail...The orgy of fact extraction in which everybody is currently engaged has, like most consumer economies, accumulated a vast debt. This is a debt of theory, and some of us are soon going to have an exciting time paying it back – with interest, I hope ⁷⁴.”

In order to mitigate this “debt of theory”, one must continually validate findings from high-throughput experiments and relate these findings to the vast amount of data already in existence. Rational vaccine design will not be guided by big data alone but must draw from decades of work that have gone into understanding the pathogenesis of disease. The next sections will provide an in-depth overview of what is known about the pathogenesis of meningococcal disease and how efforts to develop a broadly protective vaccine led to one of the most successful examples of rational vaccine design based on genomics.

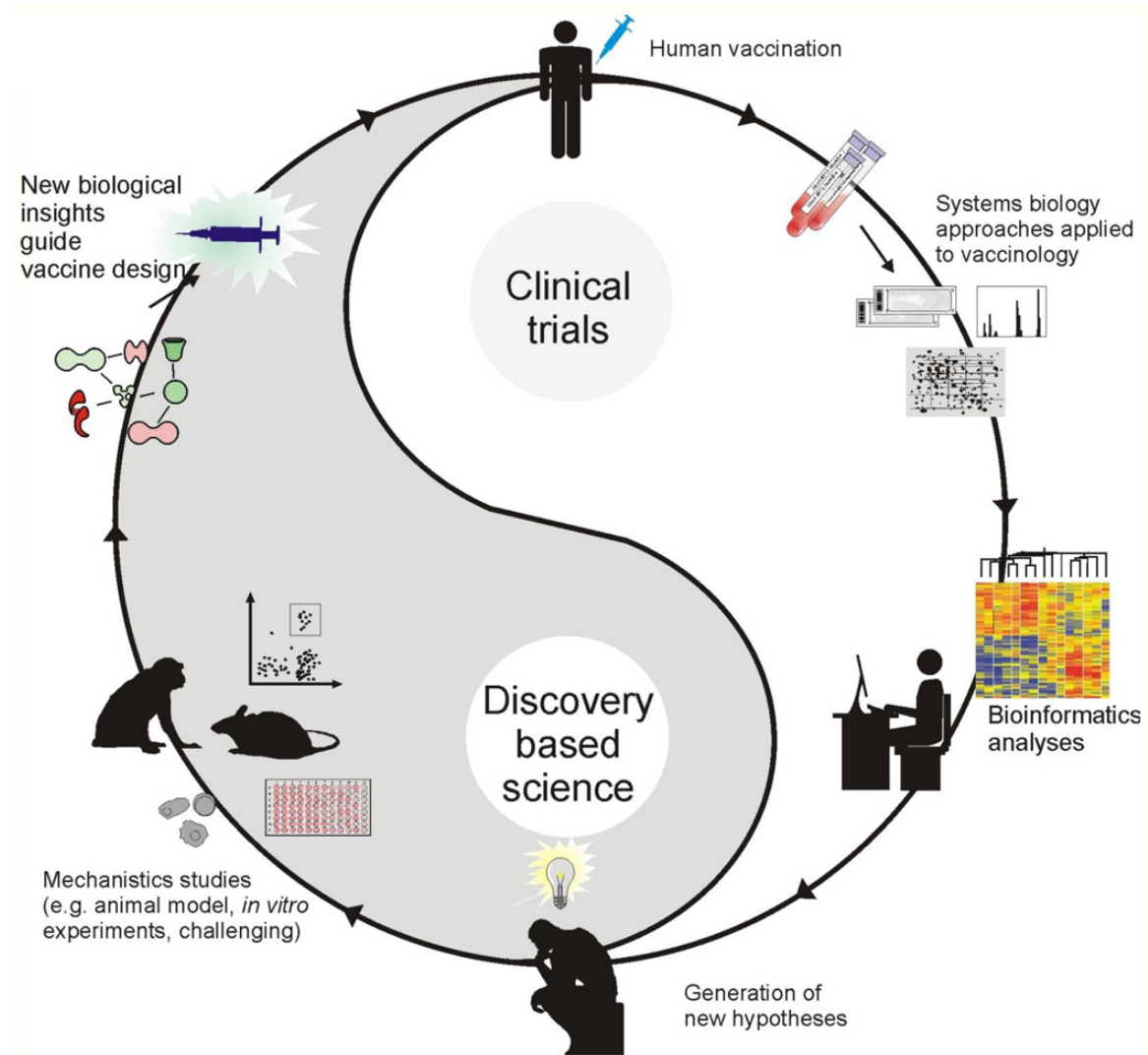


Figure 1.6. A conceptual outline of systems vaccinology. Functional genomics samples from vaccine clinical trials can be analysed alongside conventional immunological readouts to yield a variety of insights into early gene expression changes that influence later humoral and cellular immune responses, leading to the generation of novel hypotheses that can be functionally validated in animal models and potentially informing the rational design of improved vaccines. From Pulendran *et al.*, 2010 ⁷¹.

1.2 The meningococcus

Neisseria meningitidis (*N. meningitidis*), also referred to as the meningococcus, is a Gram-negative diplococcus and obligate human pathogen of global significance. It is the causative agent of invasive meningococcal disease (IMD), a condition that most commonly manifests as septicaemia and, owing to the tropism of the bacterium for brain tissue, acute inflammation of the meninges of the central nervous system (CNS). The disease is associated with a substantial proportion of infectious deaths among infants, particularly in industrialised countries, and long-term morbidity in survivors. The bacterium exhibits a diverse range of unique mechanisms for survival in and invasion of host tissues that distinguish it from other Gram-negative bacteria and are the subject of the following sections.

1.2.1 Epidemiology of invasive meningococcal disease

The vast majority of *N. meningitidis* isolates are encapsulated by a polysaccharide encoded by the *cps* locus of the bacterial genome and the nature of this outer capsule allows the bacterium to be classified as one of 12 known capsular groups, half of which are responsible for the vast majority of IMD worldwide: A, B, C, W, X, and Y ⁷⁴. The WHO do not have an official figure for the global burden of IMD, but it is considered endemic on a global scale and prone to epidemics. It is estimated that IMD claims somewhere between 50,000 and 135,000 lives annually, with up to 1.2 million cases every year ⁷⁵. The disease can occur throughout life but the incidence peaks in those less than one year old ⁷⁶.

Patterns associated with the disease exhibit considerable temporal and geographic variation with higher proportions of disease burden attributable to specific capsular groups across large areas. The highest attack rates are observed in a region known as the “meningitis belt” across 26 countries in sub-Saharan Africa, where large and frequent (~once per decade) epidemics impact on a fluctuating attack rate of 20–1,000 cases per 100,000 ⁷⁵. The dustiness and low moisture content of the air in these countries is thought to contribute to the high incidence of disease that peaks during the dry season ⁷⁷. Capsular group A (MenA) predominated in this region ⁷⁸ until the implementation of a successful immunisation campaign ⁷⁹, subsequent to which group W (MenW) began to predominate along with a rise in the incidence of group C disease (MenC) ⁷⁶. The emergence of disease caused by MenW resulted from several outbreaks of hyperinvasive strains ⁸⁰, including a strain associated with the Hajj pilgrimage in 2000 that spread across the globe and persisted for multiple years ⁸¹. A quadrivalent MenACWY tetanus toxoid conjugate vaccine is currently in an advanced stage in clinical trials for the meningitis belt in Africa ⁸². While MenA was more prevalent throughout the early 20th century, capsular groups B (MenB) and C began to predominate across Europe, the Americas, Australasia, and parts of Asia towards the latter part of the century ⁷⁵. The high burden of disease caused by MenC across Europe, particularly in the UK which had a MenC attack rate of 5 per 100,000, and the advent of effective MenC prophylaxis (section 1.2.3A) prompted several national immunisation campaign initiatives ⁸³. In the intervening years, MenB has taken centre stage (Fig. 1.7), also due to the lack of an effective vaccine for the majority of this period, and is currently responsible for up to 90% of IMD cases in the UK ⁷⁵. For this reason, efforts have focused on the

development of an efficacious preventive intervention for MenB disease since the early 2000s.

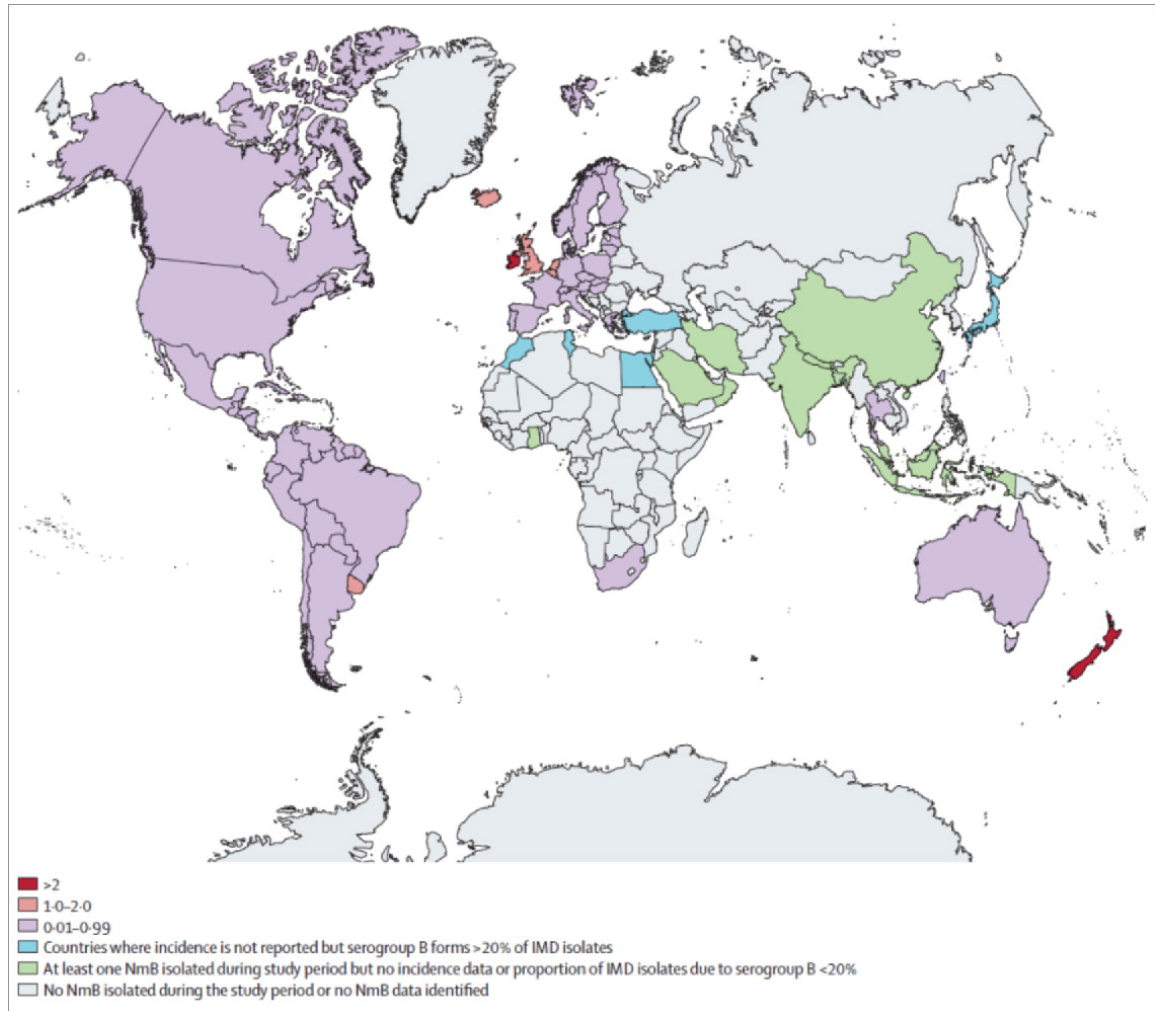


Figure 1.7. Global distribution of meningococcal disease caused by capsular group B.

Colours correspond to the categorisation of the incidence of meningococcal disease caused by *Neisseria meningitidis* capsular group B (here abbreviated as NmB) per 100,000 individuals between January 2000 and March 2015. The countries with the highest incidence during this period, highlighted in red, were the Republic of Ireland and New Zealand followed by the UK, the Netherlands, and Belgium, highlighted in orange. From Villena *et al.*, 2018 ⁸⁴.

1.2.2 Pathophysiology of invasive meningococcal disease

The meningococcus, like most invasive bacterial species, is highly adapted to surviving the host innate immune responses in the blood. Prior to the progression of invasive disease, there are requirements of the bacterium to establish colonies within host tissues, as well as host mechanisms of restricting this growth, and the mechanisms that the bacterium utilises to evade host defences.

1.2.2A Colonisation of the nasopharynx

N. meningitidis is a common commensal of the nasopharyngeal mucosa; the rates of nasal carriage are lowest in infants ⁸⁵, peak in young adults, with rates as high as 55% in the UK among students at university ⁸⁶, and decline to less than 10% later in adulthood ⁸⁷. Carriage is typically an asymptomatic and an immune sensitising event ⁸⁸, though the link between carriage and invasive disease is complicated and not proportional ⁸⁹. The first stage of colonisation of the nasopharynx requires adhesion of the bacterial Type IV pilus (Tfp) to nasopharyngeal epithelial cells ⁹⁰. This adherence is strengthened by a variety of outer membrane proteins (OMPs) including the colony opacity-associated (Opa) proteins that bind carcinoembryonic antigen cell adhesion molecules (CECAMs) on the host cells and other adhesins such as the trimeric neisserial adhesin A (NadA) autotransporter ⁹¹. Expression of NadA is variable between clinical isolates and is controlled by a transcriptional repressor encoded by *nadR* ⁹². This repression is inhibited by the activity of hydroxyphenylacetic acid in human saliva thereby inducing expression of stable NadA trimers on the surface of the bacterium

⁹². *N. meningitidis* possesses numerous other adhesins that may act in concert or at different locations to coordinate bacterial colonisation throughout infection.

The genetic and antigenic diversity exhibited by meningococcal carrier isolates is known to be greater than that of disease isolates ⁸⁵. Although the exact host and pathogen factors that influence the transition from colonisation of the nasopharynx to invasion of the bloodstream remain poorly understood, epidemiological data have associated the presence of a bacteriophage and a haemoglobin receptor gene with invasive isolates ^{93,94}. From a host perspective, infection with influenza A virus, occupying college dormitories ⁹⁵, smoking and attending crowded bars ⁹⁶, as well as genetic factors identified through genome-wide association studies have all been identified as predisposing factors for meningococcal septicaemia (meningococcaemia) ^{97,98}.

1.2.2B Survival in the blood

N. meningitidis has evolved many virulence factors that promote its survival in the blood, particularly against complement and innate immune cells. While expression of meningococcal capsular polysaccharide at the stage of nasopharyngeal colonisation would prevent adherence to and transmigration of the host epithelium by concealing the outer membrane proteins necessary for adhesion and internalisation, its expression is essential for bacterial survival during the invasive phase of disease ^{99–101}. Capsule biosynthesis proteins are encoded by the *cps* locus and expression is regulated by two types of phase variation: transposable genetic elements inserted into the DNA and slipped-strand mispairing ^{99,102,103}. Host protection takes the form of bactericidal antibodies in the serum, the titres of which are inversely proportional to disease susceptibility ¹⁰⁴.

While the polysaccharidic capsule evolved to protect the bacterium from bactericidal activity, antibodies directed at the capsule, with the exception of those generated against capsular group B, form the basis of protective immune responses induced by meningococcal polysaccharide conjugate vaccines that led to very successful immunisation campaigns ¹⁰⁵. The meningococcus is particularly well-adapted to the evasion of complement-mediated killing and exhibits superior resistance to killing by complement when compared with other Gram-negative pathogens, requiring high concentrations of human serum to prevent growth ¹⁰⁶. The importance of a functioning complement system is vital for host clearance of the *N. meningitidis* bacterium, evidenced by the dramatically increased susceptibility of individuals with complement deficiencies to invasive disease ¹⁰⁷. Therefore, the current gold standard correlate of protection against meningococcal disease is based on the ability of antibody to induce complement-mediated killing of the bacterium ¹⁰⁸.

1.2.2C The complement system

The complement system plays a critical role in the host innate immune response against bacterial pathogens and also plays an important role in the modulation of adaptive immune responses ¹⁰⁹. The cascade of small molecule proteolysis that characterises the complement system can be initiated by either the classical pathway or lectin pathway and may be further stimulated by activation of the alternative pathway (Fig. 1.8), with each converging on the formation of C3 convertase ¹⁹. In the classical pathway, binding of antibodies capable of fixing complement, such as immunoglobulin (Ig)G and IgM, to the surface of a pathogen triggers the attachment of the C1 complex, consisting of C1q, C1r, and C1s, to the Ig

fragment crystallisable (Fc) region where the autocatalytic activity of the C1r and C1s serine proteases yield the fragments for the generation of C3 convertase ¹¹⁰. The lectin pathway is activated independently of antibody-pathogen complexes and is instead initiated by the recognition by mannose-binding lectin (MBL) or ficolin of conserved antigens, PAMPs, leading to the formation of a complex with MBL-associated serine proteases (MASPs) to yield C3 convertase in an analogous manner to the C1 complex ^{111,112}. The mechanism of the alternative pathway is distinct in that it is activated by the hydrolytic conversion of C3 into C3a and C3b spontaneously on the surface of pathogens, with the deposition of the latter instigating a loop of amplified C3 cleavage ¹¹³. The latter stage of all three pathways converge on C3 hydrolysis leading to the release of C3a as a chemoattractant for phagocytic cells and the binding of C3b to receptors on these phagocytes, stimulating phagocytosis ¹¹⁴. C3b forms part of a convertase responsible for cleaving C5 into C5a, another chemoattractant, and C5b, the initial molecule required to form a multimeric structure known as the membrane-attack complex (MAC) capable of lysing cells ^{114,115}. Phagocytic uptake is the most common form of clearance for the majority of bacteria, but the susceptibility of *N. meningitidis* to MAC-mediated lysis is apparent from case studies of patients deficient in components of this pathway ¹¹⁶.

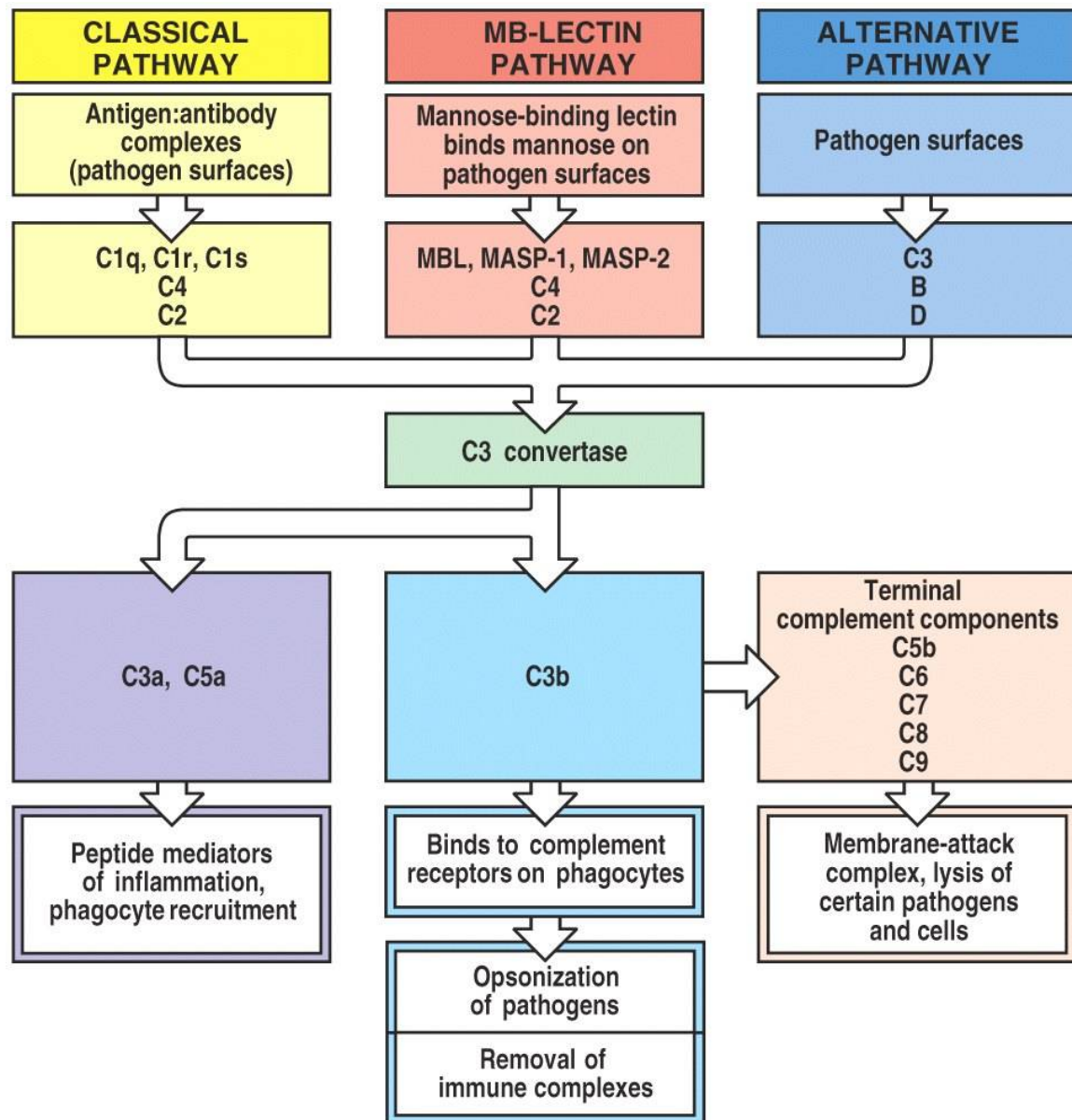


Figure 2-19 Immunobiology, 6/e. (© Garland Science 2005)

Figure 1.8. The complement system. Shows the three pathways of complement activation, the classical, mannose-binding (MB)-lectin, and alternative pathways, all of which lead to the generation of C3 convertase capable of producing inflammatory mediators, promoting complement-dependent phagocytosis, and initiating the formation of a membrane-attack complex. From Janeway *et al.*, 2005¹⁹.

1.2.2D Complement evasion

Human clearance of meningococci can occur via all three pathways. The serum bactericidal antibody (SBA) assay, the correlate of protection used to measure protection conferred by vaccine-induced antibody, is based on classical pathway bacterial killing elicited by complement-fixing Ig ¹⁰⁸. MBL deficiency is a frequently inherited condition that predisposes individuals to meningococcal disease ¹¹⁷. The bacterium has evolved a variety of defence mechanisms to regulate effectors of these pathways, particularly the alternative pathway. Sialic acid protects host cells from lysis by complement and is the main constituent of the polysaccharide capsule of encapsulated *N. meningitidis* ^{103,118}. The polysaccharide capsule also serves to prevent insertion of the MAC into the bacterial membrane and is therefore indispensable for survival of the bacterium in the blood ¹¹⁹. Another neisserial attribute that promotes resistance to serum killing is the membrane-bound lipooligosaccharide (LOS) containing α - and β -chain carbohydrates linked to the inner core and lipid A moiety, rather than the polymeric glycan O-antigen structure of LPS ¹²⁰. The LOS α -chain exhibits molecular mimicry with an epitope present on certain blood groups in humans, lacto-N-neotetraose, to which a sialic acid moiety can be added, mildly increasing resistance to complement-mediated killing ^{121,122}. In addition to the integral protection afforded by the capsule and membrane-bound endotoxin, the meningococcus expresses multiple OMPs capable of sequestering host complement regulators (Fig. 1.9). For example, the C4 binding protein (C4BP) found in the circulation dissociates C3 convertase and can be bound by porin A (PorA) and Tfp. The OMP known to bind complement factor H (fH), a negative regulator of the alternative pathway that engages C3b in tandem with markers

recognised as host to differentiate self from non-self ¹²³, is of particular interest for vaccine development and is discussed at length in section 1.2.3C. Finally, shedding of outer membrane vesicles (OMVs), formed through blebbing of the outer membrane as a stress response ¹²⁴, is a potential mechanism for directing the immune response away from the bacterium as OMVs are known to activate complement ¹²⁵. A variety of OMPs in association with membrane-bound endotoxin make OMVs immunogenic and an attractive vaccine platform for meningococcal disease (section 1.2.3B).

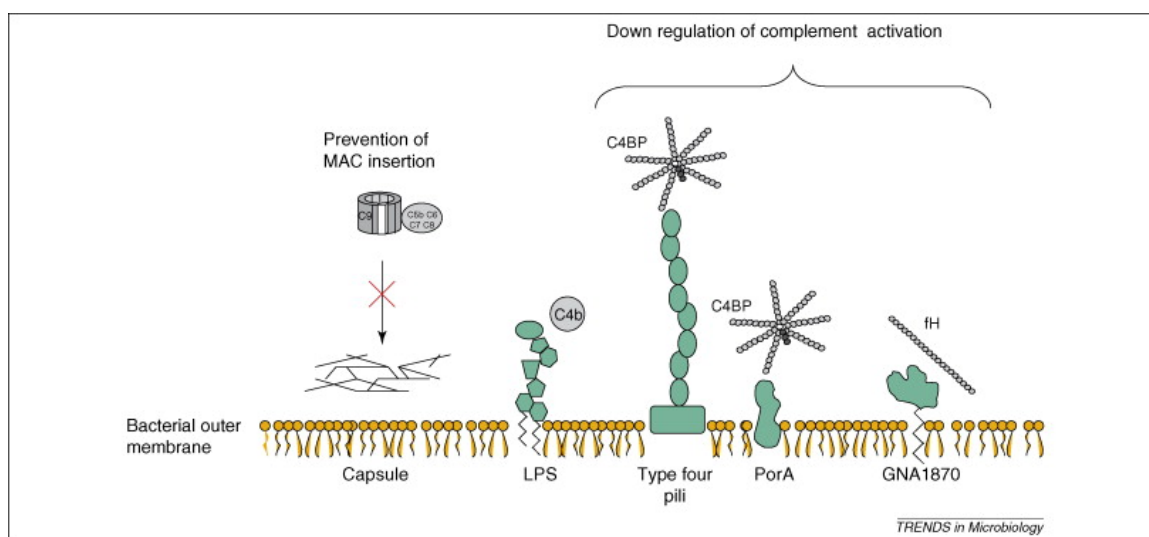


Figure 1.9. Meningococcal defences against complement-mediated killing. The meningococcus possesses a variety of surface-expressed outer membrane proteins capable of protecting the bacterium from complement-mediated killing or downregulating specific complement activators found in circulation. From Schneider *et al.*, 2007 ¹²⁶.

1.2.2E Interaction with the vasculature

The pressure exerted by the human circulation on invading bacteria must be overcome if the bacteria are to replicate and grow in the bloodstream. This requires some form of attachment to host cells and neighbouring bacteria in order to establish microbial colonies and provide protection against shear stress. Due to the requirement for low pressure environments, the bacterium has an affinity for the capillaries where the rate of blood flow facilitates adhesion ¹²⁷. Expression of the Tfp, a polymeric filament found on the surface of *N. meningitidis* and other Gram-negative bacteria, is vital for interaction of the bacterium with host cells ¹²⁸. Initial attachment to the vasculature occurs via Tfp binding to the Ig-like CD147 protein (basigin) on the luminal surface of endothelial cells ¹²⁹. Interbacterial contact via Tfp promotes modification and release of some bacteria, allowing them to migrate elsewhere to establish new colonies ¹³⁰. Initial contacts between Tfp and endothelial cells trigger a signalling pathway via a G protein-coupled receptor, the β 2-adrenoreceptor (β 2AR), which recruits proteins known as β -arrestins to the site of Tfp binding ¹³¹. Recruitment of host cellular proteins beneath the site of the newly formed colony leads to reorganisation and polymerisation of the actin cytoskeleton to the site of bacterial adhesion ¹³². This creates extensions from the cell membrane that enfold the bacteria, increasing the surface available for them to adhere and protecting them from shear stress ¹²⁷ and tight junctions between these junctions are loosened, making them accessible to bacteria ¹³². From small numbers of diplococci entering the bloodstream the concerted action of bacterial Tfp effectors can lead to the colonisation of apical surfaces of endothelia where host cell responses facilitate transmigration and rapid dissemination to other parts of

the body. These phenomena explain the progression and pathology of invasive disease.

1.2.2F Clinical manifestations

Clinical presentation of IMD varies between cases and may progress rapidly; milder symptoms can progress quickly to severe bacteraemia and multi-organ failure ¹³³. The factors that influence disease presentations and progression are mostly immunological in nature and stem from the response to the activity of the bacterium. Metrics such as the quantity of circulating endotoxin and bacterial DNA are reliable correlates of disease severity and clinical manifestation – <0.5 infectious units (IU)/mL of LOS and <1000 copies of DNA indicates meningitis only, >40 IU/mL LOS and $\sim 2 \times 10^7$ copies of DNA indicates septicaemia ¹³⁴. Host genetic polymorphisms related to the innate immune response play a significant role in susceptibility to and severity of the disease ¹³⁵. IMD presents itself most commonly in the form of meningitis, the characteristic features of which are caused by the immune response to the infiltration of bacteria in the capillaries between the arachnoid and the pia mater (Fig. 1.10) ¹³⁶. Symptoms of meningococcal meningitis are often non-specific in children, including fever, irritability and crying but may be more specific with signs such as photophobia and neck-stiffness ¹³⁷. Meningococcaemia, replication of the invading bacteria in the bloodstream and release of high quantities of endotoxin triggering a strong inflammatory response, is more readily identifiable by the appearance of purpura or petechiae on the skin ¹³⁸. The immunopathology results in purpura fulminans – thrombosis, coagulation, and severe damage to the vasculature – and myocardial depression that contributes to the multiorgan failure and mortality associated with the disease ¹³⁹.

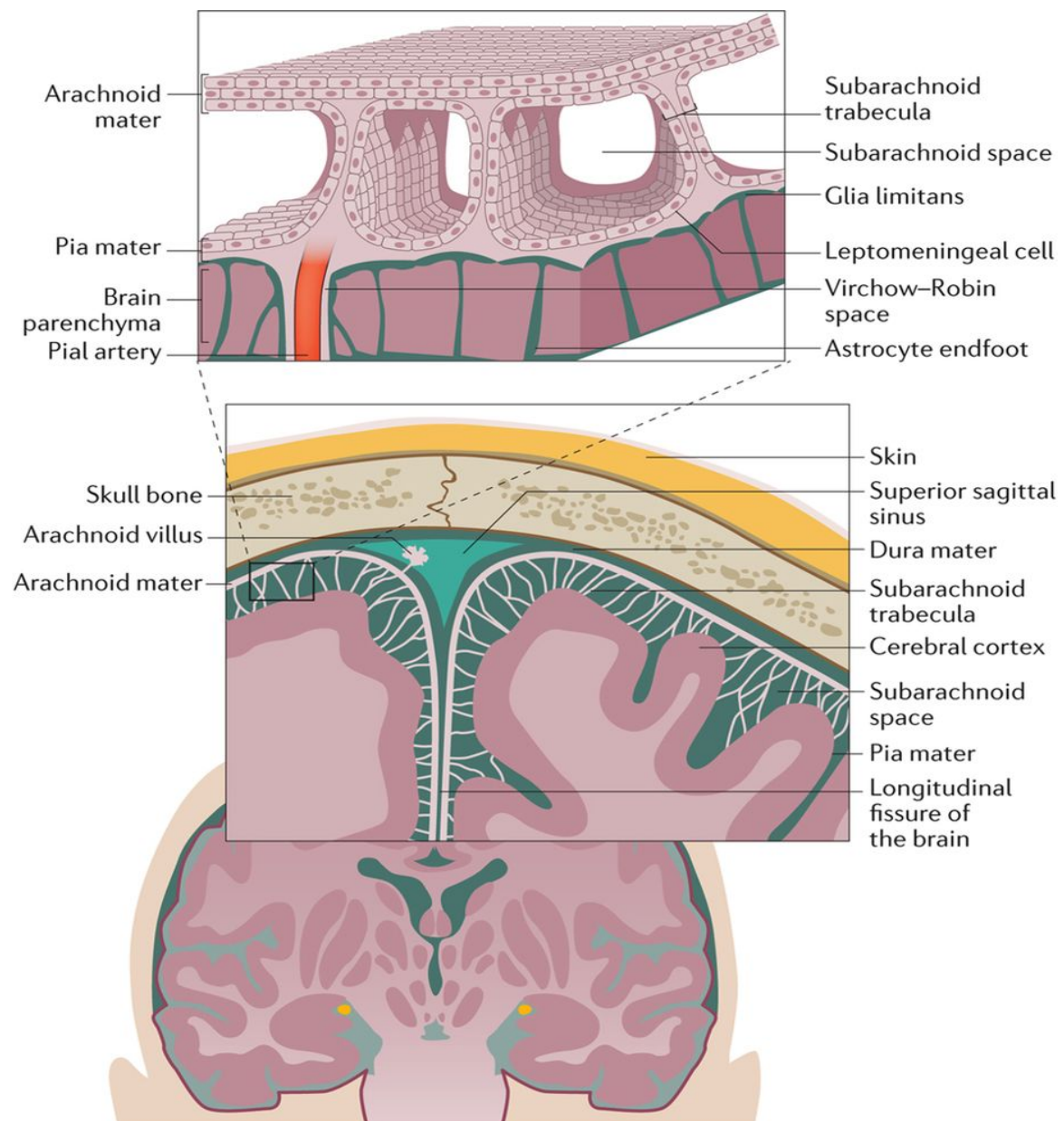


Figure 1.10. Areas of the brain pertaining to invasive meningococcal disease. The leptomeninges consist of the pia mater which continues into the arachnoid mater. The subarachnoid space is filled with connective tissue that form a trabecula structure lined with leptomeningeal cells and containing blood vessels. The continuous layer of cells formed by these trabeculae and the pia mater create a barrier that allows H_2O and low-molecular-weight solute to passively diffuse across. The glia limitans is a boundary composed of astrocyte appendages that delimits the pia mater and the basal lamina of the brain parenchyma, which envelopes the central nervous system (CNS). These tissues constitute the meninges of the human brain and represent the locations through which the meningococcus is most likely to traverse from the circulation into the CNS to cause meningococcal meningitis. From Coureuil *et al.*, 2017¹⁴⁰.

Our understanding of the interplay between the host and pathogen during invasive disease is derived mostly from the study of biopsies and post-mortem samples. It has been observed that the bacteria interact directly with the capillary endothelium where they can form large colonies and are found within purpuric lesions along with retracted endothelial cells, consistent with the effects of CD147 and β 2AR engagement ¹⁴¹. Meningococci can also be internalised by endothelial cells and found in the junctions between endothelial cells in cutaneous lesions ¹⁴². The precise location and mechanism by which *N. meningitidis* crosses the BBB remain unknown, but post-mortem analyses of brain tissue have shown the presence of bacteria attached to BECs of the choroid plexuses, parenchyma, leptomeninges, where adhesiveness was correlated with the expression of a Tfp component ¹⁴³. *N. meningitidis* most likely transmigrates the endothelium at one or more of these locations by employing the mechanisms of bacterial adhesion and signalling that disrupt endothelial cell tight junctions, outlined in the previous section, to gain access via the paracellular route ¹³².

1.2.3 Meningococcal vaccine development

The fulminant nature of IMD is such that there is a very short window for therapeutic intervention. Early symptoms of IMD are often non-specific, making it difficult to diagnose, but progress rapidly in severity and, despite technological advances in healthcare and our ability to manage the disease, the case-fatality rate remains high at about 20–50% of patients who have developed septic shock and one in ten overall, depending on the setting ¹⁴⁴. Survivors of IMD are often left with

debilitating long-term morbidities including neurological impairment, loss of hearing, scarring or loss of limbs, with such sequelae manifesting in about 20-30% of those afflicted ¹³⁷. These factors make disease prevention through immunisation the optimal strategy to reduce morbidity and mortality associated with meningococcal disease. It was determined by the pioneering work of Irving Goldschneider and colleagues that a certain quantity of bactericidal antibody in circulation is associated with reduced susceptibility to IMD and employed an SBA assay to ascertain how well antibody in sera can kill the meningococcus in a complement-dependent manner ¹⁰⁴. The subsequent determination that an SBA titre greater than or equal to 1:4 was associated with a lower risk of disease ¹⁰⁴ led to the establishment of this SBA titre as an accepted correlate of protection against IMD, now considered the gold standard for predicting vaccine effectiveness and providing the basis for licensure ¹⁴⁵. The most successful endeavours to develop a prophylactic intervention for meningococcal disease will be reviewed in the following sections.

1.2.3A Conjugate vaccines

As the main virulence factor common to encapsulated strains of *N. meningitidis*, the capsular polysaccharide represented an obvious target for a meningococcal vaccine. The first meningococcal vaccines were based on these polysaccharides alone and their use dates back to the 1970s, but this antigen is T-independent as it is not recognised by the T cell receptor and are therefore not sufficiently immunogenic in children under two ¹⁴⁶. They also failed to induce lasting protection in an adult population ¹⁴⁷. Coupling of capsular polysaccharide carrier proteins that recruit and engage T cells, such as the tetanus toxoid, was

shown to enhance the immunogenicity of these preparations ^{148,149} and several meningococcal polysaccharide-protein conjugate vaccines were licensed around the turn of the century ¹⁵⁰. This led to successful conjugate vaccine immunisation programmes against MenC, implemented first in the UK in 1999 ¹⁵¹ followed by several countries across Europe, leading to a dramatic decline in the incidence of disease caused by this capsular group (Fig. 1.11) ¹⁵². The success of MenC conjugate vaccine national programmes was due in part to their ability to extend protection to unvaccinated individuals, a phenomenon known as herd protection, by reducing the attack rate of the disease and MenC carriage in this cohort ^{153,154}. Polysaccharide conjugate vaccines aimed at providing broad protection against multiple capsular groups have also been licensed in several countries, such as the quadrivalent ACWY glycoconjugate vaccine ¹⁵⁵. However, the polysaccharide-protein conjugate strategy has proved ineffective for developing an effective MenB vaccine due to the identity between its capsular constituent, *N*-acetylneuraminic acid with $\alpha(2,8)$ -linkage, and a cell adhesion molecule found on mammalian neural tissue rendering its polysaccharide poorly immunogenic ¹⁵⁶. The disparity between the progress made with prevention of MenB and other capsular groups has led to the predominance of MenB as the cause of IMD in high-income countries such as the UK ¹⁵⁷.

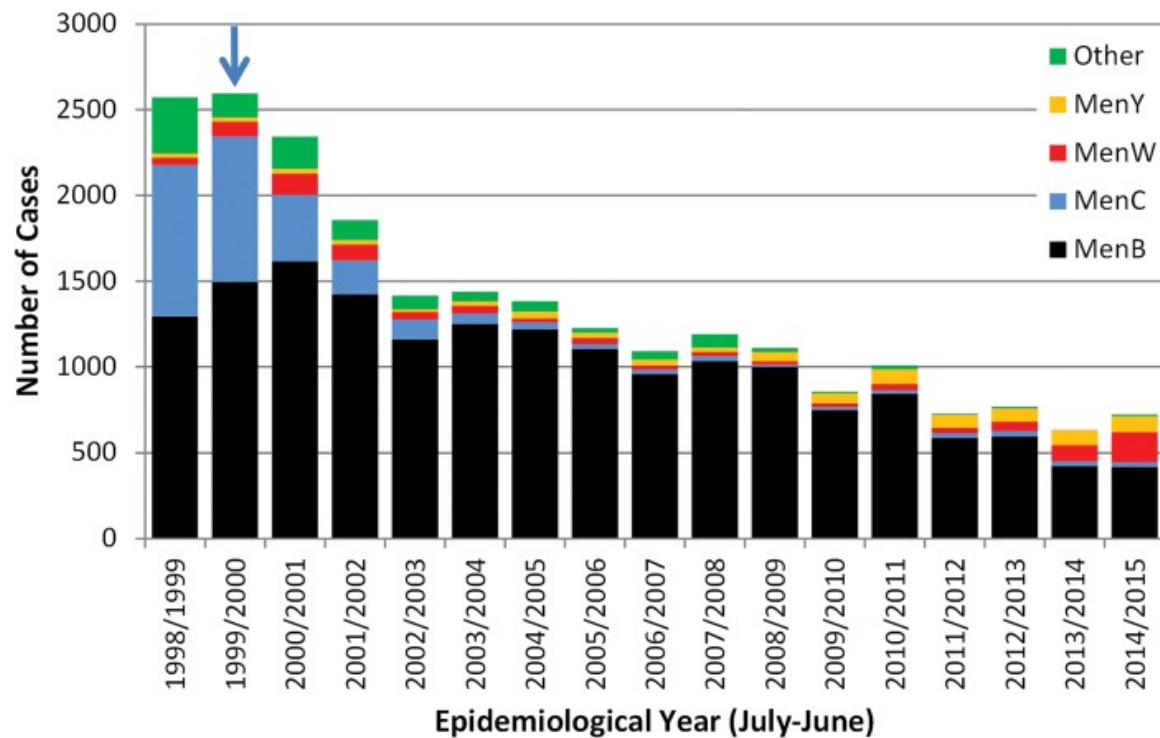


Figure 1.11. Effectiveness of the meningococcal capsular group C (MenC) conjugate vaccine in the UK. Stacked bars represent the proportion of cases of meningococcal disease attributable to each of the main disease-causing capsular groups of the meningococcus for each epidemiological between 1998 and 2015. The blue arrow indicates the year when the highly effective MenC polysaccharide conjugate vaccine immunisation programme was introduced. The number of cases associated with this capsular group have diminished considerably since then, leaving capsular group B (black) as the predominant disease-causing capsular group. From Ladhani *et al.*, 2015¹⁵⁷.

1.2.3B Outer membrane vesicles

OMVs produced by the bacterium have been discussed in the context of immune evasion, but they also lend themselves to vaccine development due to the presence of multiple immunogenic surface proteins embedded in their membrane (Fig. 1.12) maintained in a stable physicochemical state, and their self-adjuvanting properties. They can be biochemically extracted from cultured bacteria using sodium deoxycholate (detergent-extracted OMVs [dOMVs]) or allowed to bleb naturally into the culture media for subsequent concentration from supernatant (native OMVs [nOMVs]) ¹⁵⁸. The quantity of endotoxin in OMV preparations is around a quarter to half of the total membrane-bound protein but detergent extraction reduces this proportion approximately 5-fold ¹⁵⁹; a minimum of around 1% LOS must be maintained to preserve structural integrity . When membrane-bound, meningococcal endotoxin is about 100 times less toxigenic than when it is in its free form ¹⁶⁰, and adsorption onto alum further reduces the pyrogenic properties of these OMVs 5- to 10-fold by minimising the amount of free endotoxin released ¹⁶¹. OMVs are antigenically diverse and exhibit strain-to-strain variation in their antigen composition and endotoxin content, but tend to commonly express certain OMPs including porins, such as PorA and PorB, iron transporters, such as TbpA, TbpB, and FetA, abundantly on their surface ¹⁶². dOMVs have been used for the control of localised MenB epidemics in the absence of a broadly protective vaccine in countries such as Cuba ¹⁶³, where a dOMV vaccine was the first to be demonstrated as efficacious against a clonal MenB epidemic, Norway, where dOMVs derived from the H44/76 strain conferred protection in just over half of the schoolchildren immunised ¹⁶⁴, and New Zealand, where dOMVs derived from the NZ98/254 strain were administered to over three million children and adults

¹⁶⁵. While effective at preventing strain-specific disease, it was determined from SBA data from a trial in Chilean children that PorA was the immunodominant antigen in the OMV vaccine used for an outbreak in Chile ¹⁶⁶. Considering the variation exhibited by this antigen ¹⁶⁷, the results demonstrated that these wild-type OMVs are poor candidates for inducing broad protection and better suited to outbreak settings ¹⁶⁸. The clinical trials conducted prior to the use of NZ98/254 dOMVs for the New Zealand immunisation programme also provided insight on the reactogenicity of dOMVs, with a high incidence of fever ($\geq 38^\circ\text{C}$) observed in up to 37% of infants ¹⁶⁹ with rates of fever declining with age – 15% in toddlers ¹⁷⁰ and 3% or lower in those aged eight and above ¹⁷¹. This reactogenicity, which is believed to be caused by endotoxin, has important implications for the broadly protective multicomponent vaccine, 4CMenB, as described in section 1.2.3E. To mitigate the pyrogenicity of OMV preparations, an alternative to the deoxycholate-extraction has been developed based on harvesting nOMVs derived from the culture of *N. meningitidis* strains genetically attenuated to reduce the activity of membrane-bound endotoxin. The lipid A moiety of meningococcal LOS consists of six acyl groups to which the toxicity of the molecule can be attributed, but deletion of the *lpxL1* gene results in the loss of one of these acyl groups and the resultant penta-acylated endotoxin exhibits diminished ability to activate TLR4 ¹⁷² and stimulate pro-inflammatory cytokine production on human tissues ¹⁷³. Deoxycholate extraction can also dislodge membrane-bound lipoproteins, known to be key immunogens, from OMVs. This prompted efforts to generate OMVs from strains overexpressing a particular antigen ¹⁷⁴ or coupling antigen overexpression with endotoxin attenuation on *lpxL1* nOMVs ^{173,175}. These and other advances in the manufacture of OMVs have the potential to dissociate reactogenicity from

immunogenicity and even enhance the immunogenicity and cross-protection afforded by this vaccine platform.

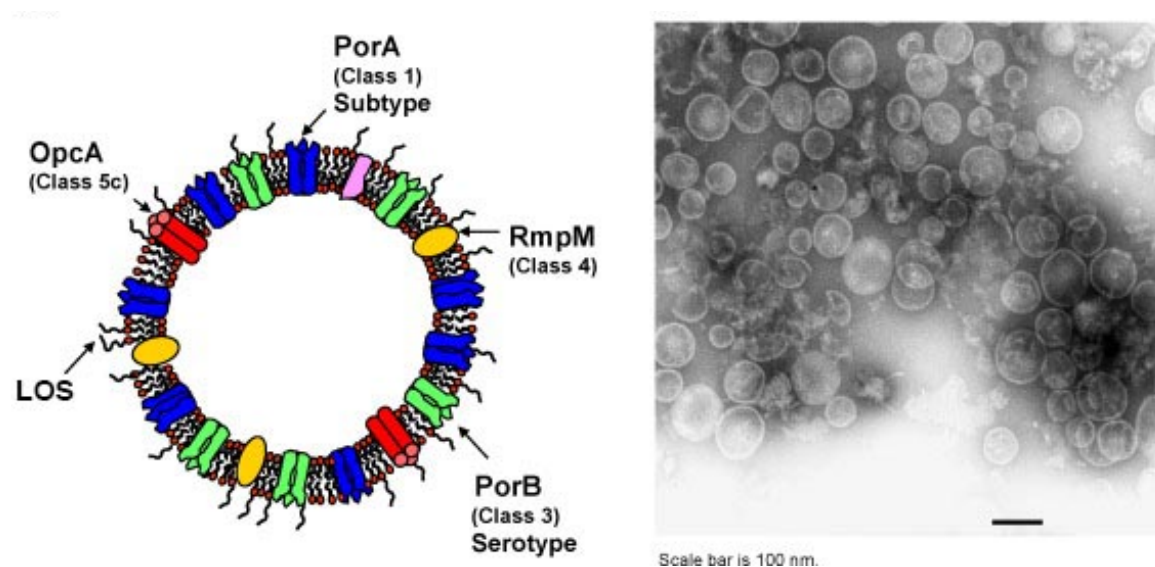


Figure 1.12. Outer membrane vesicles derived from *Neisseria meningitidis*. The left panel shows a schematic of an outer membrane vesicle (OMV) and its major outer membrane proteins, including the invasin OpcA, porins PorA and PorB, reduction modifiable protein RmpM, and lipooligosaccharide (LOS). The panel to the right displays an image of OMVs, 50-200 nm in size, taken using an electron microscope. From Holst *et al.*, 2009 ¹⁵⁸.

1.2.3C Factor H binding protein

Factor H binding protein (fHbp) has become a prominent candidate antigen in the field of MenB vaccine development since it was autonomously identified almost simultaneously by separate research groups almost two decades ago ^{176,177}. It was discovered through a conventional biochemical screening strategy employed at Pfizer (then Wyeth) in which meningococcal membranes were extracted, OMPs purified by ion exchange and chromatography, and mice were sequentially immunised to determine the most broadly protective antigen ¹⁷⁸. A novel approach

that became known as ‘reverse vaccinology’, applying algorithms to predict open reading frames (ORFs) encoding surface-exposed proteins from *N. meningitidis* reference genomes to be recombinantly expressed in *Escherichia coli* (*E. coli*) and tested for the induction of bactericidal antibodies in mice, led to the antigen’s discovery in a collaboration between the Children’s Hospital Oakland Research Institute and Novartis (then Chiron) ¹⁷⁹. One major difference between the production of the antigen by Pfizer and Novartis, initially named LP2086 and GNA1870, respectively, is the presence of a native lipid moiety in the former that increases its immunogenicity relative to its non-lipidated form ¹⁷⁸.

fHbp is expressed as an immature lipoprotein with an intact lipobox motif ¹⁸⁰ demarcated by a lysine (Lys) and cysteine (Cys) residue, LXXC, that is cleaved to Cys and modified during translation giving rise to a tri-Pam-Cys lipidated anchor residue ¹⁸¹. A flexible glycine (Gly)- and serine (Ser)-rich linker stems from the Cys at the N-terminus and connects the outer membrane anchoring region to the surface exposed N-terminal β -sheet and C-terminal β -barrel domains (Fig. 1.13) ¹⁸¹. These domains are well conserved ¹⁸² and vulnerable to antibodies ¹⁸³. The antigen exhibits sequence variability between isolates and can be classified according to two subfamilies, A and B ¹⁷⁸, or three variants, 1, 2, and 3 ¹⁷⁹, due to the different nomenclature applied by the respective groups. Epidemiological studies across Europe and the US have determined that the majority, approximately 70%, of disease-causing isolates of meningococcus belong to subfamily B with the remainder, ~30%, distributed across subfamily A ¹⁸². While there is sequence homology ranging from 60 to 75% between the subfamilies, the greater conservation of sequence within subfamilies of 83% or above ¹⁸² is thought to afford cross-protection from antibodies.

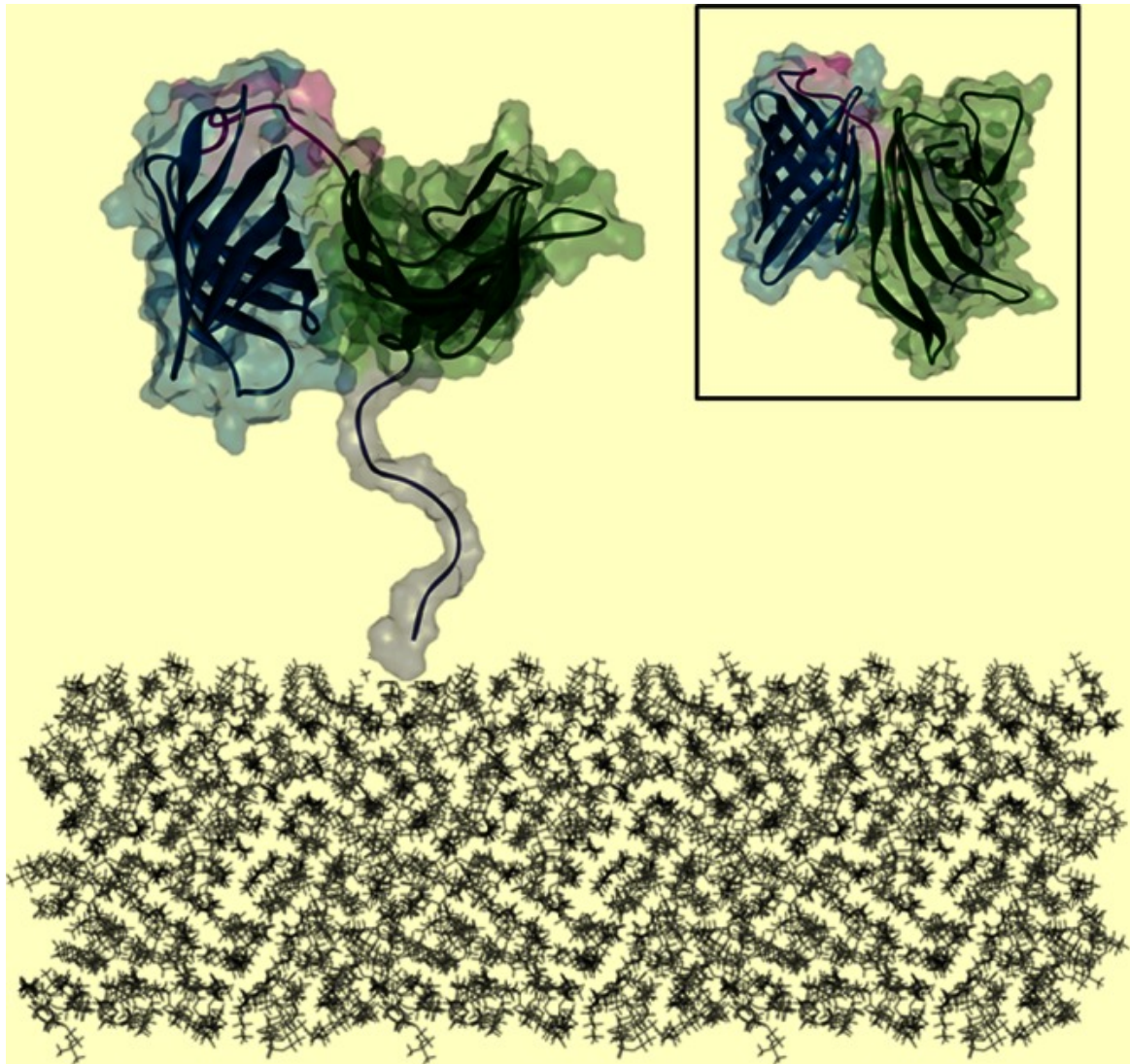


Figure 1.13. The factor H binding protein. A schematic of the meningococcal factor H binding protein (fHbp). The head of the protein is composed of two main domains, a taco-shaped β -sheet at the N-terminus, coloured in blue, and a cannoli-shaped β -barrel at the C-terminus, coloured in green. A peptide links these two domains, coloured in pink. The grey stem is the flexible, glycine- and serine-rich stem that anchors the protein to the bacterial outer membrane. From McNeil *et al.*, 2013¹⁸¹.

The functional activity and conventional name of fHbp were put forward in a study that assessed the survival of bacteria with an fHbp deletion in human complement and observed reduced binding of fH and enhanced killing of the mutant strain relative to its wild-type, suggesting a crucial role for fH binding in

meningococcal serum resistance ¹⁸⁴. The specific binding site for fHbp on human fH was later determined by flow cytometry ¹⁸⁵. From the point of view of vaccine development, binding of fH by fHbp-containing vaccines can direct antibodies to epitopes other than the fH binding site, negatively impacting bactericidal activity ¹⁸⁶; point mutations introduced in the antigen can substantially increase SBA directed to key epitopes on this antigen by reducing fH binding ¹⁸⁶. Two licensed vaccines that incorporate fHbp will be discussed in the following sections.

1.2.3D MenB-fHbp

A bivalent lipidated fHbp-based vaccine, MenB-fHbp, consisting of two variants of fHbp – one from each of the two subfamilies ¹⁸⁷ – has been licensed by the US Food and Drug Administration (FDA) ¹⁸⁸ and European Medicines Agency (EMA) ¹⁸⁹ for use in those over ten years of age. Two variants were chosen to increase the coverage and cross-protection afforded by the vaccine ¹⁹⁰. While the vaccine was determined to be effective at inducing protective titres of SBA with cross-immunity within each subfamily during clinical trials in various age groups ¹⁹¹, early trials in infants were brought to a halt due to concerns about the reactogenicity of the vaccine in this cohort with an incidence of fever as high as 80% in participants receiving half of the standard dose ¹⁶⁹. Reporting of adverse events was also high in adolescents, with roughly a third of participants experiencing some degree of reactogenicity regardless of the dose, though the incidence of fever was much lower in this age group ¹⁹². Overall, this vaccine was well tolerated and immunogenic in the age categories for which it is currently licensed and its use is indicated for those aged ten–25 in the US ¹⁸⁸, and in young

children and/or young adults in seven European countries ¹⁹³, though it is not yet a part of any national immunisation programmes.

1.2.3E 4CMenB

The most effective broadly protective MenB vaccine to date was developed through the pioneering ‘reverse vaccinology’ method outlined briefly in section 1.2.3C. It began with the whole-genome sequencing of the *N. meningitidis* MC58 reference strain by Richard Moxon and colleagues ¹⁹⁴ followed by the employment of computational methods to identify candidate antigens, recombinant expression of 350 candidate antigens in *E. coli*, purification, and serial immunisation of mice to determine antigen localisation, sequence conservation, and ability to induce sufficient SBA titres ¹⁹⁵. In addition to fHbp, two other surface-expressed antigens, NadA and the neisserial heparin binding antigen (NHBA), were chosen based on these criteria. The result was a four component MenB (4CMenB) vaccine consisting of NadA variant 3.1 and two fusion proteins, fHbp variant 1.1 fused with GNA2091 and NHBA peptide ID 2 fused with GNA1030, combined with NZ98/254 dOMVs, quantified by the relative proportion of membrane-bound PorA serosubtype 1.4 (Fig. 1.14) ^{196,197}. The final formulation of 4CMenB is adsorbed on alum and includes sodium chloride, histidine, sucrose and water ¹⁹⁷.

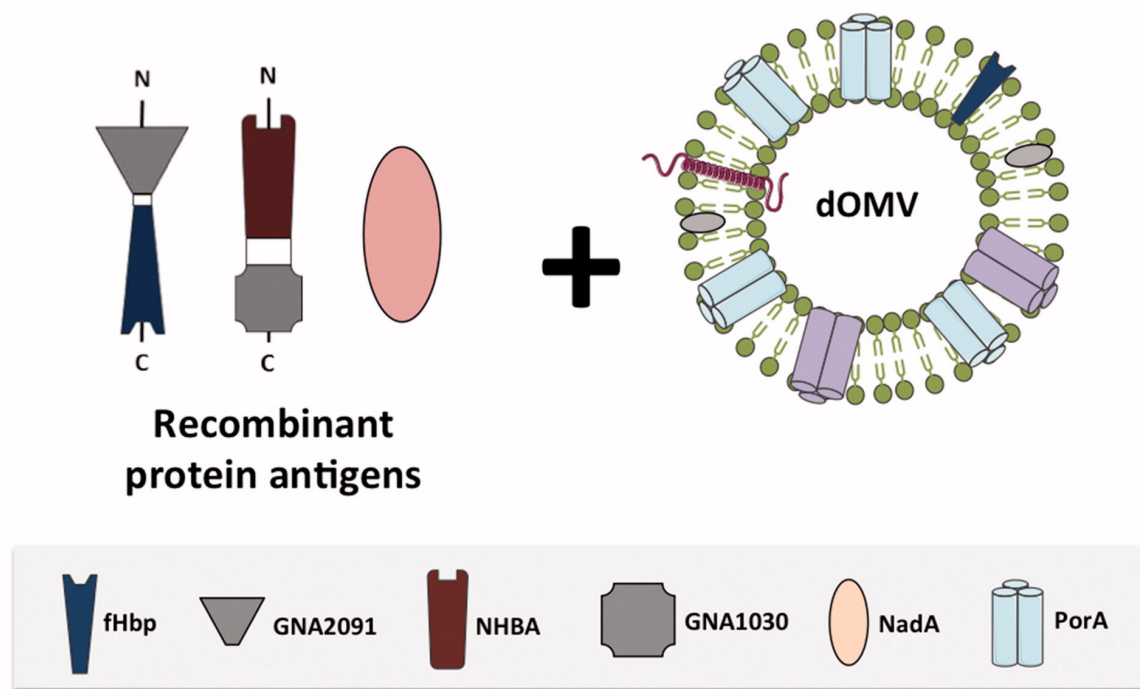


Figure 1.14. Constituent antigens of the four component meningococcal group B vaccine. The vaccine consists of two fusion proteins, the factor H binding protein (fHbp) fused to GNA2091 and the neisseria heparin binding antigen (NHBA) fused to GNA1030, the neisseria adhesin A (NadA), and detergent extracted outer membrane vesicles (dOMVs) derived from the NZ98/254 New Zealand epidemic strain. These four components are adsorbed on aluminium hydroxide. From Wang and Pollard, 2016 ¹⁹⁸.

4CMenB was demonstrated to be safe and cross-protective throughout clinical trials ^{199–203} and gained licensure for administration to those two months or older across multiple medicines agencies ^{204–206}. In the UK, the Joint Committee on Vaccination and Immunisation (JCVI) initially did not find the proposed inclusion of 4CMenB in the routine infant immunisation schedule to be cost-effective, the committee reevaluated the cost-effectiveness based on a reduced dose schedule (2 + 1 rather than 3 + 1) ²⁰⁷ and subsequently made the recommendation for its inclusion making the UK the first country to introduce 4CMenB into its immunisation schedule ²⁰⁸. Ten months after its introduction, 4CMenB was found

to be highly effective at preventing IMD related to MenB, reducing cases by 50% in the vaccine-eligible cohort, with good coverage ²⁰⁹. However, this vaccine also has the drawback of being highly reactogenic, especially when it is administered to infants in combination with other routinely-administered vaccines ²⁰² – this will be the subject of chapter 4. Uptake for the vaccine remains high in the UK, 95.5% of the vaccine-eligible cohort for the first dose and 88.6% for the two prime doses ²¹⁰, and other countries where immunisation programmes have been implemented – Austria, Ireland, Italy, and Lithuania at the time of writing (June, 2019) ²¹¹ – but the fever associated with the vaccine remains a concern for the reasons outlined in section 1.1.3B. The vaccine requires multiple doses to achieve sufficient titres of bactericidal antibody, and there is some concern about the persistence of these antibodies as they have been found to wane ^{212,213}, even with a booster dose ²¹⁴. It was also not deemed to be cost-effective for an adolescent routine immunisation programme ²⁰⁷, a cohort in which polysaccharide conjugate vaccines have proved effective at reducing carriage of the bacterium and therefore transmission ²¹⁵, as the impact this vaccine will have on carriage remains unknown. For these reasons, it is of vital importance that we continue to develop next generation vaccines against MenB if immunisation programmes are to be launched in other countries, protection is to be maintained, and adolescents are to be immunised in a cost-effective manner. In the following section, a vaccine platform that has been utilised for the induction of humoral and cellular responses against a variety of pathogens and holds promise for the development of an effective vaccine against MenB will be discussed.

1.3 Viral vectored vaccines

Viruses have been exploited for their natural capacity to infect mammalian cells and utilise the host cell machinery for the expression of their genetic material. By genetically engineering viruses to express human genes to act in lieu of defective genes within the host's cells or genes encoding pathogen-derived antigens, viral vectors can be generated for use in gene therapy or as platforms for vaccine delivery, respectively. While the immunological response to viral vectors has limited their effectiveness as permanent gene replacement therapies, this response is often beneficial in the context of their use as vaccines where the stimulation of an immune response facilitates the induction of antigen-specific immunity and can have an adjuvant effect. After cancer and monogenic diseases, the application of viral vectors in the field of infectious disease accounts for the greatest proportion of clinical trials conducted with this technology to date. Several different species of viruses have been developed as vectors and assessed in clinical trials, the majority of which have been based on various serotypes of adenovirus. Adenovirus vectors represent a particularly attractive platform for vaccine development owing to the fact that they are highly immunogenic and stimulate strong CD8⁺ T cell responses, a consideration which may be of importance when dealing with pathogens that exhibit intracellular replication cycle stages ²¹⁶. This characteristic has made them a popular choice as a candidate vaccine vector for the prevention of malaria ²¹⁷, human immunodeficiency virus ²¹⁸, and hepatitis C virus (HCV) ²¹⁹. Adenovirus vectors are also very effective at eliciting robust humoral immunity by stimulating durable antigen-specific germinal centre responses on par with the best available protein-in-adjuvant formulations ²²⁰. Their ability to induce high titres of

protective antibody in humans has recently been showcased by several adenovirus-based Ebola vaccine candidates that demonstrated immunogenicity in endemic countries ^{221,222}. By studying the kinetics and biodistribution of transgene expression from adenovirus vectors, the most favourable characteristics of an antigen or the vector may be determined for a specific disease. This section aims to provide an account of the mechanisms that underlie the immune responses to adenovirus vectored vaccines and how these have been and may continue to be harnessed in efforts to prevent a variety of global infectious diseases.

1.3.1 Adenovirus vector kinetics and transgene expression

Transgene expression kinetics are influenced by the location at which they are expressed. For most adenovirus subtypes that have been developed as vectors, entry into host cells is restricted to cells that possess the coxsackie and adenovirus receptor (CAR), present on a wide variety of tissues including muscle, liver, endothelium, and basolateral epithelium ²²³. An arginine-Gly-aspartic acid (RGD) within the loop of the virus' penton fibre is responsible for binding these receptors and clathrin-mediated endocytosis (Fig. 1.15) is facilitated by cellular αV integrins ²²⁴. Once internalised the virus traffics to the nucleus where it undergoes a replicative life cycle consisting of an early and late phase. Deletion of the early phase region E1 eliminates the virus' capacity to replicate and sustain expression of transgenes by preventing host cell lysis ²²⁵.

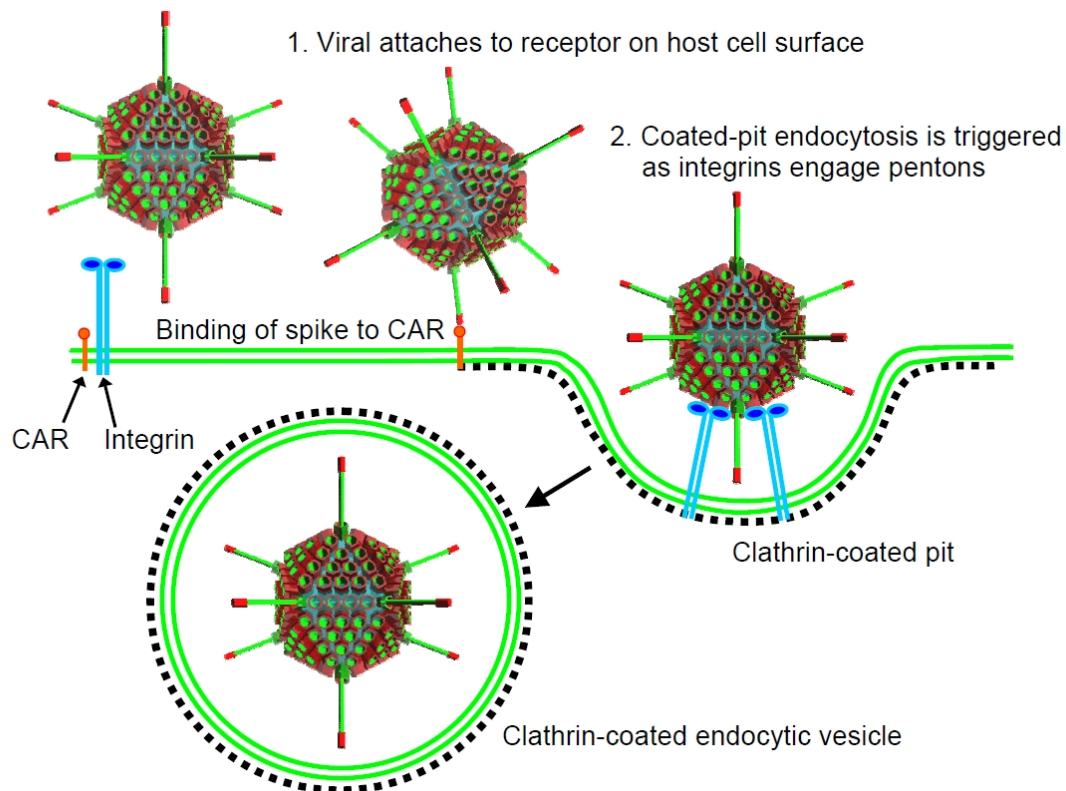


Figure 1.15. Adenovirus entry into host cells. Initial binding of the adenovirus vector to cells expressing the coxsackie and adenovirus (CAR) receptor occurs via the arginine-glycine-aspartic acid (RGD) within the loop of the virus' penton fibre. Endocytosis of the vector proceeds in a clathrin-dependent manner, facilitated by αV integrins on the cell surface, allowing for endosomal trafficking of the adenovirus. From <http://cronodon.com/BioTech/Adenovirus.html>.

The initial location of transgene expression from adenovirus vectors is the site of injection. Expression has been widely reported in muscle tissue surrounding and at the site of intramuscular injection. The vector biodistribution and transgene expression kinetics of recombinant human adenovirus serotype 5 (AdHu5) encoding a luciferase antigen have been demonstrated using *in vivo* bioimaging²²⁶. Antigen expression in myocytes at the site of inoculation persisted for one week following immunisation and it was suggested that early, high-level expression at this location may be important for the induction of long-term immunological

memory to vaccine antigens administered via this route ²²⁶. A more recent experiment compared the immunogenicity of AdHu5 with the recently developed chimpanzee adenovirus (ChAd), ChAdOx1, and examined the differential expression of transgene-encoded luciferase in muscle tissue and draining lymph nodes (dLNs) ²²⁷. Transgene expression within the first 24 hours post-immunisation was localised almost exclusively to the site of intra-muscular injection ²²⁷. The transduced cell populations in the dLNs were further analysed by using recombinant vectors encoding enhanced green fluorescent protein (eGFP). Approximately half of the cells expressing eGFP in the dLNs were B cells, 20% were conventional myeloid DCs, and 10% were pDCs ²²⁷. Accumulation of AdHu5 vector in non-lymphoid organs has been observed in the adrenal glands of mice ²²⁸ and the liver is well characterised as the main site of sequestration. Vector-encoded gene transfer has been shown to occur in murine ²²⁹ and bovine adrenal cells ²³⁰, while Kupffer cells and hepatocytes in the liver – in which transduction was also shown to occur – were found to be reservoirs for intravenously-administered vector ²³¹. An understanding of viral vector transgene expression location and kinetics is necessary to inform the development of vaccines which elicit tissue-localised CD8⁺ T cell responses.

T cell effector memory cells mediate a protective immune response by rapidly migrating to the site of inflammation and displaying effector functionality, while central memory T cells reside within secondary lymphoid organs, ready to divide and become effector cells upon antigen stimulation ²³². The proportion of effector memory and central memory phenotypes varies within T cell populations: the former predominates in CD8⁺ cells and the latter in CD4⁺ cells. Immunisation with adenovirus vectors induces a greater magnitude of CD8⁺ effector memory than CD8⁺ central memory (Fig. 1.16) ²³³. The duration of transgene expression has

been implicated in this phenomenon. The persistence of adenovirus transgene in several tissues post-immunisation has been detected in mice by real-time PCR and confirmed using GFP vectors ²³⁴. ChAd serotype 68 (AdC68) transgene sequences were detectable beyond 90 days post-immunisation and GFP expression was observed for up to five and six weeks after immunisation with AdHu5 and AdC68, respectively. In addition, antigen-specific CD8⁺ T cells were isolated from the spleen and lymph nodes at two and three months post-immunisation ²³⁴. The effect of the duration of transgene expression on CD8⁺ activation and central memory has also been analysed ²³⁵; by constructing an adenovirus vector containing a doxycycline-regulated genetic element, the expression of the transgene could be terminated at the desired timepoint. It was found that the premature termination of antigen expression prior to peak response led to a diminished primary response and impaired memory formation and protective immunity ²³⁵. For the memory response to be sustained, the antigen had to be expressed continually beyond the first two weeks post-immunisation. Taken together these data suggest that the magnitude of the antigen-specific CD8⁺ response is dependent on sustained transgene expression, and that the formation of immunological memory may result from continued priming in secondary lymphoid tissue during this time.

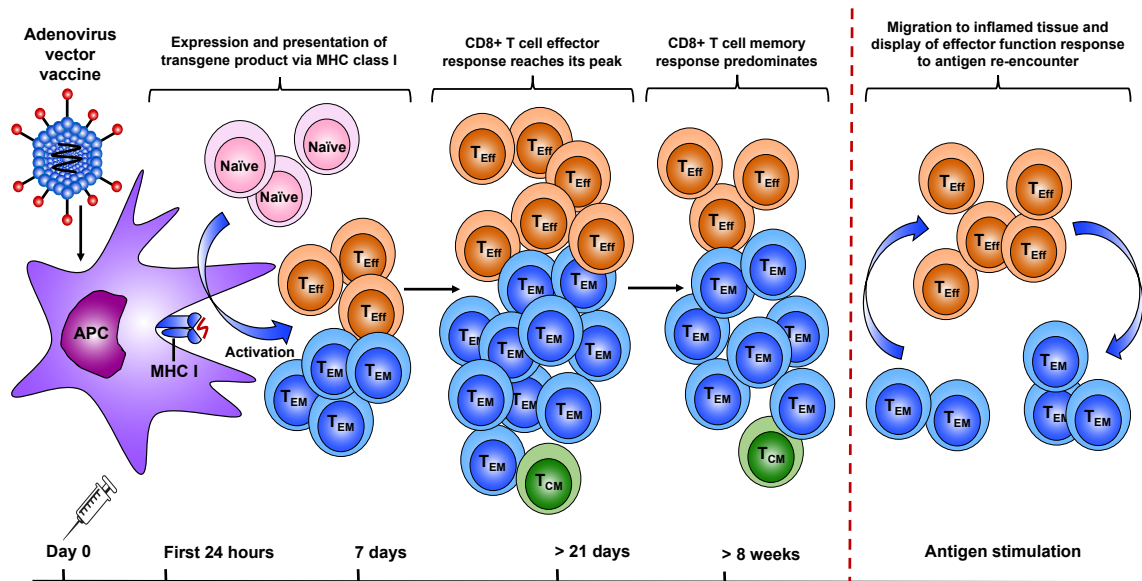


Figure 1.16. Induction of memory T cells by adenovirus vector vaccines. Adenovirus vectors are taken up by antigen-presenting cells within the first 24 hours of immunisation, allowing them to express their encoded transgene, the product of which can be processed and presented via major histocompatibility complex (MHC) class I molecules. This leads to the activation of naïve T cells and the differentiation of CD8⁺ effector or effector memory T cells (T_{Eff} and T_{EM}, respectively) within the first week post-immunisation. After three weeks the T_{Eff} cell response reaches its peak, and after eight weeks the T_{EM} phenotype predominates. Upon reencountering the same antigen, T_{EM} cells migrate to the site of infection where they switch to the T_{Eff} phenotype and engage in a more rapid cytotoxic response.

1.3.2 Overcoming pre-existing immunity to the vector

If a vector is to be successful in eliciting the desired endogenous expression, the vector itself must be able to avoid elimination by the host immune system for long enough to maintain sufficient expression of transgenes to allow immunological memory to form. Viruses are naturally adept at circumventing the host immune response due to co-evolution but pre-existing immunity in human

populations is a major concern for the clinical development of certain vectors. For example, it is likely that, by a certain stage in life, humans have been exposed to human serotypes of adenovirus and will generate a memory response. It has been shown in animal models and in humans that the presence of neutralising antibodies against a viral vector can prevent the uptake of the vector by cells due to antibody-mediated clearance, thereby reducing transgene expression and presentation ²³⁶. The administration of a second booster dose of vaccine has also been shown to overcome problems associated with immune responses directed to the vector ²¹⁶.

Much research has been conducted over the past decade into the use of replication-defective chimpanzee-derived adenoviruses as vaccine vectors for a variety of infectious agents. The reduced seroprevalence of chimpanzee serotypes relative to human serotypes makes them significantly less susceptible to antibody neutralisation in humans ²³⁷. Certain ChAds, such as AdC68 share the entry mechanism and tissue tropism of human adenovirus subtypes which were used initially and therefore do not significantly alter the location of transgene expression ²³⁸. ChAd63 has been established as an effective vector for the induction of strong cytotoxic T lymphocyte (CTL) responses, but alternative ChAd vectors are still being developed. The ChAdOx1 vector, mentioned previously, was created by insertion of the Y25 ChAd genome into a bacterial artificial chromosome allowing for deletion and manipulation of the early phase regions ²³⁹. ChAdOx1 expressing the conserved nucleoprotein and matrix protein of influenza A has proven to be safe and immunogenic in a first-in-human study ²⁴⁰ and has also been shown to improve protection against tuberculosis offered by the *Bacillus Calmette-Guérin* (BCG) vaccine ²⁴¹. Both immunisation strategies included a heterologous boost with modified vaccinia Ankara (MVA).

1.3.3 Tailoring the immune response

As mentioned previously, induction of a pathogen-specific CTL response is critical for clearance of a variety of infections and was the initial basis for the clinical development of most adenovirus vectors. It has been established that adenovirus vectors are capable of inducing persistent expression in host cells leading to strong cytotoxic CD8⁺ T cell responses, superior to those obtained using DNA immunisation ²⁴². The kinetics of the CD8⁺ response following immunisation with an adenovirus vector containing the well-characterised model antigen SIINFEKL has been studied. The antigen-specific cytotoxic responses in the lymph nodes of mice within three days of immunisation were observed, with peak interferon (IFN)- γ -producing CD8⁺ T cells in the spleen at 11 days post-immunisation. Due to the importance of the location and nature of these CD8⁺ responses for developing vaccines against HIV, studies have been conducted to determine the distribution of CD8⁺ responses to different methods of immunisation including intramuscular, intranasal, oral and rectal administration. One such study found that the route of immunisation had a distinct effect on the location of transgene expression, the phenotype of the CD8⁺ response and the properties of CD8⁺ trafficking ²⁴³. This demonstrates the importance of the initial site of transgene expression and its microenvironment in shaping the subsequent immunogenic response to the vaccine.

Recombinant ChAds have been successfully utilised in conjunction with MVA vectors in a variety of immunisation strategies at various stages of clinical trials to elicit strong CD8⁺ T cell responses to intracellular pathogens including

Plasmodium falciparum, HCV and HIV ^{244–248}. However, the strong and durable humoral response elicited by adenovirus vectors has gained considerable attention in recent years ²⁴⁹. The humoral responses induced by adenovirus-encoded antigens and the gold standard protein/adjuvant formulations have been compared in mice ²⁵⁰, providing evidence that adenovirus vector-induced IgG responses are comparable to protein with alum or protein with oil-in-water. Further elucidation of the optimal approaches for stimulating the germinal centre responses will be critical for the development of vaccines capable of inducing humoral immunity to pathogens with highly variable epitopes.

1.3.4 Tackling the Ebola epidemic: adenovirus in action

An outbreak of Ebola virus (EBOV), the causative agent of a severe haemorrhagic fever which became known as Ebola virus disease (EVD), led to the epidemic in West Africa, claiming the lives of 11,310 infected patients in the affected countries ²⁵¹. EBOV first emerged in 1976 in southern Sudan where it caused an outbreak in the town of Nzara and the surrounding area ²⁵². However, despite several small outbreaks in Central Africa, it remained relatively unknown until a larger outbreak occurred in 1995 in the Democratic Republic of Congo, then known as Zaire ²⁵³. This outbreak received much attention in the international media and identified EBOV as one of the most significant threats to human health worldwide. While vaccine research began in 1976, it was not until this outbreak that the importance of having an EBOV vaccine became apparent. The first successful immunisation of a non-human primate (NHP) against a lethal challenge with EBOV was described in 2000 when a non-replicating AdHu5-based vector

encoding the EBOV glycoprotein (GP) was found to be protective after a single priming dose ²⁵⁴. This vaccine was later found to be capable of inducing antibody responses in humans and demonstrated a good safety profile in a phase I trial ²⁵⁵. In 2014, during the West Africa epidemic, a ChAd serotype 3 (ChAd3) vector was developed encoding the same GP antigen and was found to be protective against lethal EBOV challenge in macaques ²⁵⁶. In order to elicit more durable, long-term protection, ChAd3 was boosted with MVA to increase the longevity of the antibody response. This prime-boost combination is now one of the main EBOV vaccine candidates. The results of the Phase I clinical trials have recently been published and phase II/III trials are currently ongoing in several West African countries as part of the STRIVE study ²⁵⁷. “Ebola ça suffit!” is another study which has reached the final stages of Phase III trials. Its clinical construct also incorporates the EBOV surface GP but utilises a recombinant vesicular stomatitis virus (rVSV) as a replication-competent vector; early reports from the Guinea ring phase III trial suggest that the vaccine offers substantial protection against the disease with a single administration ²⁵⁸. A third candidate viral vectored vaccine combination, consisting of a recombinant human Ad26 encoding EBOV GP as a prime and MVA encoding GPs from several HFVs as a boost (MVA-BN-Filo), has completed phase I clinical trials and is currently entering phase II/III ²⁵⁹. If successful, this vaccine strategy will provide protection against other HFVs such as Marburg virus, Sudan virus and Tai Forest virus in addition to EBOV. Taken together, the data generated from these clinical trials have highlighted the clinical effectiveness of recombinant viruses in mitigating epidemic diseases and their potential as platforms for the prevention of other outbreak pathogens. In chapter 5, a novel viral vectored vaccine based on adenovirus that is undergoing development for the prevention of disease caused by MenB is discussed.

1.4 Study aims

The primary aim of this study was to provide a comparative analysis of several licensed and candidate vaccines against meningococcal disease, in order to elucidate the host pathways and mechanisms involved in eliciting their respective immunogenic, reactogenic, or anamnestic responses. Central to fulfilling this aim was the use of a mouse immunisation model to which a ‘systems vaccinology’ approach was applied. RNA-seq is central to this methodology, but computational choices involved in the processing of such data may introduce variation in the analysis that impact on the biological interpretation. Thus, the initial aim of the study was to establish a novel method to identify and remove this computational ‘noise’. This method was applied to subsequent analyses of the 4CMenB vaccine and its components in mice. The goal of this part of the study was to identify molecular determinants of fever induced by the vaccine, given on its own or in combination with routine vaccines, and to attribute the reactogenicity to a specific component of the vaccine to inform the design of better-tolerated vaccines.

A novel meningococcal vaccine, based on an adenovirus vectored delivery technology, was developed at the Oxford Vaccine Group (OVG). The novel viral vectored vaccine provides early and persistent bactericidal antibody titres in mice. This study sought to explore the factors that underlie the expression and immune-engagement of the encoded antigen by evaluating this candidate vaccine in mammalian cells and mice. Finally, this novel vaccine was compared with a range of other vaccine platforms, both established and experimental, for the engagement of immunological pathways with the aim of establishing those that may be most important for the establishment of protective immunity against MenB.

1.5 Outline of thesis

- **Chapter 2** provides a detailed description of the materials and methods used throughout this project.
- **Chapter 3** introduces a novel method for the quantification and elimination of technical noise introduced by computational choices in RNA-seq data analysis.
- **Chapter 4** compares the reactogenicity of the combined administration of 4CMenB and routinely-administered vaccines between human infants and mice and further explores the reactogenicity of the individual components of 4CMenB in mice.
- **Chapter 5** assesses the immune mechanisms underlying a novel candidate MenB vaccine antigen encoded by a viral vectored vaccine in mice.
- **Chapter 6** contrasts the transcriptional changes underlying the immune responses to adenoviral and poxviral vector vaccines and in mice.
- **Chapter 7** discusses and makes conclusions based on the results obtained from the present study.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Genome annotation

The complete genomic sequences and annotation files for *Homo sapiens* (*H. sapiens*) and *Mus musculus* (*M. musculus*) were downloaded from the Ensembl genome browser (www.ensembl.org). The latest (release 96 at the time of writing, May 2019) FASTA-formatted *H. sapiens* GRCh38 and *M. musculus* GRCm38 chromosomal DNA sequences were downloaded individually and concatenated to generate a complete genome sequence FASTA file for each species; the human and mouse genome assemblies corresponded to the GCA_000001405.27 and GCA_000001635.8 GenBank assembly IDs, respectively. These assemblies were used for probabilistic/semi-probabilistic RNA-seq read alignment. Human and mouse protein-coding nucleotide sequences (cDNA FASTA files) based on Ensembl predictions were used for RNA-seq deterministic read alignment and pseudoalignment. The gene transfer format (GTF) annotation files, based on the GENCODE gene set, were also obtained from Ensembl for both species for the estimation of gene abundances.

2.1.2 Datasets

The human infant dataset GSE131929 was used for chapter 3 and can be accessed from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>). The datasets used in chapter 4 are available from GSE131914 and GSE132199. The dataset used in chapter 6 is not yet publicly available.

2.1.3 R packages

Package name	Installation command	Citation
BiocManager	<i>install.packages("BiocManager")</i>	260
biomaRt	<i>BiocManager::install("biomaRt")</i>	261,262
ClusterMap	<i>install_github('ClusterMap','xgao')</i>	263
clusterProfiler	<i>BiocManager::install("clusterProfiler")</i>	264
cowplot	<i>install.packages("cowplot")</i>	265
devtools	<i>install.packages("devtools")</i>	266
dplyr	<i>install.packages("dplyr")</i>	267
edgeR	<i>BiocManager::install("edgeR")</i>	268,269
EnsDb.Hsapiens.v86	<i>BiocManager::install("EnsDb.Hsapiens.v86")</i>	270
ensembladb	<i>BiocManager::install("ensembladb")</i>	271
factoextra	<i>install.packages("factoextra")</i>	272
gplots	<i>install.packages("gplots")</i>	273
ggplot2	<i>install.packages("ggplot2")</i>	274
ggpubr	<i>install.packages("ggpubr")</i>	275

ggsignif	<i>install.packages("ggsignif")</i>	276
gridExtra	<i>install.packages("gridExtra")</i>	277
limma	<i>BiocManager::install("limma")</i>	278
magick	<i>install.packages("magick")</i>	279
Matrix	<i>install.packages("matrix")</i>	280
plyr	<i>install.packages("plyr")</i>	281
preprocessCore	<i>BiocManager::install("preprocessCore")</i>	282
reshape2	<i>install.packages("reshape2")</i>	283
rowr	<i>install.packages("rowr")</i>	284
scales	<i>install.packages("scales")</i>	285
Seurat	<i>install.packages("Seurat")</i>	286
tidyverse	<i>install.packages("tidyverse")</i>	287
UpSetR	<i>install.packages("UpSetR")</i>	288

2.1.4 Reagents

Reagent	Source	Cat. No.
1 kb DNA ladder	New England BioLabs	N3232
10-cm Petri dish	Fisher Scientific	FB0875713
10X PBS	Life Technologies	70011-044
2-Mercaptoethanol	Gibco	31350-010
26 G Needle 5/8 inch, single use, sterile	BD Biosciences	305115
35- x 10-mm Cell culture dish	Corning	430165
4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)	Life Technologies	D1306
40 µm cell strainer	Falcon	352340
6-well tissue culture plates	Corning	TKT-520-010c
Agarose	Sigma-Aldrich	A9539
Alhydrogel® adjuvant	Invivogen	vac-alu-250
Ampicillin	Sigma-Aldrich	A5354
autoMACS® Rinsing Solution	Miltenyi Biotec	130-091-222
autoMACS® Running Buffer	Miltenyi Biotec	130-091-221
BD Medical™ BD Micro-Fine™ Insulin Syringe	BD Biosciences	13161931
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A7030
Brefeldin A solution	eBioscience	00-4506-51
Buffer RLT	QIAGEN	79216

Carbonate-bicarbonate (Carb-bicarb) buffer capsules	Sigma-Aldrich	C3041
Chloramphenicol	Sigma-Aldrich	C7795
Chromium Single Cell 3' Reagent Kit	10X Genomics	PN-1000075
Collagenase/dispase	Sigma-Aldrich	11097113001
Columbia agar with horse blood, 90mm ready poured plate	Fisher Scientific	12917158
Cover glasses, 22 x 22 mm	Sigma-Aldrich	C9802
Cryogenic tube with screw cap, volume 2 mL	Nalgene	BR114841
DH5α Competent <i>E. coli</i> (High Efficiency)	New England BioLabs	C2987
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
DNase	Worthington	2139
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich	D5671
EASYseal™ plate sealer, transparent	Grenier Bio-One	676001
Eppendorf Safe-Lock Tubes, 0.5 mL	Eppendorf	022363611
Eppendorf Safe-Lock Tubes, 1.5 mL	Eppendorf	022363212
Eppendorf Safe-Lock Tubes, 2 mL	Eppendorf	022363379
Ethanol	Ethanol	32221
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	E7889

Fluorescence-activated cell sorting (FACS) [™] collection tubes, 5-mL polypropylene round-bottom	Falcon	352063
FACS [™] Lysing Solution	BD Biosciences	349202
Falcon [™] 15 mL tubes	Corning	CLS430791
Falcon [™] 50 mL tubes	Corning	CLS430829
Falcon [™] 5mL round bottom polypropylene test tube with snap cap, 12 x 75 mm	Corning	352063
Fixable Viability Stain 780	BD Biosciences	565388
Fixation/Permeabilization Solution Kit	BD Biosciences	554714
Foetal Bovine Serum (FBS)	Sigma-Aldrich	F0392
Gateway [®] LR Clonase [®] II Enzyme Mix	Life Technologies	11791-020
Gel Loading Dye, Purple	New England BioLabs	B7024S
gentleMACS [®] C Tubes	Miltenyi Biotec	130-096-334
GLOBINclear [™] Kit, mouse/rat, for globin mRNA depletion	Life Technologies	AM1981
Glycerol	Life Technologies	17904
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Life Technologies	A-11001
Hank's Balanced Salt Solution (HBSS)	Life Technologies	14175-095

Heparin sodium 100 IU/ml I.V. flush solution	Fannin	RJAP108FUA
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems	4368813
Illumina™ TotalPrep™-96 RNA Amplification Kit	Illumina	4393543
Isoflurane (Isoflo)	University of Oxford	N/A
JAR5 monoclonal antibody	Absolute Antibody	Ab00445-1.4
Kanomycin	Sigma-Aldrich	K0254
L-glutamine (L/G)	Sigma-Aldrich	G7513
L-shaped cell spreaders	Fisher Scientific	15625467
Lysogeny broth (LB) agar, vegitone	Sigma-Aldrich	19344
LB broth, vegitone	Sigma-Aldrich	28713
LigaFast™ Rapid Ligation Buffer	Promega	9PIM822
LPS-EB	Invivogen	tlrl-eblps
LPS-EB Ultrapure	Invivogen	tlrl-3pelps
Luer-Lok™ 1 mL syringe	BD Biosciences	309628
MicroAmp® Fast Optical 96-Well Reaction Plate, 0.1	Life Technologies	4346907
MicroAmp® Reaction Tube with Cap, 0.2 mL	Life Technologies	N8010540
MILLIPLEX® Mouse Cytokine/Chemokine Magnetic Bead Panel	Millipore	MCYTOMAG-70K
Mouse IFN-γ/IL-17A FluoroSpot kit	Mabtech	FS-4144-2

Mouse RiboPure™-Blood RNA Isolation Kit	Life Technologies	AM1951
Muse® Count and Viability Reagent	Millipore	MCH600103
NotI-HF	New England BioLabs	R3189S
Nunc™ EasYFlask™ 175 cm ² with filter cap, sterile	Sigma-Aldrich	F7677
Nunc® MicroWell™ 96 well polystyrene plates	Sigma-Aldrich	P7491
PacI	New England BioLabs	R0547S
Polymerase chain reaction (PCR) plate, 96-well, low profile, skirted	Life Technologies	AB0800
Penicillin-streptomycin (P/S)	Life Technologies	15140-122
PeqGreen	VWR International	PEQL37-5010
Percoll	Sigma-Aldrich	P1644
Peroxidase AffiniPure Goat Anti-Mouse IgG	Jackson ImmunoResearch Laboratories	115-035-003
Peptidoglycan (PGN)-EB	Invivogen	tlrl-pgneb
Phosphate-buffered saline (PBS) tablets	Sigma-Aldrich	D-8537
ProLong Gold Antifade Mountant	Life Technologies	P10144
QIAGEN Plasmid Midi Kit	QIAGEN	12143
QIAquick Gel Extraction Kit	QIAGEN	28704
Qubit® RNA BR Assay Kit	Life Technologies	Q10210
Qubit® RNA HS Assay Kit	Life Technologies	Q32855

Rat Anti-Mouse Anti-CD31-APC (clone: MEC 13.3)	BD Biosciences	550274
Rat Anti-Mouse Anti-CD45-PE (clone: 30-F11)	BD Biosciences	562420
Rat Anti-Mouse Anti-Ly6C-PE (clone: AL-21)	BD Biosciences	560593
Rat Anti-Mouse Anti-Ly6G-BV711 (clone: 1A8)	BD Biosciences	563979
Rat Anti-Mouse Anti-CD11b-V450 (clone: M1/70)	BD Biosciences	560455
Red Blood Cell (RBC) Lysis Buffer	BioLegend	420302
RNA ^{later} [™] Stabilization Solution	Life Technologies	AM7022
RNeasy Plus Micro Kit	QIAGEN	74034
Shaker flask Erlenmeyer, baffled bottom, vented	Fisher Scientific	11755263
SiR-DNA kit	tebu-bio	CHF260.00
Slides, microscope, 25 x 75 mm	Sigma-Aldrich	S8400
SOC Medium	New England BioLabs	B9020S
Sulphuric acid	Sigma-Aldrich	339741
T4 DNA Ligase	New England BioLabs	M0202
Tris-acetate-EDTA (TAE) Buffer, RNase-free	Invitrogen	AM9896
TaqMan [™] Gene Expression Master Mix	Life Technologies	4369016
TaqMan [™] PreAmp Master Mix	Life Technologies	4391128

TaqMan™ Primers	Life Technologies	4331182
Tris-EDTA (TE) Buffer	Invitrogen	12090015
Tetramethylbenzidine (TMB) liquid substrate	MP Biomedicals	0215234680
Trypsin-EDTA solution	Sigma-Aldrich	T3924
TWEEN®-20	Sigma-Aldrich	9005-64-5
UltraPure DNase/RNase-free distilled H ₂ O	Life Technologies	10977035
μ-Slide 8 well, ibiTreat, tissue culture treated, sterile	ThistleScientific	IB-80826

2.1.5 Solutions

- **1% BSA in PBS:** 2 PBS tablets were dissolved in 400 mL of distilled (d)H₂O. 4 g of BSA powder was dissolved in this PBS solution, filtered, and autoclaved.
- **Carb/bicarb buffer:** 15 mM sodium carbonate plus 35 mM sodium bicarbonate dissolved in dH₂O and autoclaved.
- **DMEM complete medium:** 50 mL of the 500 mL DMEM was replaced 50 mL of FBS and 5 mL of P/S (100 U penicillin + 100 μg streptomycin) antibiotic and 5 mL of 2 mM L/G were added.
- **LB agar:** 14 g of LB agar, vegitone, powder was dissolved in 400 mL of dH₂O and autoclaved.
- **LB broth:** 10 g of LB broth, vegitone, powder was dissolved in 500 mL of dH₂O and autoclaved.

- **PBS-Tween (PBST):** A 1 L solution of was made using the following concentration of reagents: 8 mM Na₂HPO₄, 150 mM NaCl, 2 mM KH₂PO₄, 3 mM KCl, 0.05% TWEEN®-20. The solution was adjusted to pH 7.4 and autoclaved.

2.1.6 Vaccines and antigens

Vaccine	Trade name	Manufacturer
4CMenB	Bexsero®	GlaxoSmithKline plc
DTaP (acellular)-IPV-Hib	Pediacel®	Sanofi Pasteur Ltd
13-valent PCV (PCV13)	Prevnar®	Wyeth Pharmaceuticals Inc
NZ98/254 dOMVs (PorA), rfHbp, rNHBA, rNadA	[Components of] Bexsero®	GlaxoSmithKline plc
H44/76 dOMVs (MenPF-1)	N/A	OVG
H44/76 lpxL1 nOMVs	N/A	OVG
LPS-EB (from <i>E. coli</i> O111:B4)	N/A	InvivoGen
MVA vectors	N/A	OVG/Vector Core
Adenovirus vectors	N/A	OVG/Vector Core
LPS-EB Ultrapure	N/A	InvivoGen
PGN-EB (from <i>E. coli</i> O111:B4)	N/A	InvivoGen

All non-commercial vaccines were prepared to double the required concentration and diluted 1:2 with alum.

2.1.7 Cell lines and culture

HeLa and C2C12 (both acquired from Public Health England) cell lines were used for *in vitro* expression assays. Both cell lines were cultured in DMEM complete medium. Cells were grown in T175 flasks at 37 °C in a humidified 5% CO₂ atmosphere to a confluency of approximately 70–80%. At this point, cells were harvested by washing twice with 20 mL of autoMACS® rinsing solution, adding 8 mL of Trypsin-EDTA, and incubating for 5 mins at 37 °C. Complete DMEM was added to the cells to neutralise the trypsin and, if needed for an experiment, cells were transferred to a 50 mL tube to be spun at 300 x *g*. The supernatant was discarded, and the pellet resuspended in 1 mL of complete DMEM using a 1 mL pipette and topped up to 5 mL with more media. 20 µL of resuspended cells were added to 380 µL of Muse® Cell Count and Viability Assay Solution in a 2 mL tube and counted using a Muse® Cell Analyzer instrument.

2.2 Methods: Animal procedures

All procedures were performed in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee. General anaesthesia was induced using 3.5% isoflurane mixed with 2 L/min of O₂ released into the mouse anaesthetic chamber and then via direct inhalation for each mouse through a tube while procedures were being performed. Cardiac bleeds (followed by cervical dislocation), immunisations and terminal bleeds were performed under

general anaesthesia. All mice were female and aged between six- and eight-weeks of age at the beginning of each experiment.

2.2.1 Mouse immunisations

Once anaesthetised, vaccines were administered to the mouse by injecting no more than 50 μ L into each of the *musculus tibialis* at the back of the leg, using a 29-gauge insulin syringe. For vaccine combinations requiring more than two injections, 33 μ L of each of two vaccines were injected into one leg and 33 μ L of the third vaccine was injected into the other.

2.2.2 Mouse bleeds

Tail bleeds were performed to obtain blood samples prior to terminal bleeds. Using a 37 °C heating box, mice were pre-warmed and restrained to facilitate nicking of the tail vein to collect 8–10 drops of blood in a 2 mL tube. For terminal bleeds, mice were put under general anaesthetic and cardiac bleeds were performed followed by cervical dislocation to confirm death. Blood for RNA-seq experiments was transferred to pre-filled RNeasyTM blood collection microcentrifuge tubes containing. For downstream neutrophil isolation, blood was transferred to cryovials containing of heparin sodium anti-coagulant at a ratio of 15 μ L per mL of blood.

2.2.3 Mouse temperature measurement

Mouse surface temperatures were measured in triplicate by infrared thermometry using a No Touch Thermometer NTF3000 (Braun), as outlined by Mei *et al.*²⁸⁹ (Fig. 2.1), and averaged to obtain timepoint temperature readings for each mouse.



Figure 2.1. Mouse surface temperature measurement. The red circle indicates the perianal area where temperature measurements were taken with an NTF3000 No Touch Thermometer. The mouse was allowed to grip the bars of the cage while the base of the tail was lifted to expose the anogenital region for temperature measurement. Readings were taken in triplicate.

2.2.4 Isolation of mouse neutrophils

Serum was separated from heparin-stabilised blood tubes by microcentrifugation at maximum speed for 10 mins. Sera were stored at -20 °C for cytokine analysis. Pellets were resuspended in 1X BD FACS™ Lysing Solution, transferred to a 15 mL tube, topped up to 10 mL with lysis solution, and left to incubate for 15 mins at room temperature. Tubes were then centrifuged at 500 x *g* for 10 mins. Supernatants were discarded and pellets were washed twice by resuspending in 1 mL of autoMACS® rinsing solution, centrifuging at 300 x *g* for 10 mins, and discarding the supernatant. After the second wash was complete, the pellet was resuspended in 100 µL rinsing buffer and the appropriate concentration of antibodies were added: 1:200 anti-Ly6C-PE, 1:10 anti-Ly6G-BV711, 1:10 anti-

CD11b-V450, 1:50 BD Horizon™ Fixable Viability Stain 780. Samples were incubated in the dark at 4 °C for 30 mins, washed twice with rinsing buffer, transferred to 5 mL Falcon™ Round-Bottom Polystyrene Tubes and then sorted using a BD FACS Aria™ cell sorter. Neutrophils were identified as Ly6C⁻ CD11b⁺ Ly6G⁺ live cells. These cells were sorted directly into 2 mL microcentrifuge tubes) containing 2% FBS in 1X PBS and transferred to ice. A Muse® Count & Viability Assay Kit was used to measure the final cell count and viability of each sample on a Muse® Cell Analyzer instrument (Millipore).

2.2.5 Isolation of mouse brain endothelial cells

Mouse brains were harvested, enzymatically digested, and stained for FACS isolation of BECs according to the protocol developed by Crouch and Doetsch ²⁹⁰. Briefly, brains were dissected into smaller pieces in a 10 cm Petri dish and transferred into a 15 mL Falcon tube containing 2% (vol/vol) FBS in PBS to be centrifuged at 300 x *g* and 4 °C for 5 mins. 0.5 mL of collagenase/dispase stock solution was added to 14.5 mL of 2% FBS in PBS for each sample. The supernatants were discarded, and pellets resuspended in 3 mL of this collagenase/dispase solution. Each tube was split into two 15 mL Falcon tubes, topped up to 5 mL with collagenase/dispase solution, and incubated on a shaking incubator set to 37 °C for 30 mins. Tubes were centrifuged at 300 x *g* and 4 °C for 5 mins, supernatants were discarded, and pellets resuspended in 1 mL of 2% FBS in PBS containing 0.5 µL of DNase. Each tube was again split into two 15 mL Falcon tubes containing 4 mL of 22% Percoll (diluted in UltraPure DNase/RNase-free dH₂O) and centrifuged at 560 x *g* and 4 °C for 10 mins. The supernatants were discarded, and pellets resuspended in 1 mL HBSS. Tube contents were pooled into a single tube

corresponding to their original sample and centrifuged at 300 x *g* and 4 °C for 10 mins. The pellets were resuspended in 400 µL HBSS, or 100 µL for controls. Antibodies were added at the appropriate dilution: 1:50 (4 µg/mL) for anti-CD31-APC and 1:200 (1 µg/mL) for anti-CD45-PE. Cells were incubated at 4 °C for 20 mins, spun down at 300 x *g* and 4 °C for 5 mins. Cell pellets were resuspended in an appropriate volume of HBSS containing 1:500 (vol/vol) DAPI stock solution, transferred to FACS tubes, and CD31⁺CD45⁻ live cells were isolated using a BD FACS Aria™ (BD Biosciences) cell sorter. Cells were sorted directly into 2 mL microcentrifuge tubes containing 350 µL of RLT buffer containing 2-mercaptoethanol at a concentration of 1:50 and stored on ice. RNA was extracted from sorted cells using a RNeasy Plus Micro Kit, as per manufacturer's instructions, and stored at -20 °C.

2.2.6 Harvesting mouse splenocytes

Spleens were removed by incising the left-hand side of the abdomen, after cardiac bleed and cervical dislocation of the animal, and transferred to gentleMACs™ C tubes containing autoMACS® running buffer. Spleens were macerated in a gentleMACS™ Dissociator (Miltenyi Biotec) using the mouse spleen pre-set program and then tubes were centrifuged at 250 x *g* for 10 mins. The supernatants were discarded, and pellets resuspended in RBC lysis solution for 5 mins. Sterile PBS was added to quench the lysis solution after 5 mins and the cell suspensions were passed through 70 µm filter into 50 mL Falcon tubes using Pasteur pipettes. The filtered solutions were centrifuged again at 250 x *g* for 10 mins, supernatants discarded, and pellets resuspend in 10 mL of complete DMEM. A Muse® Count & Viability Assay Kit was used to measure the final cell count and

viability of each sample on a Muse® Cell Analyzer instrument (Millipore). Samples were diluted to 4×10^6 in complete DMEM.

2.3 Methods: RNA sequencing

2.3.1 RNA extraction from mouse whole blood

Mouse whole blood was stored in RNAlater™ RNA stabilisation solution pre-filled microcentrifuge tubes. For small volumes of blood, $\leq 250 \mu\text{L}$, $350 \mu\text{L}$ of RNAlater™ was discarded prior to addition, otherwise $\leq 0.5 \text{ mL}$ of blood was added directly to the pre-filled tube. RNA-stabilised blood samples were spun at $\geq 15,000 \times g$ for 3 mins in a microcentrifuge and the supernatant was removed and discarded by pipetting. RNA was extracted from mouse whole blood pellets using a Mouse RiboPure™-Blood RNA Isolation Kit according to manufacturer's instructions. The concentration of RNA in this solution was measured using a Qubit™ 2.0 Fluorometer (Life Technologies) and a Qubit™ RNA Broad Range (BR) or High Sensitivity (HS) for high and low anticipated concentrations, respectively. For RNA samples due to for depletion of ribosomal (r)RNAs prior to library preparation, $30 \mu\text{L}$ of each sample was added directly to a well of a 96-well skirted PCR plate which was then stored at -80°C . For downstream qPCR (section 2.3.4), sample aliquots were stored at -20°C . For RNA samples due for depletion of globin messenger (m)RNAs, samples were immediately processed using a GLOBINclear™ mouse/rat Kit according to manufacturer's instructions. RNA concentration was once again measured for each sample before transferring the remaining volume of each aliquot to a 96-well skirted PCR plate and storing at -80°C .

°C. When ready for processing, samples for RNA-seq were allowed to thaw and RNA integrity was measured using a 2100 Bioanalyzer Instrument (Agilent Technologies) prior to library preparation.

2.3.2 Library preparation and bulk RNA-sequencing

Polyadenylated transcripts were oligo (dT) bead-selected, reverse transcribed, amplified, and labelled with an Illumina TotalPrep™-96 RNA Amplification Kit (Life Technologies). High-throughput sequencing was performed by the Oxford Genomics Centre functional genomics core facility team at the Wellcome Centre for Human Genetics (WCHG), University of Oxford, where sample fragments were either 75 or 150 bp size-selected and multiplexed prior to paired-end sequencing with an Illumina HiSeq4000 or NovSeq system (Illumina), respectively.

2.3.3 Single-cell RNA-sequencing

Droplet-based single-cell partitioning and single cell-RNA-seq (scRNA-seq) libraries were generated using the Chromium Single-Cell 3' Reagent v3 Kits as per the manufacturer's protocol. Briefly, a maximum volume (46.4 µL) of single-cell suspensions at densities of 100–350 cells/µL were mixed with RT master mix and immediately loaded together with Single-Cell 3' Gel Beads and Partitioning Oil into a Single-Cell Chip B. The chip was then loaded onto a Chromium Controller (10x Genomics) for single-cell gel bead-in-emulsion (GEM) generation and barcoding. Upon partitioning, the gel beads release unique oligos containing 10x cell barcodes, unique molecular identifiers (UMIs), and poly(dT) sequences. RNA transcripts

from single cells were reverse-transcribed within droplets to generate barcoded full-length cDNA. cDNA molecules from each sample were recovered and amplified. Finally, amplified cDNA was fragmented, and adapter and sample indices were incorporated to make libraries compatible with Illumina sequencing. The size profiles of the amplified cDNA and sequencing libraries were assessed by an Agilent 2200 TapeStation using High Sensitivity D5000 and D1000 ScreenTapes, respectively (Agilent Technologies). Indexed libraries were pooled in equimolar ratios and sequenced on the Illumina HiSeq 4000 system with a customised paired-end (28, 8, 98 bp) format according to the recommendation by 10x Genomics.

2.3.4 Real-time quantitative PCR

Isolated RNA was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit and pre-amplified using pooled TaqMan™ primers (Table 2.1) at a concentration of 0.4X and a TaqMan™ PreAmp Master Mix Kit. Thermocycling parameters can be found in Table 2.2A. Pre-amplified cDNA samples were diluted 1:20 in 1X TE buffer and combined in the appropriate ratio with TaqMan™ Gene Expression Master Mix, nuclease-free H₂O, and the appropriate primer on a MicroAmp Fast Optical 96-Well Reaction Plate. Samples were assayed in triplicate for each primer and real-time-quantitative PCR (RT-qPCR) was performed using a StepOnePlus™ Real-Time PCR System. Thermocycling parameters are can be found in Table 2.2B. The data obtained were normalised by subtracting the normalised threshold cycle (ΔCT), value averaged across triplicates, for the reference gene (*Pgk1*) from that of the target gene for test and control groups.

Target Gene	Primer
<i>Il1r1</i>	Mm00434237_m1
<i>Il1rap</i>	Mm00492638_m1
<i>Il6ra</i>	Mm00439653_m1
<i>Tnfrsf1a</i>	Mm00441883_g1
<i>Tnfrsf1b</i>	Mm00441889_m1
<i>Ptges</i>	Mm00452105_m1
<i>Ptgs2</i>	Mm00478374_m1
<i>Pgk1</i>	Mm01225301_m1

Table 2.1. Primers used for real-time quantitative PCR.

A

Step	Temperature (°C)	Time	Repeat
Enzyme activation	95	10 mins	-
Denaturation	95	15 s	14 times
Anneal/extend	60	4 mins	
Enzyme inactivation	99	10 mins	-
	4	∞	Hold

B

Step	Temperature (°C)	Time	Repeat
UNG incubation	50	2 mins	-
Enzyme activation	95	10 mins	-
Denaturation	95	1 s	40 times
Anneal/extend	60	20 s	

Table 2.2. PCR Thermocycling parameters. A. Parameters for preamplification of cDNA. **B.**

Parameters for StepOnePlus™ real-time quantitative PCR.

2.4 Methods: General computational biology

2.4.1 Read quality control

Raw FASTQ-formatted reads were assessed for sequence quality using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Sequences were assessed based on Phred nucleotide quality scores, GC content, number of over-represented sequences (such as globin or ribosomal), and k-mer content (Fig. 2.2). FastQC results for each file were combined into a single report using the MultiQC tool ²⁹¹. Poor quality sequences were trimmed, where necessary, using Trimmomatic v0.3.3 ²⁹².

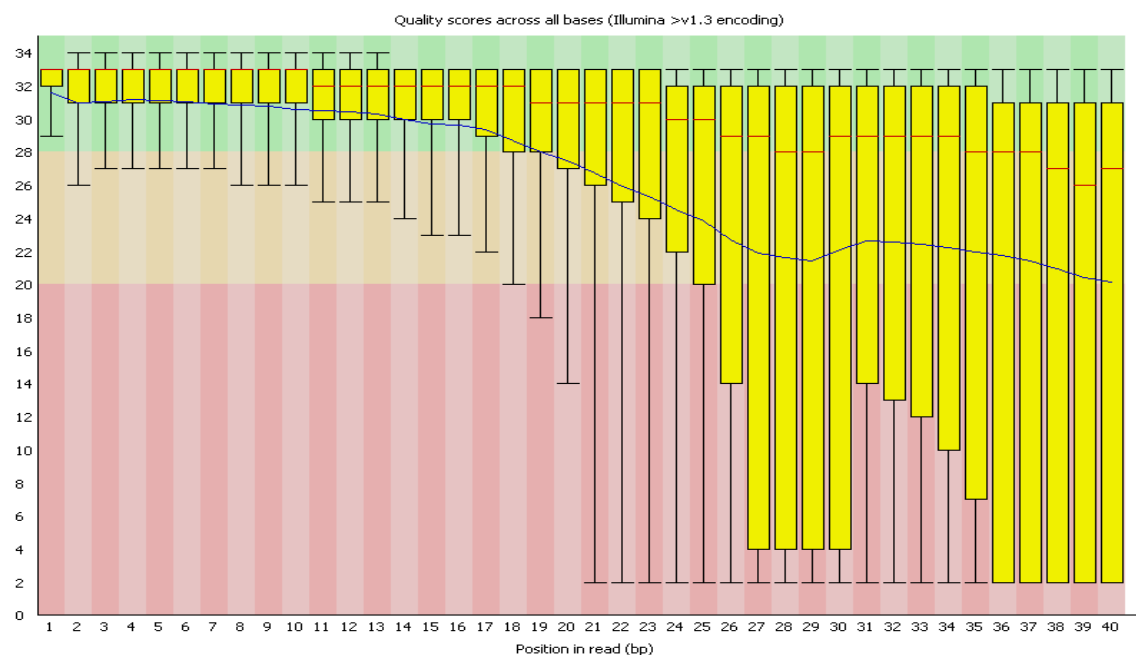


Figure 2.2. An example of a FastQC result. Phred nucleotide quality scores (y-axis) are plotted against the base pair position (x-axis). The shaded green region indicates quality scores deemed good, the orange region indicates acceptable scores, and the red region indicates poor quality sequence. Yellow boxes represent the interquartile range at each position, with median values highlighted with a red line, and upper and lower whiskers corresponding to 10% and 90% points. The blue line going across the boxplot indicates the mean quality score.

2.4.2 Read alignment

HISAT2 v2.1.0 ²⁹³ was used for the probabilistic alignment of raw RNA-seq FASTQ read sequences to the reference genome as follows:

```
-p 32 --dta -x /path/to/genome -1 forward_read.fastq.gz -2 reverse_read.fastq.gz -S  
sample_name.sam
```

The output of HISAT2 alignment is a sequence alignment map (SAM) file, containing the formatted alignment result, that can be converted to its binary equivalent binary alignment map (BAM) file, sorted by coordinate, for subsequent processing using the SAMtools (v0.1.18) algorithm ²⁹⁴:

```
samtools sort sample_name.bam -o sample_name.sorted.bam
```

The spliced transcript alignment to reference (STAR) tool ²⁹⁵ (v2.4.0.1) was also used for the semi-probabilistic alignment of read sequences to the reference genome and, in some cases, simultaneous gene quantification. Genome indexes, either consisting solely of the reference genome FASTA file or combining the reference genome with gene annotation for concurrent alignment and quantification (optional), were generated as follows:

```
--runThreadN 32 --runMode genomeGenerate --genomeDir /path/to/genome_dir --  
genomeFastaFiles /path/to/genome.fa
```

Optional: *--sjdbGTFfile /path/to/annotation.gtf --sjdbOverhang 100*

RNA-seq FASTQ read sequences were then aligned to the genome index to generate a BAM file output and gene abundances were quantified (optional) as follows:

```
--runThreadN 32 --genomeDir /path/to/genome --readFilesCommand zcat --readFilesIn  
forward_read.fastq.gz reverse_read.fastq.gz --outStd BAM_SortedByCoordinate --  
outFileNamePrefix sample_name
```

Optional: *--quantMode GeneCounts*

The pattern matching in nucleotide databases (PatMaN) tool ²⁹⁶ (v1.2.2) was used for the fully deterministic alignment of FASTA-formatted RNA-seq reads to the cDNA reference assembly. Its usage is integrated with the noise detection pipeline outlined in the next section.

2.4.3 Noise detection

Using the transcript coordinates of the aligned reads as input, the expression profile for each individual transcript is built as an algebraic point sum of the abundances of reads incident to any given position; if the alignment is performed for each read, the corresponding abundance for every entry is set to +1. For each sample, j , and for each transcript, T , the point-to-point Pearson correlation between the expression profile in j and the one in all other samples is calculated. The noise detection is based on the relative location of the distribution

of the point-to-point Pearson correlation coefficient (p2pPCC) ²⁹⁷ versus the abundances of genes and is specific for each individual sample. For low abundance transcripts the stochastic distribution of reads across the transcript leads to a low p2pPCC; the aim of the approach is to determine the range where the distribution of correlation coefficients (used as proxy for the distribution of reads across a transcript) are above a user-defined threshold; to approximate the signal-to-noise threshold a binning on the abundances is performed. For all examples presented in this study, the binning is done on $\log_2$ ranges; the signal-to-noise thresholds were defined as the abundance at which the median of the p2pPCC distribution within a bin exceeds 0.7. A range for the signal-to-noise can be defined on: [lower bound] the first occurrence of a distribution for which the median exceeds 0.7 and [upper bound] the last distribution for which the median is lower than 0.7. The correlation threshold, the localisation of the distribution and the binning range are user-defined parameters. The localisation parameter can be chosen as the lower/upper quartile (25/75%) or top 5%, 95%. The usage of local extremes (min/max) is not recommended due to the high inter-sample variability. A log-base binning approach should be applied that will assign at least 25 transcripts per bin for the bins around the mode of the abundance distribution.

2.4.4 Gene quantification

Gene abundances were estimated from BAM files using either transcript-based or union exon-based quantification tools. Transcript-based quantification was performed using StringTie v1.3.5 ²⁹⁸ to generate a sample-specific GTF from a reference annotation GTF:


```
-e -B -p 32 -G /path/to/annotation.gtf -o sample_name.gtf -l sample_name  
sample_name.bam
```

Sample GTFs were consolidated into a single count matrix by running the Python prepDE.py script (<http://ccb.jhu.edu/software/stringtie/dl/prepDE.py>) in the output file directory.

Union exon-based gene quantification was performed using either the htseq-count function (HTSeq v0.10.0 ²⁹⁹) or the featureCounts (v1.6.2) tool ³⁰⁰:

```
HTSeq: -f bam -r pos -t exon sample_name.sam /path/to/annotation.gtf > result.txt
```

```
FeatureCounts: -t exon -g gene_id -a /path/to/annotation.gtf -o result.txt sample1.bam  
sample2.bam etc...
```

Kallisto v0.45.0 ³⁰¹ was used to pseudoalign reads, quantifying gene abundances directly based on an index created from a cDNA FASTA file:

```
kallisto quant -i index -o output forward_read_1.fastq reverse_read_1.fastq  
forward_read_2.fastq reverse_read_2.fastq etc...
```

2.4.5 Differential gene expression analysis

Count matrices were read into the RStudio environment, running on the latest version of R, in a manner specific to the output of gene quantification. Comma-separated variable (.csv) gene count matrix files produced from StringTie quantification GTF files by the prepDE.py script were read using the *read.csv* function (*header = T, row.names = I*). The STAR *--quantMode GeneCounts* function

generates gene count matrices for each sample stored as a ReadsPerGene.out.tab file. These can be read using the *list.files* function was used to list the .tab files in the output directory, the *read.table* function to read each .txt files using *lapply* (*skip* = 4 as the first 4 rows contain unnecessary information), and producing a data frame using *sapply* to concatenate the fourth column of each .tab file containing the gene counts. The gene names were added as row names to the data frame by selecting a single first column, and the *list.files* vector was used to apply sample information as column names. The same approach is used to read the text (.txt) files generated for each sample by htseq-count with the minor exception that no rows are skipped when *read.table* is applied and it is the second column of each file that contains the gene counts. As featureCounts generates a single .txt file from all samples, this could be read in by simply using the *read.table* function. Finally, the kallisto output is a tab-separated variable (.tsv) file for each sample that contains Ensembl transcript names that must be converted to corresponding gene names. This is done by loading the EnsDb.Hsapiens.v86 package (kallisto was only used for human data in this study) and using the *keys* function to select the *keytype* = “TXNAME” information. The *select* function can then be used to match the input “TXNAME” with its “GENEID” from the Ensembl database. The .tsv files were imported using *tximport* and supplying the *tx2gene* vector in the argument (plus *ignoreTxVersion* = T) to generate a gene count data frame. For the comparison of unfiltered data with and without noise adjustment, rows with zero values for all samples were excluded prior to further processing.

For the comparison of RNA-seq alignment/quantification tool combinations (chapter 4), each data frame was duplicated to facilitate the side-by-side comparison. Counts per million (CPM) filtering was applied to one matrix,

taking genes with a sum of greater than one CPM across the number of samples in smallest group size, in the case of the samples analysed in chapter 3; both count matrices were normalised using the trimmed mean of m-values (TMM) method ³⁰². The matrices were again duplicated, and the inferred noise thresholds were applied to one set of matrices from each pair; briefly, within a sample j , all entries below the signal-to-noise threshold were set to the threshold level. Entries with no value above the signal-to-noise thresholds, in any sample, were excluded from further analyses.

The subsequent differential gene expression (DGE) analysis was conducted using the Bioconductor package *limma* ²⁷⁸. Weights were calculated using the *limma* *voom* function ³⁰³. The signal-to-noise thresholds obtained on the raw data were factor-transformed to correspond to the TMM normalised distribution of values.

Applying noise thresholds to voom-transformed data:

```
for(i in 1:ncol(v_nc$E)){
  v_nc$E[,i] <- ifelse(v_nc$E[,i] < v_nc$E[nrow(v_nc),i], v_nc$E[nrow(v_nc),i],
v_nc$E[,i])
}
```

Removing rows with sums corresponding to the sum of noise thresholds:

```
rownames(v_nc$weights) <- rownames(v_nc$E)
noise_genes <- rownames(v_nc$E[rowSums(v_nc$E) == sum(v_nc$E[nrow(v_nc),]),])
noise_counts <- v_nc$E[noise_genes,]
v_nc$weights <- v_nc$weights[rowSums(v_nc$E) != sum(v_nc$E[nrow(v_nc),]),]
v_nc$E <- v_nc$E[rowSums(v_nc$E) != sum(v_nc$E[nrow(v_nc),]),]
```

Linear models were fitted to the data using the `lmfit` function, and a trended empirical Bayes (eBayes), *eBayes*, method was applied to account for errors in the $\log_2$ fold change (LFC) estimations ²⁷⁸. Differentially expressed genes (DEGs) were defined as those with a false-discovery rate (FDR) adjusted *p*-value, calculated using the Benjamini-Hochberg method ³⁰⁴, of < 0.01 , and an absolute LFC greater than one.

2.4.6 Single-cell analysis

Raw fastq files – I1 = 8-base MPX index, R1 = Unique molecular identifier, R2 = insert/transcript sequence – were analysed for sequence quality using Fastqc software, as in section 2.4.1, and trimmed where necessary using Trimmomatic software (<https://github.com/timflutre/trimmomatic>) ²⁹². These files were aligned, filtered, barcode and UMI counted using the *cellranger count* function of the Cell Ranger (10x Genomics) pipeline to yield a barcodes.tsv, genes.tsv, and matrix.mtx file for each sample. These data were analysed using the Seurat pipeline ²⁸⁶, creating objects for each experimental condition to include features detected in at least three cells and then filtering the resultant object to keep only cells with fewer than 2% mitochondrial transcripts. An integrated, canonical correlation analysis (CCA) was then performed to compare each group for neutrophil cluster-specific responses to immunisation.

2.5 Methods: General molecular biology

2.5.1 Plasmid construct design

The nucleotide sequence for the desired antigen was obtained from the GenBank sequence database (<https://www.ncbi.nlm.nih.gov/genbank/>). The sequence was run through a glycosylation site finder (<http://www.cbs.dtu.dk/services/NetNGlyc/>) to identify putative glycosylation sites and remove them if the probability of glycosylation exceeds 50%. This is done to remove sequences that may be post-translationally modified if expressed from mammalian cells. The polyA tail was removed from the sequence and the GeneArt® Gene Synthesis tool (Invitrogen) (<https://www.thermofisher.com/en/home/life-science/cloning/gene-synthesis/geneart-gene-synthesis.html>) was used to upload the sequence, optimise codons for mammalian tissue, and add restriction sites. The HindIII (AAGCTT at the start of the sequence) and NotI (GCGGCCGC at the end of the sequence) restriction sites were added to the construct.

2.5.2 Restriction digest

To cut out the transgene from the plasmid backbone, a restriction digest reaction was set up as a 50 µL reaction in a PCR tube as follows.

Reagent	Volume/concentration
Restriction enzyme (HindIII-HF, NotI-HF)	1 μ L of each
10X CutSmart [®] buffer	5 μ L
Plasmid DNA	1 μ g
dH ₂ O	Remaining volume (up to 50 μ L)

The reaction was run for four hours at 37 °C on a thermocycler.

2.5.3 Agarose gel electrophoresis

DNA samples were run on a 1% agarose gel after restriction digest. The gel was stained using peqGreen DNA dye and placed in an electrophoresis cassette filled with 1X TAE buffer. 10 μ L of purple loading dye was added to the 50 μ L samples to achieve a 1X concentration. Samples were loaded into the wells of the gel along with a 1 kb DNA ladder. The electrophoresis apparatus was connected to a voltage box set to 100V and the gel was run for 40 mins. The gel was analysed using a UV light box and bands corresponding to the size of the insert were excised from the gel using a gel extractor tool and placed in a 2 mL Eppendorf tube. DNA was extracted from the gel section using a QIAquick Gel Extraction Kit. Briefly, kit buffer was added to the 2 mL tube at a ratio of 3:1 (100 mg gel ~ 100 μ L) and incubated at 50 °C on a heating block for 10 mins or until dissolved, one volume of isopropanol was added and the gel digest was filtered and washed as per kit instructions, eluting the DNA into a final volume of 100 μ L dH₂O. The DNA concentration was measured using a NanoDrop[™] 2000 Spectrophotometer (Thermo Scientific).

2.5.4 DNA ligation

A 20 μL DNA ligation reaction was set up, to place the insert sequence into a pMONO expression plasmid with Kanomycin resistance, in a PCR tube as follows:

Reagent	Volume/concentration
2X LigaFast™ buffer	5 μL
pMONO 164 backbone DNA	2 μL
Insert DNA	2 μL
T4 DNA ligase	1 μL

The reaction was run overnight on a thermocycler set to 16 °C.

2.5.5 Transformation

After overnight ligation, 5 μL of sample was added to 50 μL of thermocompetent DH5 α cells in a 2 mL Eppendorf tube on ice. A negative control was set up by adding 5 μL of dH₂O to the same volume of cells. Samples were incubated at 4 °C for 30 mins, heat-shocked at 42 °C for 30 s, and returned to 4 °C for 2 mins. 250 μL of pre-warmed (37 °C) SOC recovery medium was added to each tube and samples were placed on a 37 °C shaking incubator for 1 hour before plating 200 μL on LB agar containing 30 $\mu\text{g}/\text{mL}$ Kanomycin using an L-shaped spreader. Plates were incubated at 37 °C overnight. The following day, colonies

were picked from the plates using a pipette tip and dropped in an Erlenmeyer shaker flask containing 50 mL of LB broth with 30 µg/mL Kanomycin to be placed in a 37 °C shaking incubator overnight.

2.5.6 DNA extraction

Samples were transferred from the Erlenmeyer flasks for 50 mL Falcon tubes and centrifuged at 300 x *g* for 10 mins. The supernatant was poured off and DNA was extracted from the pellets using a QIAGEN Plasmid Midi Kit, as per manufacturer's instructions. DNA concentrations were measured using the NanoDrop™ and subsequently restriction digested as outlined in section 2.5.2 (using insert DNA rather than plasmid DNA). Aliquots of the digested DNA samples were run on a 1% gel and, if the band corresponded to the appropriate insert size, sent to Source Bioscience for sequencing, using forward and reverse primers for the 3' cytomegalovirus (CMV) promoter and bovine growth hormone (bGH) polyA region sequences, respectively, to ensure the presence of the correct antigen sequence in these samples.

2.5.7 Cloning into adenovirus

Sample sequence files, forward and reverse, were aligned to the plasmid containing the transgene insert reference file using SeqMan Pro (DNASTAR) software. For samples with 100% sequence identity to the reference, DNA was treated with a Gateway™ LR Clonase™ II kit. The DNA samples, containing the antigen sequence of interest, were diluted in TE buffer to yield a solution containing 150 ng of DNA which was then combined with 1 µL of an AdHu5

destination vector (at a concentration of 150 ng/ μ L) in a PCR tube. 2 μ L of LR Clonase™ II Enzyme Mix was added to each sample and incubated at room temperature for 10 mins. 1 μ L of proteinase K was added to each sample the following day and incubated at 37 °C for 10 mins. The proteinase K-digested samples were transformed using DH5 α cells as outlined in section 2.5.5, but this time plating 200 μ L on LB agar containing 100 μ g/mL ampicillin and adding 10 μ L streaks of up to four samples per plate of LB agar containing 15 μ g/mL chloramphenicol. Plates were incubated overnight and the following day samples with colonies on LB ampicillin plates that did not grow on LP chloramphenicol plates were picked and cultured overnight in a 37 °C shaking incubator in Erlenmeyer flasks containing 50 mL of LB broth with 100 μ g/mL ampicillin. DNA was extracted the following day as per section 2.5.6, the concentration was measured, and samples were again sent for sequencing with Source BioScience using the same primers. Samples with 100% sequence identity relative to a reference sequence (AdHu5 destination vector containing the antigen insert) were selected for linearization. 1 μ g of DNA was restricted by 3 μ L of PacI restriction enzyme in 10 μ L of CutSmart® buffer and made up to 100 μ L total volume with dH₂O in a PCR tube. The reaction was run for four hours at 37 °C on a thermocycler and the reaction was stopped by exposing to 65 °C for 25 mins. 15 μ L of sample was run on an agarose gel (section 2.5.3) to ensure linearization was successful and the remaining volume (85 μ L) was sent to the Viral Vector Core Facility (VVCf, Jenner Institute, University of Oxford) for production.

2.6 Methods: *In vitro/ex vivo* assays

2.6.1 Cytokine quantification

A 6-plex MILLIPLEX® mouse cytokine/chemokine panel consisting of IFN- γ , TNF α , IL-1 β , IL-3, IL-5, IL-6 was used to quantify pro-inflammatory cytokines in mouse sera. The assay was run according to manufacturer's instruction and MFI values were obtained using a Luminex MAGPIX® instrument with Exponent software (Invitrogen).

2.6.2 Expression assays

Cells were harvested at approximately 70–80% confluency. Where brefeldin stimulation was required, cells were resuspended in media containing 3 $\mu\text{g/mL}$ of brefeldin A solution. 1×10^6 cells per sample were infected with the appropriate volume of adenovirus construct to obtain a concentration of 5×10^8 . Infected cells were incubated for the required time (overnight or specific time points), harvested and surface stained and/or stained intracellularly with anti-fHbp monoclonal antibody JAR5 (National Institute of Biological Standards and Controls) and GFP-tagged IgG detection antibody using a Fixation/Permeabilization Solution Kit. Cells were resuspended in 400 μL of permeabilization buffer and filtered before running on a FACSCalibur flow cytometer (BD Biosciences). The percentage of cells expressing GFP was measured and analysis was performed using FloJo software (TreeStar).

2.6.3 Indirect enzyme-linked immunosorbent assays

To quantify antibodies against fHbp in animal sera, 2.5 µg/mL recombinant fHbp 1.1 (plasmid gifted by Dr Peter Beernink, CHORI, and protein produced at Oxford Protein Production Facility) in carb/bicarb buffer was coated onto 96-well flat-bottomed microtitre plates overnight at 4 °C. Plates were washed with PBS containing 0.05% (v/v) Tween-20. Plates were blocked with 1% BSA in PBS and serial dilutions of sera or positive control (JAR5 antibody) were added. After incubation, anti-goat anti-mouse horseradish peroxidase conjugate was added at a 1:20,000 dilution. Plates were incubated for 20 mins with 100 µL per well TMB enzyme-linked immunosorbent assay (ELISA) substrate. 1M sulphuric acid was added to stop the reaction. Absorbance were read at 450 nm and 630 nm using a Multiskan MS plate reader (Biotek). Interpolated IgG concentrations were calculated for each serum sample using a standard curve.

2.6.4 Serum bactericidal antibody assays

H44/76-SL (provided by Public Health England, PHE) strain *N. meningitidis* group B bacteria were used for SBAs. Test sera (20 µL) were heated for 30 mins at 56 °C to inactivate endogenous complement. Equal volumes (10 µL) of bacterial suspension (optical density (OD)₆₀₀ = 0.1 bugs diluted to 1:5000), and human complement were added to twofold serial dilutions of test serum sample. The SBA titre was the serum dilution resulting in 50% survival of the bacteria compared to the number of colony-forming units (CFU)/mL in the variable complement control, containing inactivated complement and no sera.

2.6.5 FluoroSpot assays

A Mouse IFN- γ /IL-17A FluoroSpot kit was used to measure antigen-specific T cell responses in mouse spleen tissue. Anti-IFN- γ and anti-IL-17A monoclonal capture antibodies were diluted in PBS to a concentration of 15 μ g/ml and 10 μ g/ml, respectively. The IPFL plate membrane was washed with 15 μ L of 35% ethanol per well for no more than 60 s and then washed three times with 200 μ L of sterile H₂O per well. 100 μ L of capture antibody was added to each well and the plate was sealed for overnight incubation at 4 °C. The plate was washed three times with 200 μ L of sterile PBS per well the following day and the wells were then blocked with DMEM containing 10% FBS for 30 mins. Stimuli, either DMSO (1:100) as a negative control, ConA (1:200) as a positive control, or reconstituted peptide pools (1:100) corresponding to the vaccine antigen, were added to the appropriate wells, followed by 2×10^5 splenocytes from the appropriate sample to each corresponding well. The plate was sealed and incubated overnight at 37 °C in a 5% CO₂ incubator. Cells were removed from the wells the following day and the plate was washed five times with 200 μ L of PBS per well. Anti-IFN- γ -R4-6A2-BAM and anti-IL-17A-MT2270 (biotinylated) detection antibodies were diluted in PBS containing 0.1% BSA in the same tube to a concentration of 1:200 and 1:250, respectively. 100 μ L of detection antibody mixture was added to each well and incubated for two hours at room temperature. Anti-BAM-490 and SA-550 fluorophore conjugates were both diluted to a concentration of 1:200 in the same tube with PBS containing 0.1% BSA and, after washing the plate five times with PBS, 100 μ L of this mixture was added to each well. The plate was wrapped in aluminium foil and incubated for one hour in the dark at room temperature. The plate was washed five times with PBS before adding 50 μ L of fluorescence enhancer

to each well and incubating in the same manner for 15 mins. The plate was emptied of all liquid and the underdrain was removed. Plates were completely dried in the dark at room temperature prior to spot counting with an IRIS™ FluoroSpot reader (Mabtech). Excitation 490 nm/emission 510 nm (FITC) and excitation 550 nm/emission 570 nm (Cy3) wavelengths were used to measure IFN- γ and IL-17A, respectively.

2.7 Methods: Cell imaging

2.7.1 Confocal microscopy

Complete DMEM containing 1×10^6 HeLa cells were seeded in 300 μ L volumes per well on glass coverslips placed at the bottom of six-well plates. Cells were infected overnight with 1.5×10^8 IU (proportional to the number of cells infected) of each AdHu5 vaccine containing an antigen-eGFP transgene or appropriate control. Cells were washed the following day using sterile PBS and fixed and permeabilised using 250 μ L of Fixation/Permeabilization solution from and incubated at 4 °C for 20 mins. After washing with PBS, 300 μ L of 300 nM DAPI (5 mg/mL stock solution diluted to 300 nM in PBS) was added to the fixed/permeabilised cells for 5 mins at 4 °C, washed again and slowly transferred, using forceps and dabbing off excess liquid, to microscope slides with a drop of ProLong Gold Antifade Mountant in the centre. Slides were cured on a flat surface overnight in the dark and imaged the following day using a Zeiss 780 inverted confocal microscope (Zeiss) with ZEN Lite image acquisition software (Zeiss). Cells were located in brightfield and then the interface was switched to ‘acquisition

mode' where 'smart settings' were applied. The fluorophore-specific settings were manually refined, and images were taken across multiple planes of focus for each sample triplicate.

2.7.2 Time-lapse imaging

Complete DMEM containing 1×10^6 HeLa cells were seeded in 100 μ L volumes per well (1×10^5 cells per well) overnight in each well of an eight-well chambered coverslip. The following day the cells were stained using far-red fluorogenic SiR-DNA 2 hours prior to infection. Cells were washed with PBS and subsequently infected with 5×10^7 IU (proportional to the number of cells) of each eGFP-expressing AdHu5 vaccine. The chambered coverslip was then secured on the stage, within a live cell stage incubator set to 37 °C and supplemented with 5% CO₂, of a ZEISS Spinning Disc microscope (Zeiss). Three coordinates were set for each sample using ZEN Blue image acquisition software (Zeiss) and imaged every 10 mins over the course of 14 hours. The time-lapse for each sample was then constructed from these images using ImageJ software (Fiji).

Chapter 3

Assessment of the effects of technical noise on RNA-seq differential gene expression pipelines

3.1 Introduction

The increase in sequencing depth for RNA-seq experiments ³⁰⁵ allows a higher sensitivity for the detection of perturbations in gene expression levels between samples ³⁰⁶. This increased accuracy facilitated the characterisation of differential gene expression (DGE) at tissue and cellular levels ³⁰⁷. However, non-biological/technical variation can be introduced at different stages of the RNA-seq library preparation and sequencing and varies from amplification/sequencing bias ³⁰⁸ to random hexamer priming during the sequencing reaction ³⁰⁹; these technical alterations of the biological signal can affect the precision of the downstream DGE call. Statistical methods focused mainly on batch/background correction, normalisation, and evaluation of DGE have been developed to mitigate the impact of known biases on DGE analyses ³¹⁰; however these approaches are not designed to account for unknown sources of technical noise which contribute to calling highly variable, low abundance genes as DE. Additionally, the choices of pre-processing tools also influence the output (and relative quantification accuracy) of gene expression ³¹¹. These analytical biases mainly arise from differences in the detection and handling of isoforms or processing of unmapped and multi-mapping reads ³⁰⁷. In this chapter, we compare a selection of commonly-used alignment and

quantification tools to assess the contribution of computational choices corroborated with the existing technical noise to the downstream DGE call.

A recent benchmarking study of widely-used RNA-seq aligners ³¹² compared their relative accuracy when applied to simulated data of varying sequence complexity. The results suggest a poor correlation between the performance of these tools and their relative frequency of use, i.e. widely-cited tools appear to underperform based on the metrics used in the study, particularly when default parameters are applied. Several other benchmarking studies have also evaluated the landscape of RNA-seq aligners under different dataset-specific criteria ^{313–316} allowing the users to make informed decisions as to which tool to use based on the particularities of their datasets. In practice, transcript- and gene-level estimation tools are often used in combination with HISAT2 ²⁹³ and STAR ²⁹⁵. The HISAT2 and STAR alignment tools differ mainly in their use of compression; HISAT utilises an extension of the Burrows-Wheeler transform compression algorithm ³¹⁷, reducing its memory requirement to <8GB, while STAR is more RAM-intensive due to its use of uncompressed suffix arrays for ultrafast alignment. However, speed and memory usage are not the sole considerations when choosing an alignment tool; an important criterion when deciding which of the ever-growing number of read alignment tools to use is the ability to integrate it within pipelines leading to downstream estimation of expression and subsequent call of differential expression.

Both transcript- and gene-level quantification tools were used in combination with HISAT2 and STAR. StringTie ²⁹⁸ performs a transcript-level estimation using a network flow algorithm, in combination with optional *de novo* assembly methods; it is a recommended tool for quantifying HISAT2-aligned reads ³¹⁸. Genomic overlap between isoforms hinders the StringTie quantification on this

particular set of transcripts; in comparison, the gene-level estimation (e.g. union exon methods) are based on the simpler merging of overlapping exons and quantification based on algebraic sum of incident reads. However, union-exon methods result in significant underestimation of gene expression levels when compared with transcript-based approaches ³¹¹. HTseq (htseq-count) ²⁹⁹ and featureCounts ³⁰⁰ are two similar union exon-based estimation tools; featureCounts has been shown to out-perform the htseq-count in speed and accuracy ³⁰⁰. The use of the *--quantMode GeneCounts* option in STAR produces an identical output as the one from htseq-count. As the accuracy of quantification relies heavily on the identification of the transcripts from which reads originate, more than the precise characteristics of the alignment of these reads to the transcript, recently developed pseudoalignment tools, such as kallisto ³⁰¹, utilise hash tables to build the index, bypass the alignment step, and base transcript identification on the length of k-mers and the mean of the distribution of fragments, increasing computational speed while maintaining accuracy.

In this chapter, a novel method for the detection of genes showing random, low-level variation (technical noise) is applied to an RNA-seq dataset (GSE131929) with samples from participants that received a stimulus that induced profound perturbation of the transcriptome relative to baseline as part of a European childhood life-threatening infectious disease study (EUCLIDS, detailed further in section 4.1). This method offers a data-driven alternative to the *ad hoc* (and sample-independent) filtration of low abundance genes, based on the counts per million (CPM) ²⁶⁸. The inferred thresholds are used to compare the proportion of “noisy” genes for combinations of the aforementioned read alignment and gene quantification tools and assess the impact on the downstream DGE call. Finally, it is demonstrated that the variation derived from protocol-specific features can be

reduced by the exclusion of genes with abundance values below user-defined noise thresholds (specific for individual samples), leading to the convergence in the identity of differentially expressed genes (DEGs) between method combinations.

3.2 Methods

3.2.1 Dataset

The RNA-seq dataset GSE131929 (EUCLIDS) was used for the analyses presented in this chapter.

3.2.2 Computational analysis

The pipelines for the processing of RNA-seq reads, noise detection, and DGE analysis can be found in sections 2.4.1–2.4.5.

3.3 Results

3.3.1 Variation in the quantification of gene expression for combinations of pre-processing tools is attributable to the choice of gene quantification tool

Run summaries were generated for the input data and on the read alignment output comprising total and unique number of reads, for the former, and of percentages of mapped reads for each method, for the latter. The proportions of uniquely-mapping reads varied in the 87.69–97.78% (median 91.63%) range for STAR and 83.24–90.23% (median 87.5%) range for HISAT2. STAR captured a greater number of reads mapping to the reference genome, with as few as 2.28–6.62% (median 3.14%) not aligned compared with 7.09–12.94% for HISAT2 (median 8.07%), but also retrieved a higher percentage of multi-mapping reads (3.55–9.23%, median 5.03%) than HISAT2 (2.59–6.77%, median 3.72%). The distributions of the mapping proportions are presented in Fig. 3.1.

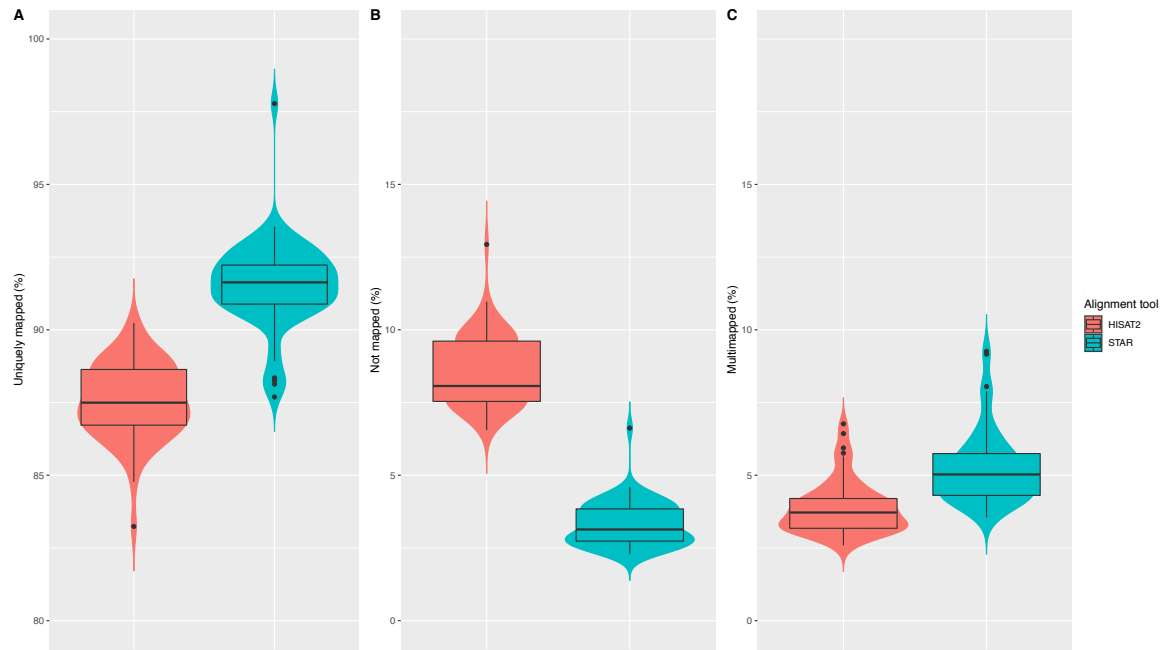


Figure 3.1. Run summaries for HISAT2 and STAR alignment tools. The violin plots show the distribution of percentages of total reads mapping to the reference genome. **A.** uniquely mapped, **B.** not mapped, and **C.** multi-mapped. The overlaying boxplots indicate the median and interquartile ranges of the distributions.

The total number of genes called (with non-zero expression level) by each alignment/quantification combination was consistent between HISAT2- and STAR-aligned outputs (37814–39370, median of absolute differences: 222 genes). The variation in total numbers of expressed genes appears to be attributable mainly to the choice of quantification tool since it remains relatively consistent between combinations with the same quantification tool even when different alignment tools are used (e.g. 39370 and 39592 for HISAT2-HTSeq and STAR-HTSeq options, respectively). A density plot of the abundances of genes uniquely called as expressed (i.e. called as expressed by one method only) by each tool combination shows that the genes in the specific differences are predominantly low abundance genes (Fig. 3.2, 83.44% genes with abundance less than the median [blue line], 16.56% genes with abundance greater than the median).

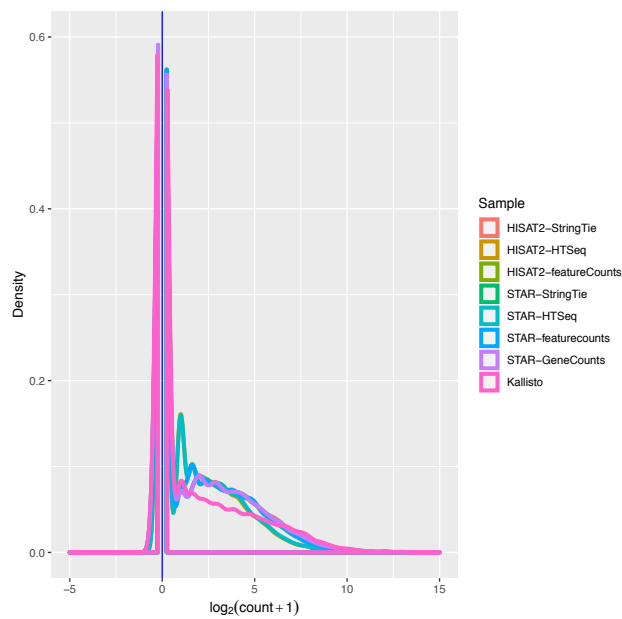


Figure 3.2. Gene count density plots of uniquely-called differentially expressed genes from each tool combination. Distribution of expression levels, based on initial pre-normalized pseudocounts from non-zero matrices of expressed, unique genes as called by each read alignment/gene quantification tool combination for a randomly selected sample. The horizontal blue lines indicate the median $\log_2$ (pseudo)count.

The MA plots of genes called as expressed by all methods are presented in Fig. 3.3; in black we represent boxplots of the differences between expression values of genes for a given abundance; in red we represent genes identified as outliers for each boxplot; the central blue line indicates a 0 log-ratio i.e. identical values in the compared samples. Tight MA plots along the median line indicate consistency in gene expression values between tool combinations (the analysis was conducted per sample; the plots shown correspond to a single sample – 0002-V03). A good concordance was observed between combinations employing similar gene quantification tools, i.e. HTseq, featureCounts and GeneCounts, even when a combination with a different alignment tool is considered.

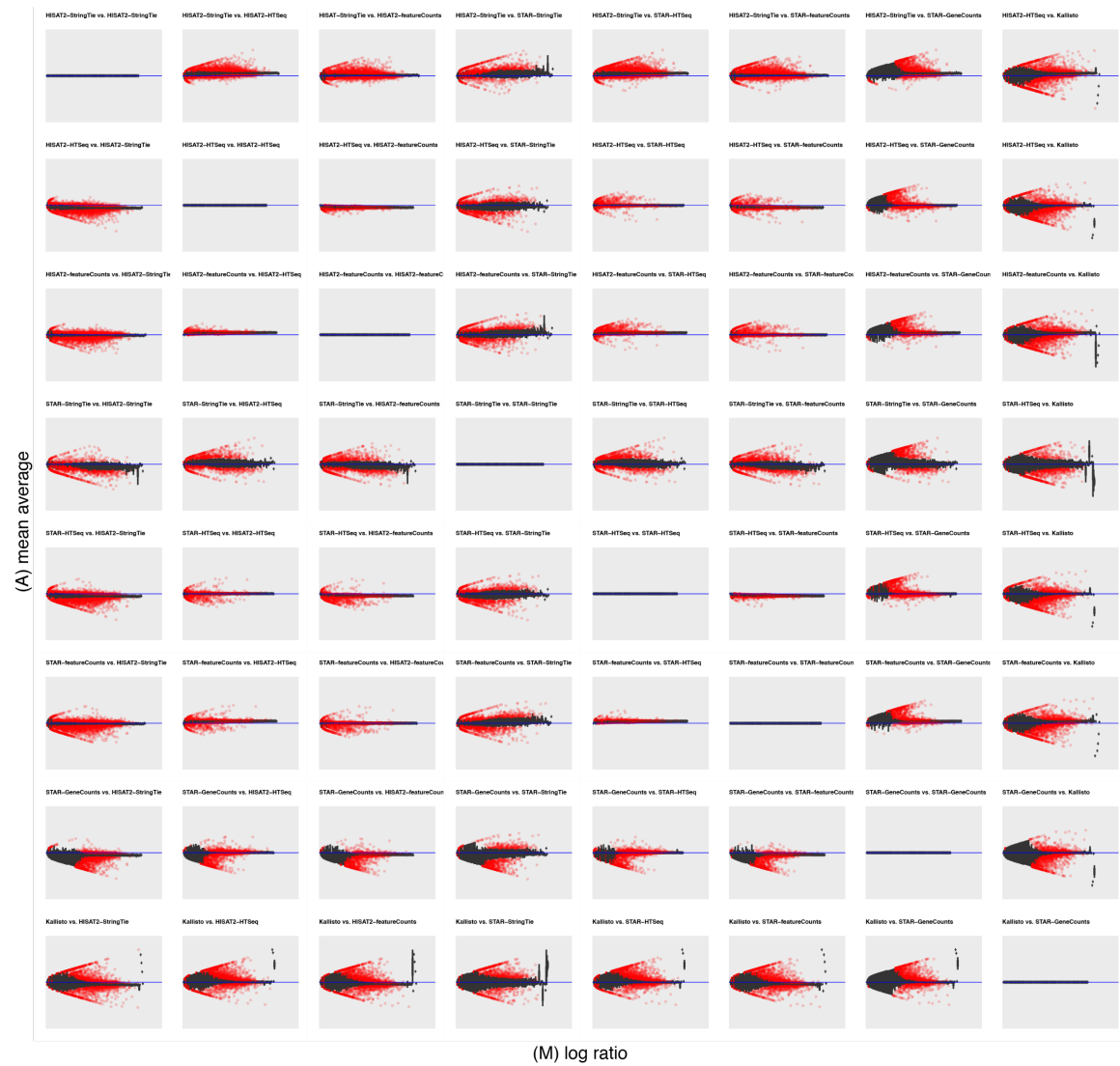


Figure 3.3. MA plots of pairwise comparisons of the expression levels of genes called by each tool combination. The log ratio (M) and mean average (A) values were compared between each pair of combinations for a single sample. The blue lines correspond to an M value of 0. In red, we indicate the genes which would be called differentially expressed (DE) if the two pseudo-quantifications of one sample were to be compared. This analysis illustrates the variability specific to the analytical noise and the deviation from what should be a standard non-DE example.

When the similarity analysis is restricted to the top 100 most variable genes, based on the sample variance for genes in the pairwise intersection of tool combinations (Jaccard approach), the consistency in response drops (0–5%) as shown in the heatmaps in Fig. 3.4. The similarities between method combinations, as computed

for a hierarchical clustering on expression vectors indicate a grouping by read alignment tool for similar gene quantification tools, whereas the transcript-level estimation (StringTie) combinations cluster separately. These findings suggest that the choice of gene quantification method contributes more to quantified variation in gene expression levels than the choice of read alignment tool.

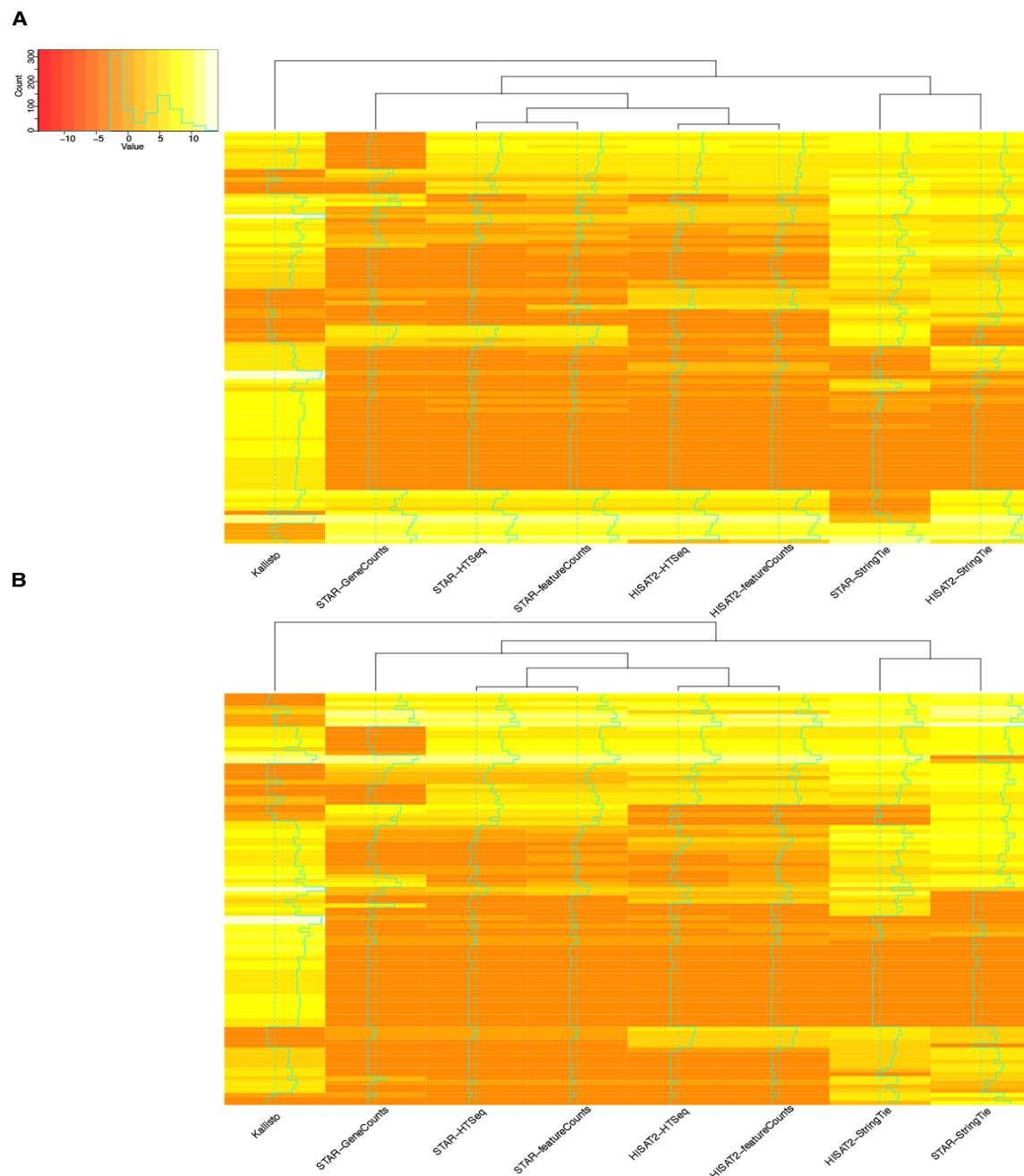


Figure 3.4. Clustering of gene expression levels from each tool combination based on the top 100 most variable genes across all combinations. Heatmaps showing the absolute and relative variation in expression levels of the top 100 most variable genes for different choices of read alignment/gene quantification tools for **A.** random sample one and **B.** random sample two. The variability is measured using the standard deviation between each measurements. The expression levels are represented on a $\log_2$ scale. The clustering of the method combinations is represented by the dendrograms on the x-axis. The grouping indicates that the alignment method drives the differences observed between the tool combinations. These observed similarities are consistent between samples.

To illustrate the changes in the distribution of gene expression values throughout the various stages of each pipeline, a set of density plots for a single tool combination are presented in Fig. 3.5. Density plots of gene expression to normalisation (Fig. 3.5A) highlights the high shape-similarity of distributions of abundances for all tested combinations. CPM filtering (genes with a CPM above one in more than 15 [minimum group size] samples) marked for exclusion from the subsequent steps of the analysis) was performed to compare the effect of the noise adjustment pipeline. This filtering step reduced the number of expressed genes included in the analysis by approximately 50% (16953–20199, median 19589, Fig. 3.5B). Corresponding TMM-normalised and *voom*-transformed density plots are presented in Fig. 3.5C-D and Fig. 3.5E-F, respectively. These analyses indicate that the resulting distributions of gene counts are relatively consistent between methods.

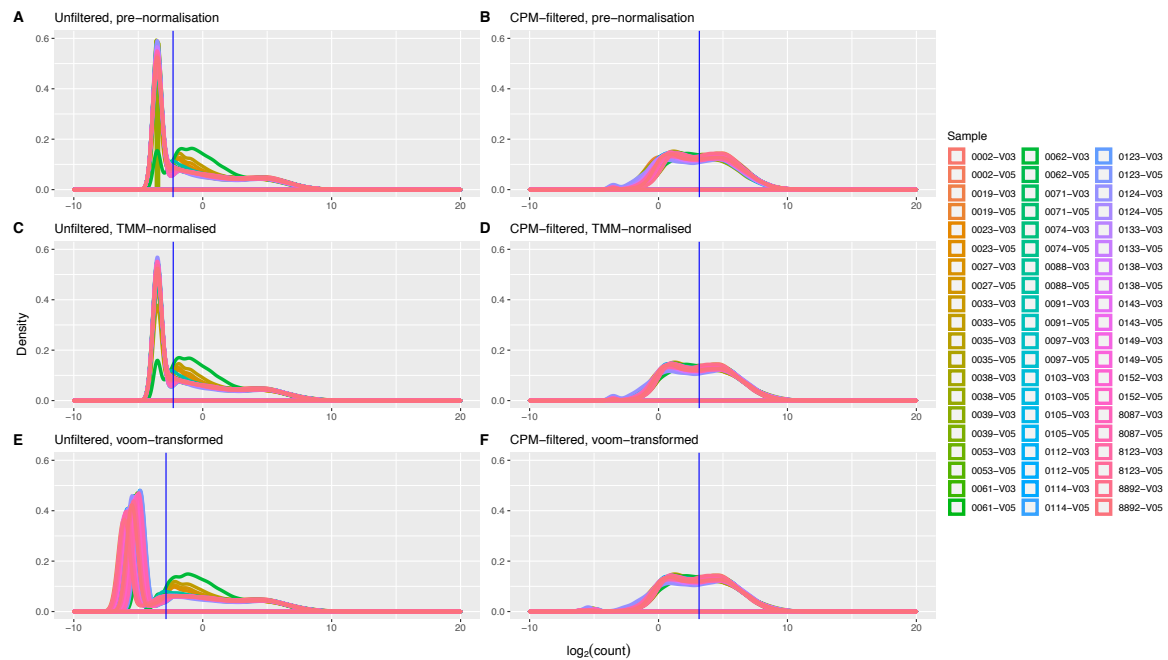


Figure 3.5. Read count density plots for the HISAT2-StringTie tool combination comparing unfiltered data with counts-per-million-filtered data at each stage of the pipeline. Distribution of gene expression values when count matrices are **A.** unfiltered, prior to normalisation, **B.** counts-per-million (CPM)-filtered, prior to normalisation, **C.** unfiltered and trimmed mean of M-values (TMM)-normalised, **D.** CPM-filtered and TMM-normalised, **E.** unfiltered, TMM-normalised, and *voom*-transformed, and **F.** CPM-filtered, TMM-normalised and *voom*-transformed. These plots highlight the effects of the initial stages of the differential gene expression pipeline on the comparison of gene expression values between samples.

3.3.2 Computational choices influence the number of genes with expression in the user-defined noise range

User-defined thresholds were inferred based on the distribution of point-to-point Pearson correlations versus the abundance of genes, for each sample. An example of three expression profiles, generated from a mixture of pre- and post-vaccination test and control samples so as not to bias for similar conditions, for a high abundance transcript (RPUSD1-201) that would generate a high value for the

p2pPCC is shown in Fig. 3.6A; an example of a low abundance transcript from a similar set of samples is shown in Fig. 3.6B (SLC7A9-201), for the latter the distribution of signal across the transcript is not consistent, due to the stochasticity of the sequencing process.

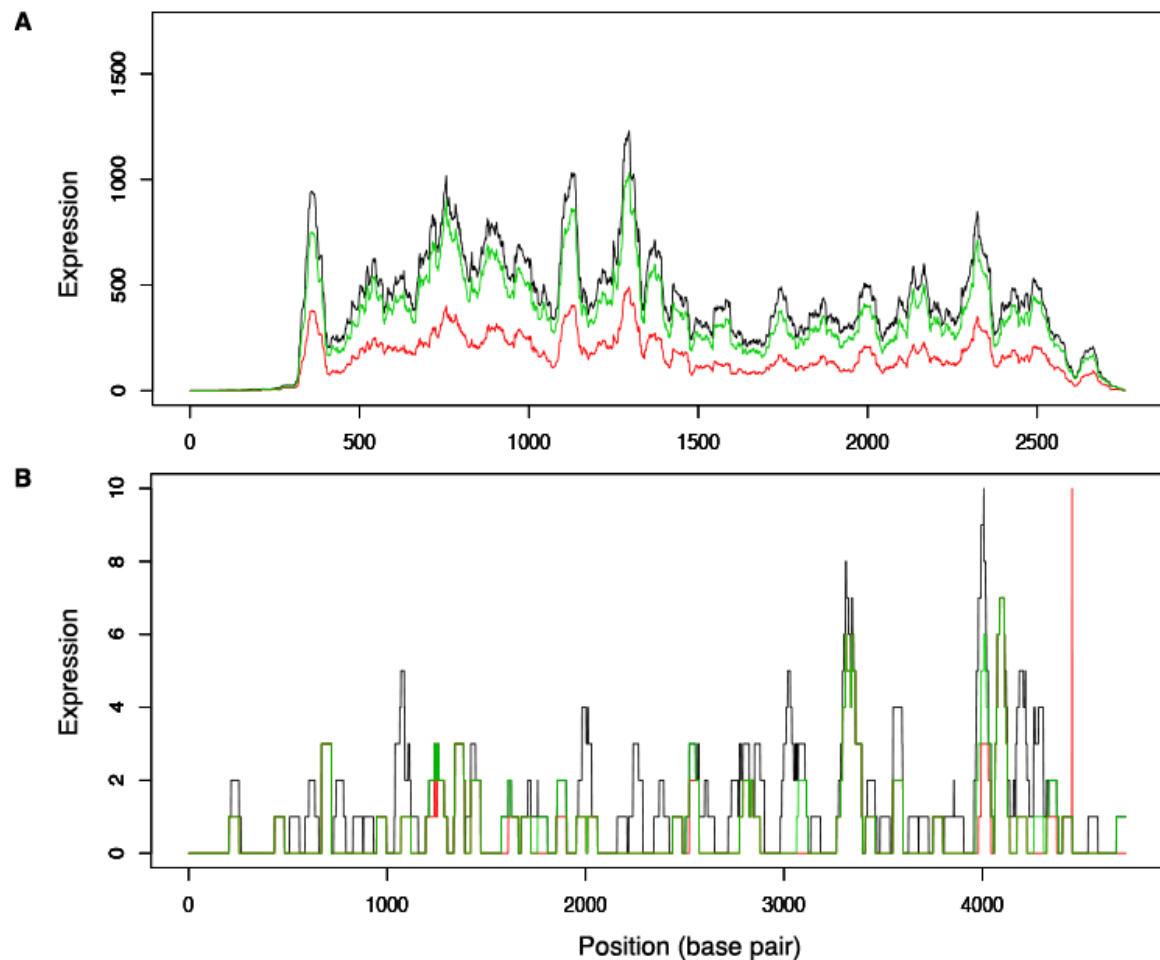


Figure 3.6. Expression profiles. Examples of expression profiles for **A.** a high abundance (RPUSD1-201) and **B.** a low abundance (SLC7A9-201) gene, generated from the noise detection pipeline. The expression profiles are built as a point sum across the length of a transcript of the abundances of all reads incident to a particular position.

A good sample is characterised by high variability in the p2pPCC values for low abundance genes and a consistently high and tight distribution of p2pPCC values

for medium and high abundances. An example of a good sample, with a consistently high distribution of correlation values is presented in Fig. 3.7A; the example presented in Fig. 3.7B shows a sample with a drop in p2pPCC at the high abundances indicating an inconsistency in distribution of signal that could be due to heavy alternative splicing or significant differential expression of a high number of highly abundant genes; Fig. 3.7C shows a high proportion of low p2pPCC transcripts over a wide range of medium abundances suggesting the occurrence of a fundamental change in the distribution of signal, across the transcript. The latter can also indicate the presence of technical issues with that particular sample that lead to a generalised pattern. Further investigation into the identity and overarching properties of the transcripts with low p2pPCC value (Fig. 3.7B and Fig. 3.7C) revealed little in the way of shared gene ontology between these transcripts.

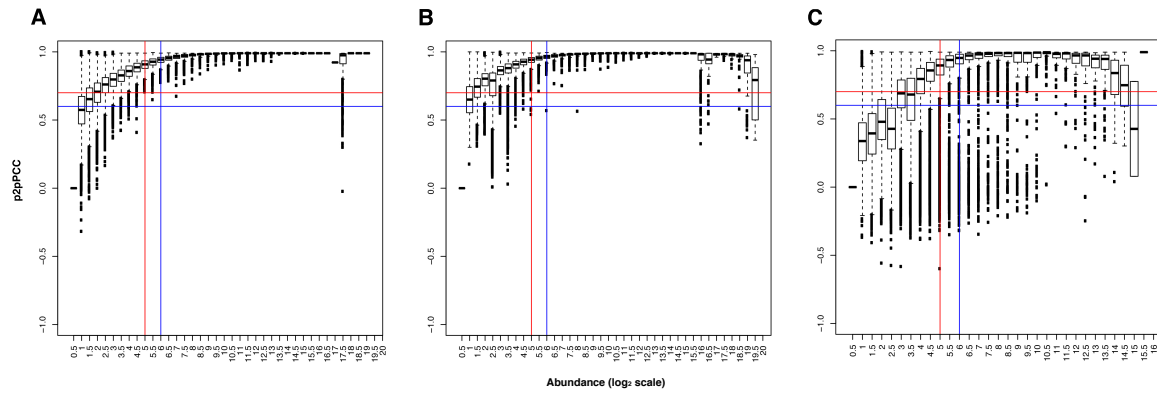


Figure 3.7. Distributions of point-to-point Pearson correlation coefficient (p2pPCC) against average abundance. In these plots, the distributions of p2pPCC values (y-axis) for bins of abundances are presented on $\log_2$ scale (x-axis). **A.** Example of a good sample for which the distribution of p2pPCCs increases steadily, proportional with the increase in abundances to a plateau that is maintained all the way through to the highest abundances. **B.** Example of a sub-optimal distribution of p2pPCCs where the distributions of correlation coefficients drop at the highest abundances indicating either extensive degradation or significant alternative splicing. **C.** Example of a poor distribution of p2pPCCs with numerous outliers across the abundance range. For these examples the signal-to-noise threshold was defined on the abundance bin for which the median of the p2pPCC was above the selected threshold. The red lines indicate a lower user-defined noise threshold, defined as the abundance at which the median of the p2pPCC distribution exceeds 0.7. The blue lines indicate a higher user-defined noise threshold, with a user-defined threshold of 0.9. Both thresholds are “first-occurrence” i.e. the first abundance for which the distribution of p2pPCCs is above the user-defined threshold. The implemented method provides the option of “last-occurrence” threshold i.e. the last abundance for which the distribution of p2pPCC is below the user-defined threshold.

The proportions of genes in the noise range are consistent between alignment tools (18581–25357 [median 20317] and 18619–25096 [median 20362] for HISAT2 and STAR alignments, respectively) but vary depending on the quantification tool used with approximately 34% of genes falling in the noise range for StringTie (25357 for HISAT2-StringTie and 25096 for STAR-StringTie

combinations) and ~56% (28960) when using the STAR *--quantMode GeneCounts* function (see Fig. 3.8). This observation indicates that more computational noise is introduced by the choice of the gene/transcript estimation tool than by the choice of read alignment tool.

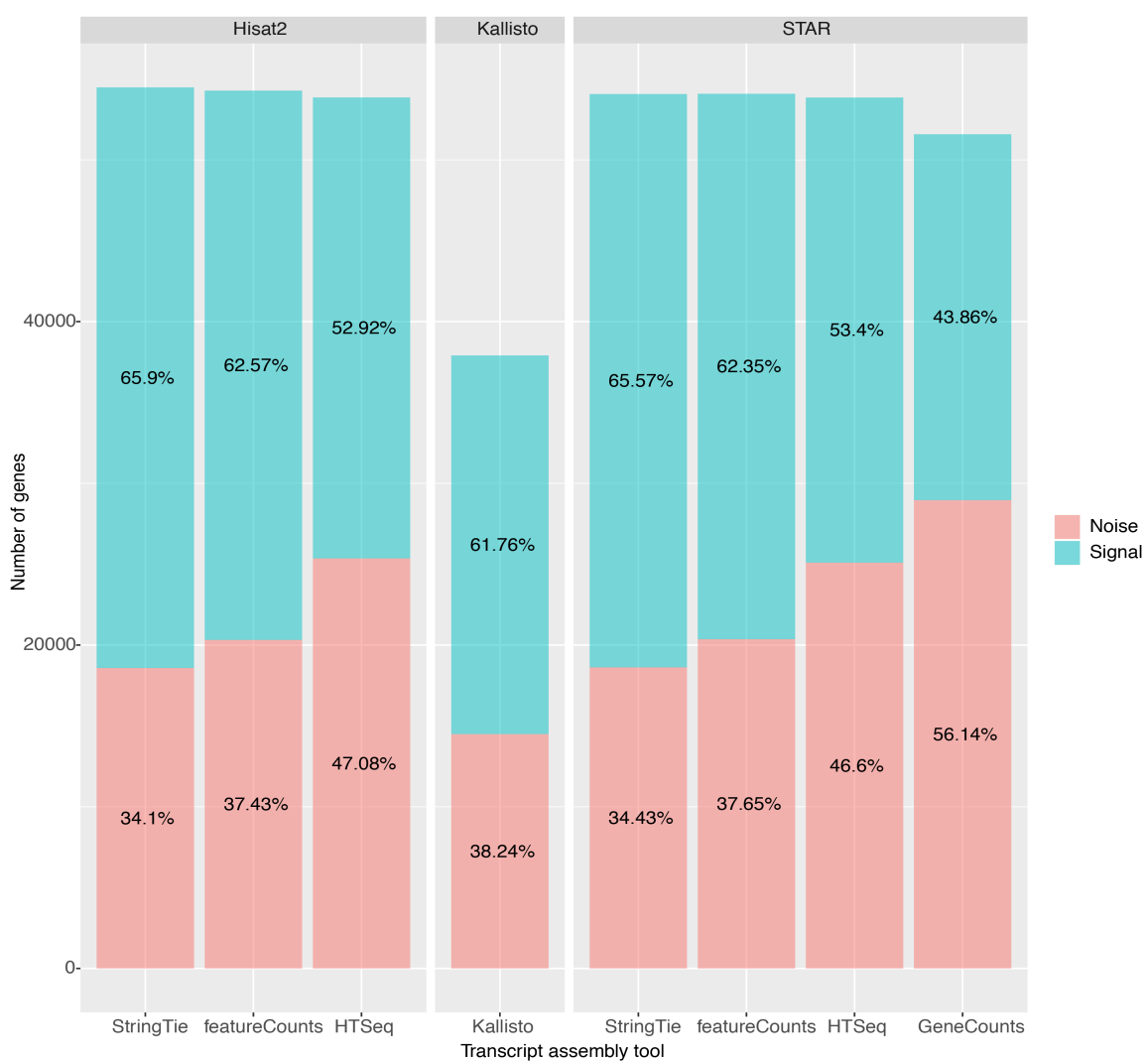


Figure 3.8. Proportion of genes in the signal/noise range for each tool combination.

Stacked bar plot showing the relative proportion of genes with expression levels above/below the signal-to-noise for each read alignment/gene quantification tool combination. Proportions are presented as percentages of total expressed genes for each tool combination. StringTie generates a lower number of entries for the noisy range (18851 and 18619 for HISAT2 and STAR combinations, respectively), whereas HTseq and GeneCounts produce a higher number of genes with low, and highly variable abundances (for the latter >50% of expressed genes).

Density plots of genes with expression levels below the user-defined noise threshold are shown in Fig. 3.9. The expression values of “noisy” genes were increased to the signal-to-noise threshold, corresponding to each sample to reduce the number of DGE calls for genes with values below the noise threshold in a subset of samples. Genes with all expression values below the noise thresholds, for each sample, were excluded from the analysis (Fig. 3.9).

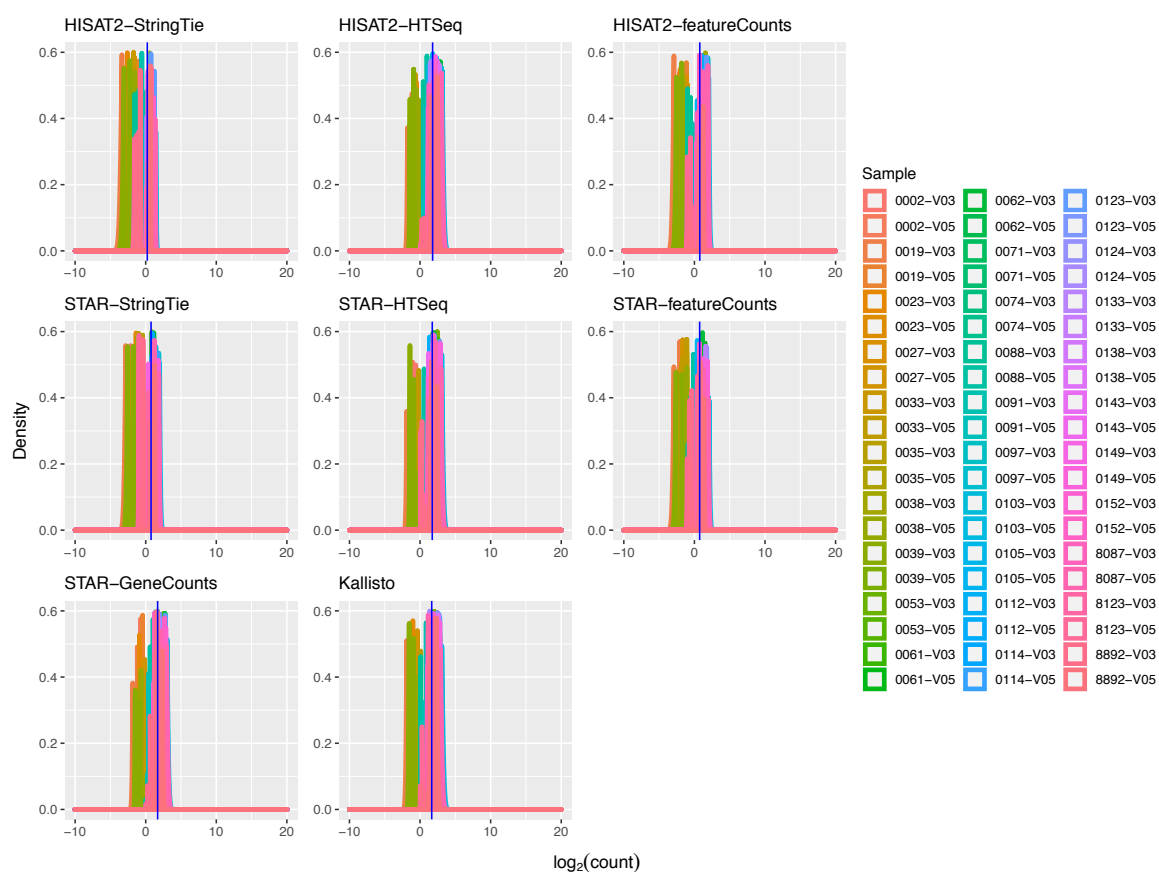


Figure 3.9. Read count density plots of genes with expression levels less or equal to the noise threshold for each sample and each tool combination. The median is represented by the vertical blue line. These genes were subsequently removed from the analysis to mitigate the interference of highly variable, low abundance genes in the final differential gene expression call.

Noise adjustment reduced the number of expressed genes included in the analysis by 5.1–40.6% (22627–35902 genes remaining, median 33108), depending on the relative proportion of noise associated with a particular tool combination, with the remaining lowly expressed genes treated in a sample-specific manner. The resultant representative noise-adjusted, *voom*-transformed count density plots for noise-adjusted and above-the-threshold genes, with and without prior CPM filtration, are presented in Fig. 3.10A and B, respectively. The former (Fig. 3.10A) exhibits greater variation in between density peaks below the median across all samples due to sample-specific noise adjustment, while in the latter (Fig. 3.10B) these differences are less apparent; the density distributions are uniformly aligned above the median for both methods.

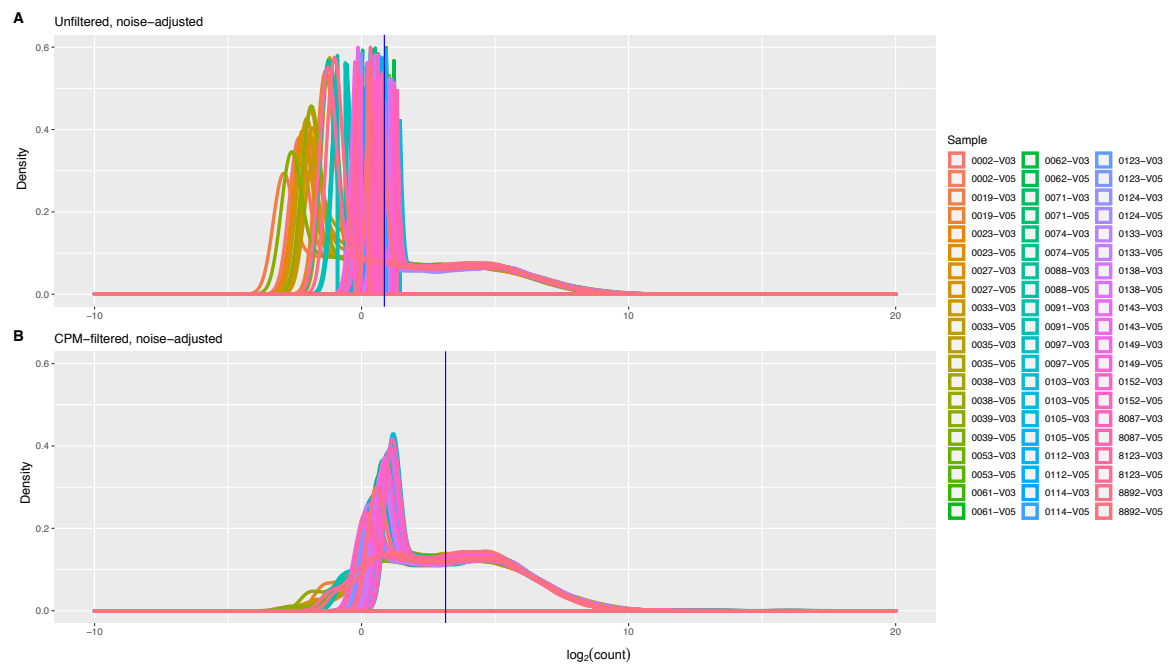


Figure 3.10. Read count density plots for the HISAT2-StringTie tool combination comparing the effects of *ad hoc* filtration on noise-adjusted data. **A.** Density plots of median gene abundances for genes with abundances below the user-defined noise threshold for a single tool combination (HISAT2-StringTie). The expression values of these genes are subsequently increased to the sample specific signal/noise threshold and genes with expression values less than the signal-to-noise thresholds in all samples are removed. **B.** Density plot of noise-adjusted, *voom*-transformed read counts for the same tool combination (HISAT2-StringTie). The vertical blue lines indicate the median $\log_2$ count across all samples.

3.3.3 Removing genes in the noise range reduces the effect of pipeline-specific differences on the final set of differentially expressed genes

MA plots were generated after the DGE call for each tool combination; those from a single tool combination, with/without CPM filtration and with/without noise adjustment, are shown in Fig. 3.11A–D. CPM filtration without noise adjustment (Fig. 3.11B) gave rise to a greater number of low abundance, high $\log_2$

fold change (LFC) outliers – genes with highly variable distribution of signal across the transcript and for which the calculated LFC on the low number of incident reads does not reflect a transcript of biological relevance. These outliers (artefacts of differential expression analyses derived from low abundance transcripts) were absent from the corresponding noise-adjusted MA plot and reduced the call as differentially expressed by 250–1168 (median 509) transcripts, with LFC values varying between -1.016653 and 6.717757 ($|\text{median}|$ 2.31) and abundances in the 1.3261–74120.038 (median of medians: 30.9832) range. The CPM-filtered, noise-adjusted method gave rise to the same distribution of abundances as the unfiltered, noise-adjusted MA plot, suggesting that the noise-adjustment performs equally well on filtered and non-filtered data. The noise-adjusted MA plots showed a more consistent distribution of abundances across the entire range in comparison to their noise-included counterparts, highlighting yet again a high degree of variability at the lower abundances. This suggests a converging effect of the noise adjustment on a variety of analytical backgrounds.

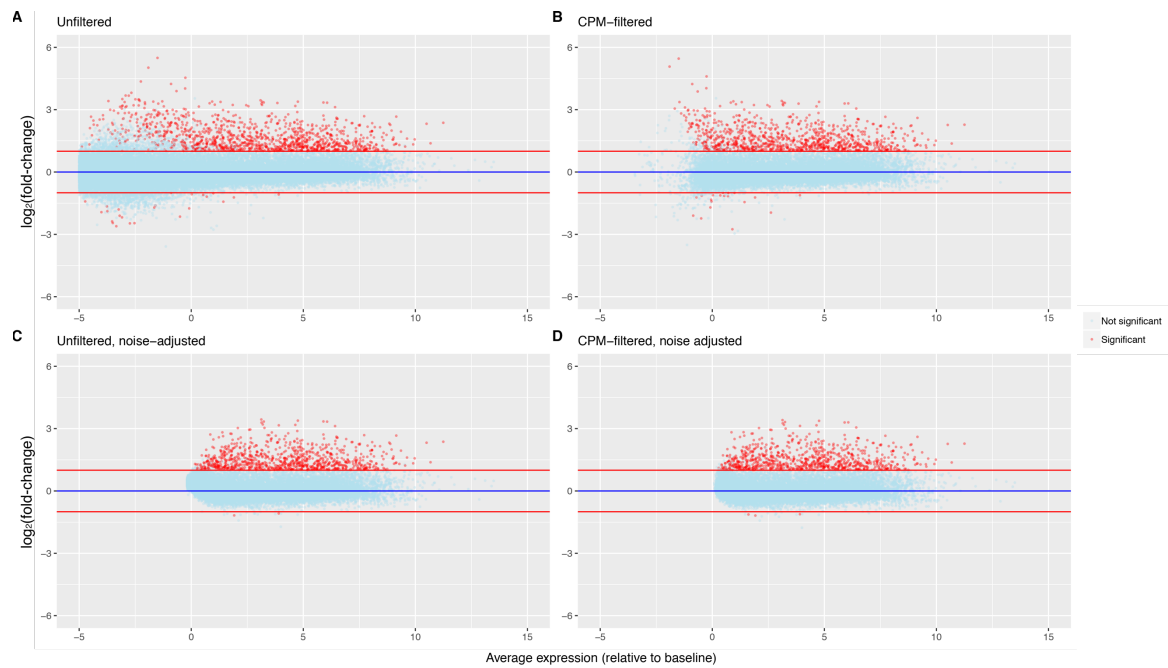


Figure 3.11. Distributions of genes called as differentially expressed subsequent to each filtration method. The MA plots show the pairwise comparison between the pre-vaccination and the post-vaccination samples; the significantly differentially expressed genes (DEGs, $FDR < 0.1$, absolute $\log_2$ fold-change > 1) are highlighted in red. The input for each MA plot is: **A.** unfiltered input, **B.** counts-per-million (CPM)-filtered input, no noise adjustment, **C.** unfiltered, noise-adjusted, and **D.** CPM-filtered, noise-adjusted. For each plot, the horizontal blue line corresponds to a $\log_2$ fold-change of 0; the horizontal red lines correspond to a $\log_2$ fold-change of ± 1 . Due to the nature of the compared datasets and the significant biological changes occurring after vaccination, the predominant identification of up-regulated genes is correct.

Finally, histograms of the unique DEGs ($FDR < 0.01$, $|LFC| > 1$) associated with each filtration method for each tool combination revealed a low density of overlapping medium abundance genes between the CPM-filtered methods (see Fig. 3.12). Conversely, the unfiltered methods had a higher density peak associated with low abundance genes (Fig. 3.12). These plots highlight the specific differences remaining between the different tool combinations following DGE analysis.

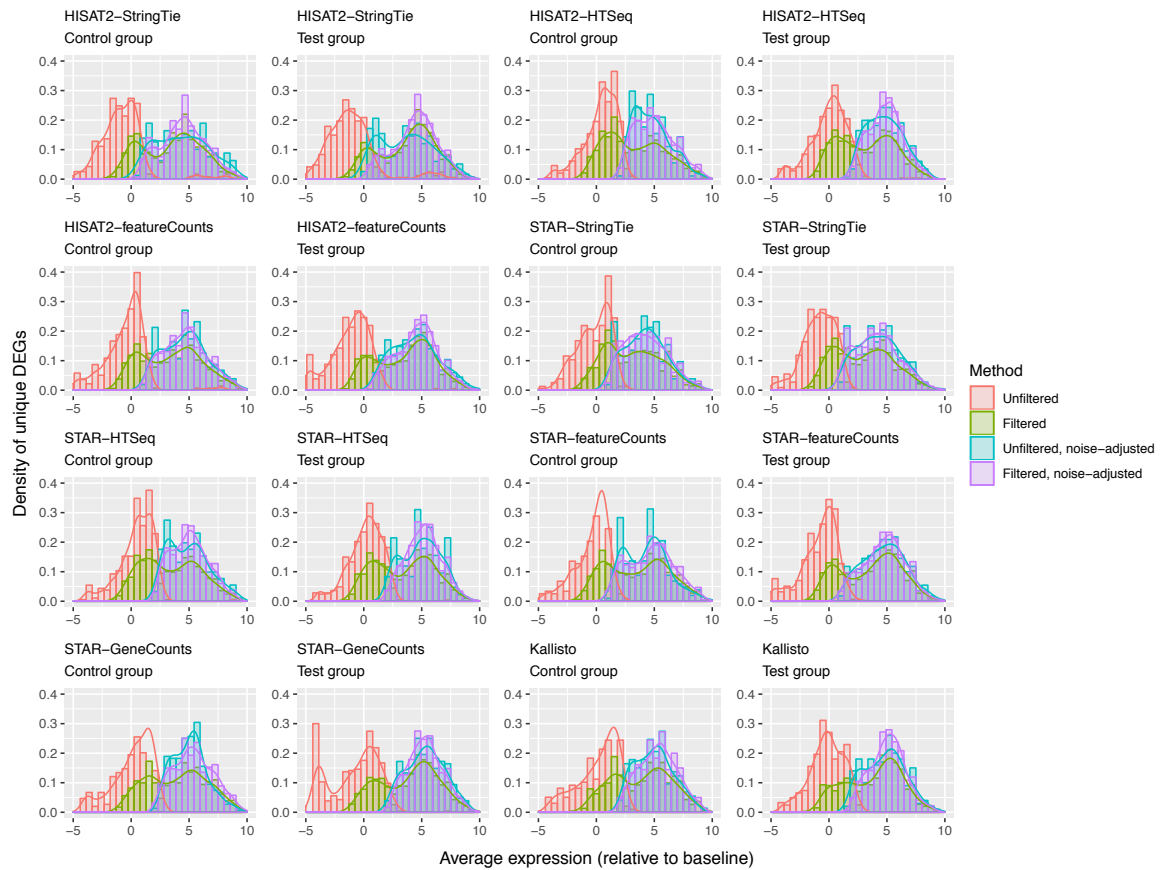


Figure 3.12. Properties of genes uniquely called as differentially expressed subsequent to each filtration method. Density histograms of average expression of genes uniquely called as significantly differentially expressed (DE) for each filtration method on the same tool combination. This plot indicates that the unfiltered data leads to more differentially expressed genes with low expression values being called significantly DE compared with the same data that have been counts-per-million (CPM)-filtered and/or noise-adjusted. CPM-filtered, noise-adjusted data call fewer lowly expressed genes as significantly DE but capture more significantly DE genes with high average expression values than other methods.

3.4 Discussion

This analysis highlights the specific differences between eight different read processing pipelines (as combinations of frequently used pre-processing tools) in relation to empirically-determined noise thresholds; it also illustrates the effect of a novel data-driven filtration method for the removal of outlying low abundance, highly variable genes. While most studies comparing RNA-seq analysis pipelines have focused on benchmarking the normalisation and statistical testing of DGE^{297,310,319,320}, the study presented in this chapter instead focuses on the understudied effects of alignment and count estimation methods on DE calls. A previous study evaluating the impact of alignment methods on the DGE analysis, without taking into account the presence or extent of technical noise, explored these methods in combination with a single gene quantification tool³²¹. This work suggests a minimal impact of the read alignment tool on the final DGE call³²¹, in accordance with the work presented here. Another study introduced Zseq, an algorithm that reduces the impact of sequence bias, duplication, and mapping ambiguity on the efficiency of gene quantification, and benchmarked this novel tool against the Tophat2 and Cufflinks transcript gene quantification tools³²². This study highlighted the importance of this stage of RNA-seq read processing on the final DGE call³²². The present analysis provides further evidence that a vast proportion of variability in the final gene expression output introduced by computational choices in the processing of reads is attributable to the transcript/gene quantification tool and specifically assesses the combined effects of multiple commonly-used read alignment and gene quantification tools on the final DGE call. The effect of the choice of algorithm at this stage of pre-processing

may be explained by the differential handling of multi-mapping reads and assessment of transcript variants identified in the alignment stage by the chosen tool. However, it is encouraging to observe that a data-driven filtering of genes with inconsistent distribution of signal, reduces the effect of tool choice.

The nature of the data being assessed can further influence the outcome of DGE analysis due in part to the handling of reads arising from these data by read processing algorithms, thereby compounding technical noise prior to read generation with computational noise subsequent to read generation. For example, it has been suggested that bias driven by GC content can negatively impact on assembly of a genome *de novo* ³²³, though it is not clear to what extent. Sequences with low complexity have an increased likelihood of mapping exactly one time to the genome and therefore increase the frequency of multi-mapping ³²². Intronic regions must be handled appropriately in the context of RNA-seq data, particularly for rRNA-depleted samples that retain a greater number of intronic reads than polyA-selected samples ³²⁴; the Bowtie algorithm was developed for DNA alignment ³²⁵ and therefore tools based on it do not deal with intronic regions adequately. Additionally, alignment tools exhibit variation in their ability to accurately identify splice junctions, a finding that warrants attention when exploring conditions where transcript isoforms may play an important role ³¹⁶. These factors are often unaccounted for in benchmarking studies and must be appropriately simulated for informative comparisons ³¹². The computational choices that are made prior to DGE analysis should preference tools that accurately handle these potential sources of variability in real data. The analyses presented here were conducted on an experimentally-generated dataset in *H. sapiens* in which both experimental groups were given a highly immunogenic stimulus that would cause a significant perturbation in the host transcriptome (GSE131929,

EUCLIDS). The high variability between individuals observed in *H. sapiens* allowed for the evaluation of the robustness of the noise detection method on samples likely to exhibit considerable intra- and inter-condition inconsistency. The complexity of reads is taken into account in this pipeline by restricting the input to non-redundant reads, thereby increasing the complexity of the input data to reduce multi-mapping and the computational requirements of the alignment. The technical factors that contribute to apparent variation in the sequenced data are then accounted for by defining sample-specific noise thresholds and identifying potentially problematic transcripts based on their associated expression profiles. The performance of this data-driven method was compared with the *ad hoc* fixed CPM filtration procedure (applied uniformly across samples) that has been suggested for RNA-seq DGE analysis ²⁶⁸. While this method is practical for bulk-excluding low abundance genes, the criteria for filtration should be sample-specific; it has been demonstrated that filtering methods that make use of group conditions, such as minimum sample size of a test group, may increase the true false positive rate in the downstream in the analysis ³²⁶, this effect becomes particularly visible with the increase in complexity of the experimental design of the dataset. Although the noise adjustment pipeline performed similarly on unfiltered and CPM-filtered data, we observed that the noise adjustment of unfiltered data results in a higher number of uniquely significantly DEGs that have low average expression values compared with those uniquely identified by noise adjustment of CPM-filtered data. However, both methods demonstrated an improvement on the CPM filtration without noise adjustment method.

The noise analysis is provided as a standalone pipeline, accessible via a wrapper script that invokes all the components, in the correct order and with a seamless linking of parameter transfer; the speed of the processing is achieved via

a *divide et impera* split of the initial dataset that allows the execution of this pipeline without extensive memory or disk usage. The individual scripts that make up this pipeline are accessible and could be replaced by synonymous components, e.g. that make use of alternative alignment tools. One limitation of the current implementation of our novel noise detection pipeline is its use of the slow PatMaN algorithm²⁹⁶ chosen for its high mapping accuracy but at the cost of prolonging the alignment time. We aim to improve this step of the pipeline by adapting the scripts for faster deterministic alignment tools to reduce the trade-off between accuracy and time. Additionally, the implementation of the noise thresholds in the DGE analysis is thus far only adapted to the limma *voom* method. This is due to difficulties that are encountered with adjusting the functions for methods that employ scaling-based normalisation methods with negative binomial distributions^{268,327}.

The characterisation of the novel pipeline introduced in this chapter provides accurate quantification of the degree to which specific read processing tools and their combinations introduce or capture technical noise in high-throughput sequencing data and demonstrates the impact that this low-level variation has on determining DE. In addition to this, the pipeline provides a means for eliminating such noise in an empirical and sample-specific manner as opposed to an *ad hoc* intervention across all samples. This pipeline was used to improve the accuracy of the gene expression analyses presented in chapters 4 and 6.

Chapter 4

Comparative transcriptomics between species attributes reactogenicity pathways induced by the capsular group B meningococcal vaccine, 4CMenB, to the membrane-bound endotoxin of its outer membrane vesicle component

4.1 Introduction

Early post-implementation surveillance in the UK indicates that 4CMenB has been effective at preventing invasive disease caused by MenB, with an effectiveness of 82.9%, resulting in a halving of all cases in the vaccine-eligible group within ten months of the introduction of the two-dose schedule ²⁰⁹. However, the reactogenicity associated with the vaccine, which is exacerbated when 4CMenB is administered concomitantly with vaccines such as the standard DTP-containing vaccine that is routinely-administered to infants in the UK, warrants continual monitoring and further investigation if high uptake of the vaccine is to be maintained. Fever rates (≥ 38.5 °C) in a 4CMenB + routine immunisation cohort were nearly double (50–60%) those observed in infants receiving routine immunisations alone in phase III trials ²⁰³. Other studies have reported somewhat contradictory findings; a recent European study comparing concomitant and

separate administration of 4CMenB and routine immunisations employed a risk interval analysis to assess the effects of co-administration on the incidence of AEFI and found no evidence of interaction underlying the reactogenicity of the combination ³²⁸. Regardless of whether vaccine interactions exacerbate this reactogenicity, 4CMenB is associated with a high incidence of fever when administered alone ²⁰², as are DTP-containing vaccines, so the phenomenon of an increased incidence of fever following co-administration is likely a cumulative effect of the respective AEFI associated with each intervention.

Paracetamol prophylaxis was found to significantly decrease fever after concomitant 4CMenB immunisation without negatively impacting on the immunogenicity ³²⁹, and PHE have since made the recommendation that infants in the UK be given paracetamol after receiving the first and second dose of the vaccine ³³⁰. While post-immunisation fever is typically a benign event, with mild to moderate and transient manifestations ³³¹, it represents a challenge for clinicians who are faced with the difficulty of differentiating AEFI from significant intercurrent bacterial infection in infants ³³². Data are beginning to emerge on the healthcare burden of 4CMenB-related AEFI and highlight a small but significant increase in emergency department attendances in the region of 2–3% ^{333–337}. Under the current guidelines of the National Institute for Health and Care Excellence (NICE), presentation with AEFI in those under three months of age may result in unnecessary hospitalisation, invasive diagnostic procedures such as lumbar punctures, or the administration of antibiotics (Fig. 4.1) ³³⁸. Therefore, it is of clinical importance to understand the aetiology of post-immunisation fever.

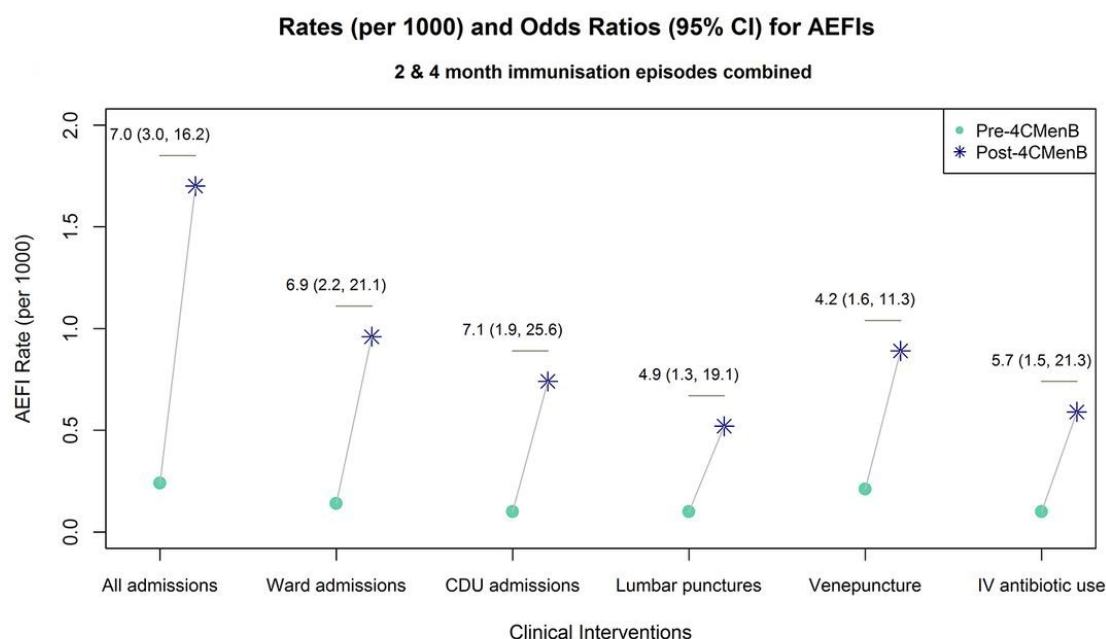


Figure 4.1. Increases in the number of clinical interventions associated with the introduction of 4CMenB to the UK infant immunisation schedule. The rate of adverse events following immunisation (AEFI) per 1,000 cases across Oxford University Hospitals National Health Service Trust’s emergency departments associated with the inclusion of the 4CMenB vaccine in the UK infant immunisation schedule in 2015. The rate of AEFI associated with the first and second doses of the vaccine at two and four months of age presented here has led to a small but significant increase in the number of clinical interventions being performed to rule out bacterial infection. Odds ratios and 95% confidence intervals are presented above each pair of points. CDU, clinical decision unit. From Nainani *et al.*, 2017³³³.

The vaccine has been reported as safe following mass administration and appears to be well accepted by parents. Post-licensure and -implementation surveillance of the vaccine’s safety in the UK, using the Medicines and Healthcare Products Regulatory Agency (MHRA)’s Yellow Card scheme for the reporting of side effects, determined no major safety concerns associated with co-administration of 4CMenB with routine immunisations in infants, although 40% of reports mentioned fever³³⁹. When interviewed, parents still cite the possibility of fever as a source of anxiety when having their child immunised against 4CMenB

but most would still have their child immunised and recognise the severity of the disease relative to the potential AEFI according to a qualitative study performed in the UK ³⁴⁰. Communication between health professionals and parents is crucial to allay these concerns and will be key to ensuring continued success of MenB immunisation programmes. However, as outlined in section 1.1.3B, parental concerns relating to vaccine safety can persist as the incidence of infection begins to decline and the occurrence of AEFI becomes more prominent in the public's perception than the devastation of disease. With this in mind, gaining a better understanding of the mechanisms, management, and prevention of fever and other related AEFI is imperative to safeguarding against future hesitancy that may impact upon efforts to eradicate the disease.

In order to elucidate the host molecular pathways associated with the immunogenicity and reactogenicity of 4CMenB, a European childhood life-threatening infectious disease study (EUCLIDS) was conducted at OVG. The clinical study focused on investigating changes in gene expression in infants after their second dose of vaccines routinely-administered to UK infants (at the time of study), at four months of age, with or without concomitant 4CMenB, and relating these data to continuous temperature readings measured using an iButton® monitoring device ³⁴¹. A significantly higher incidence of fever ($p < 0.001$, Fig. 4.2) was observed in infants receiving 4CMenB in addition to routinely-administered vaccines (4CMenB + routine group, 59.6% [95% CI: 48.6%, 69.8%]) than those receiving routine immunisations only (routine only group, 27.3% [95% CI: 18.3%, 37.8%]), in agreement with most published studies investigating this phenomenon.

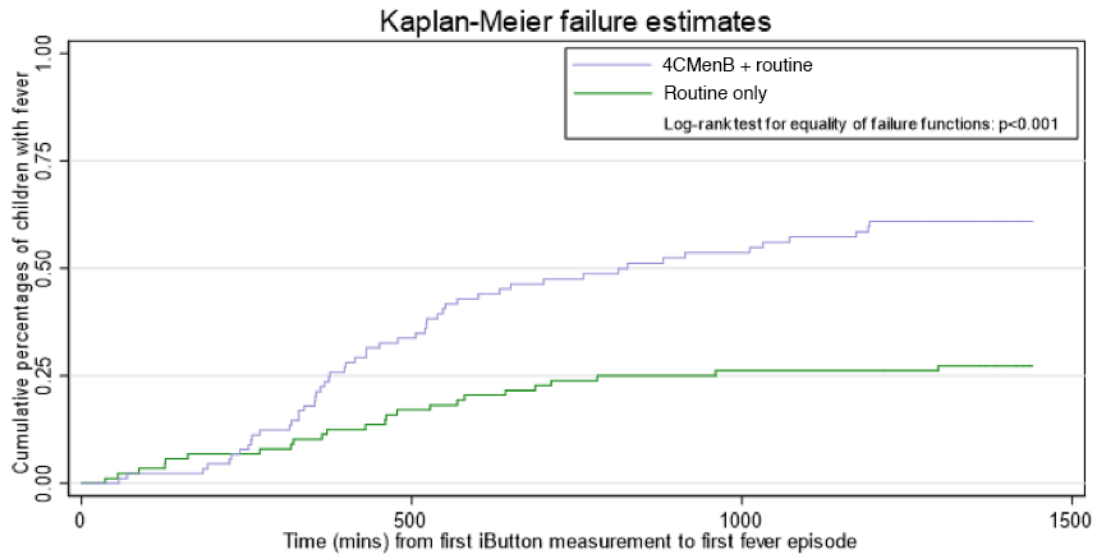


Figure 4.2. Kaplan-Meier failure curve estimates of time to first episode of fever in human infants. Temperature was measured continuously in infants over 24 hours after their second dose of either 4CMenB + routine immunisations (purple) or routine immunisations only (green) using a wireless iButton® temperature monitoring device. From O'Connor *et al*, (unpublished as of June 2019).

Post-immunisation whole blood gene signatures were characterised and correlated with temperature and immunogenicity data (unpublished observations). Peak DGE was observed within the first 24 hours post-immunisation for both vaccine groups. Both groups displayed significantly elevated levels of neutrophils based on whole blood counts relative to baseline that were highly correlated with the enrichment of the neutrophil fraction of the whole blood transcriptome signature at 24 hours ($r = 0.9$, $p < 2.2 \times 10^{-16}$), indicating the importance of this cell type in the early immune response to these vaccine combinations. While there were very few genes identified as significantly differentially expressed (DE) between the two groups at each time point, several genes were found among the top significantly DEGs for each group based on time course expression profiles, including the

peptidoglycan receptor gene *Pglyrp1* which was found to be upregulated in the 4CMenB + routine group at four hours post-immunisation.

To explore and identify mechanisms involved in the reactogenicity of concomitant 4CMenB immunisation, and to determine the relative contribution of each of its components to these reactions, temperature and gene expression changes were assessed in groups of mice immunised with 4CMenB, on its own or with routine vaccines, or one of its four alum-adsorbed components (full list of vaccines/antigens and corresponding doses for this experiment are presented in Tables 4.1–4.3). There is evidence that the OMV component of the vaccine contributes to the reactogenicity of the vaccine (section 1.2.3B); this is likely due to quantities of membrane-bound endotoxin found in OMV preparations ¹⁵⁸. Early studies of 4CMenB recombinant proteins –fHbp, NHBA, and NadA – in infants found a slight increase in local and systemic reactogenicity associated with the inclusion of OMVs ^{200,201}. The role of OMVs and membrane-bound endotoxin in driving this reactogenicity was therefore examined by comparing the 4CMenB NZ98/254 dOMVs with those of another strain, H44/76, and with nOMVs from this strain genetically-engineered to express an attenuated form of endotoxin (lpxL1). The comparative analysis of OMV preparations and recombinant protein components of 4CMenB presented herein sheds light on their differential propensities to engage pro-inflammatory pathways that culminate in the induction of fever in mice.

4.2 Methods

4.2.1 Mouse procedures

The mouse procedures used in this chapter, including immunisations, bleeds, temperature measurement, neutrophil isolation, and brain endothelial cell isolation are outlined in sections 2.2.1–2.2.5. A maximum total volume of 100 µL of vaccine/antigen was administered to each animal intramuscularly (IM) in accordance with the Project License, hence vaccines with a 500 µL administration volume for humans were restricted to 1/15 of the human dose if administered as part of a three-vaccine combination or 1/5 of the human dose in mice if administered as a single vaccine. For experiments testing three-vaccine combinations 33 µL of each vaccine was injected into the left, lower right, and upper right thigh of each mouse (Table 4.1) and for experiments testing a single vaccine/antigen per group, 50 µL of vaccine was injected into each leg of each mouse (Table 4.2 and Table 4.3).

Vaccine	Dose
4CMenB + DTaP-IPV-Hib + PCV13	1/15 human dose (33 µl each)
DTaP-IPV-Hib + PCV13 + PBS	1/15 human dose (33 µl each)
PBS + PBS + PBS	33 µl each
4CMenB + PBS + PBS	1/15 human dose (33 µl each)

Table 4.1. Vaccines and corresponding doses for the concomitant 4CMenB versus routine only experiment mouse immunisations.

Vaccine	Dose
4CMenB	1/5 human dose (50 µL per leg)
rfHbp + alum	10 µg (50 µL per leg)
rNadA + alum	10 µg (50 µL per leg)
rNHBA + alum	10 µg (50 µL per leg)
NZ98/254 OMVs + alum	5 µg (50 µL per leg)
H44/76 OMVs + alum	5 µg (50 µL per leg)
H44/76 LpxL1 OMVs + alum	5 µg (50 µL per leg)
LPS-EB (from <i>E. coli</i> O111:B4) + alum	1 µg (50 µL per leg)
MVA	10 ⁷ viral particles (50 µL per leg)

Table 4.2. Vaccines and corresponding doses for 4CMenB component comparison experiment mouse immunisations.

Vaccine	Dose
4CMenB	1/5 human dose (50 µL per leg)
LPS-EB + alum	1 µg (50 µL per leg)
LPS-EB (no alum)	1 µg (50 µL per leg)
LPS-EB Ultrapure + alum	1 µg (50 µL per leg)
PGN-EB (from <i>E. coli</i> O111:B4) + alum	1 µg (50 µL per leg)
LPS-EB + PGN-EB + alum	1 µg of each (50 µL per leg)
Alum	50 µL per leg

Table 4.3. Vaccines and corresponding doses for the endotoxin experiment mouse immunisations.

4.2.2 RNA sequencing

The protocols for RNA extraction from whole blood, library preparation and bulk RNA-seq, scRNA-seq, and qPCR are detailed in sections 2.3.1–2.3.4.

4.2.3 Computational methods

The whole blood RNA-seq pre-processing steps are covered in sections 2.4.1–2.4.4. The DGE analysis pipelines for whole blood RNA-seq and scRNA-seq are outlined in sections 2.4.5 and 2.4.6, respectively.

4.2.4 Cytokine quantification

Details of the multiplex bead array are given in section 2.6.1.

4.3 Results

4.3.1 4CMenB immunisation increases temperature in mice when administered on its own or concomitantly with routine immunisations

Groups of six mice were immunised with 1/15 of the human dose of each of four three-vaccine combinations: 4CMenB, DTaP-IPV-Hib, and PCV13 (routine + 4CMenB group), DTaP-IPV-Hib, PCV13, and PBS to control for the third injection (routine only group), 4CMenB with two doses of PBS to control for the second and third injections (4CMenB only), or three doses of PBS (PBS control group) – at day 0 and day 21 (Fig. 4.3).

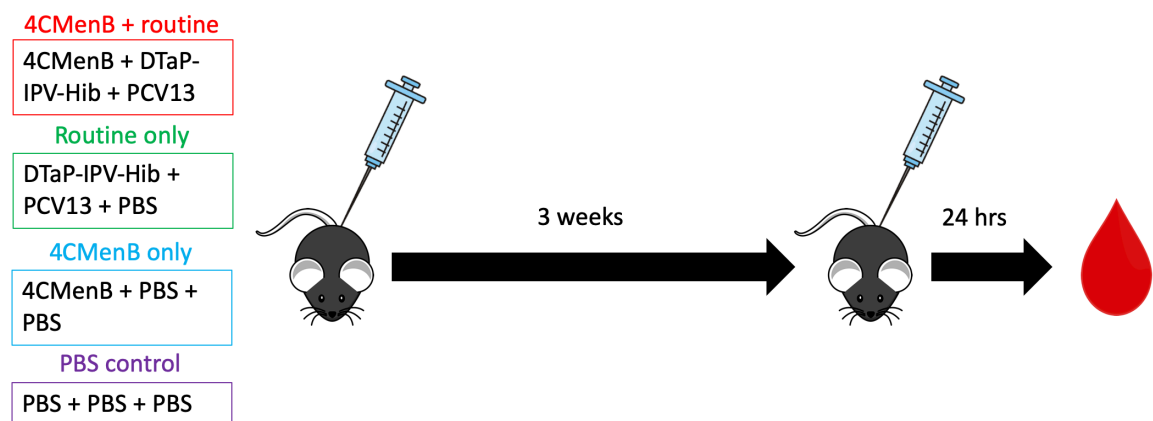


Figure 4.3. 4CMenB and routine immunisation mouse immunisation strategy.

Schematic of the mouse immunisation strategy for comparing the effects of 4CMenB administered with or without routine immunisations with those of routine immunisations only. Groups of six C57BL/6 mice were immunised with 1/15 of the human dose of one of the three-vaccine combinations listed on the left at day 0 and received a second shot of the same immunisation at day 21. Mice were bled 24 hours after this second dose.

Non-contact infrared thermometry was used to measure the surface temperature of mice at baseline on day 21 (60 mins before second dose), and every 90 mins after the second dose up to six hours, and 24 hours after the second dose (Fig. 4.4). A statistically significant rise ($p < 0.05$) in temperature occurred as early as three hours after the second dose in the 4CMenB groups, with and without routine immunisations continuing to rise up to 24 hours after this dose ($p < 0.01$). No such rise in temperature was observed for the routine or PBS control groups. This finding demonstrates an increased incidence of fever associated with 4CMenB, with or without routine immunisations, in the mouse immunisation model, recapitulating what is observed in human studies. This also demonstrates that the majority of this fever is driven by 4CMenB immunisation in mice, with only a modest increase in temperature observed when the vaccine is combined with routine immunisations.

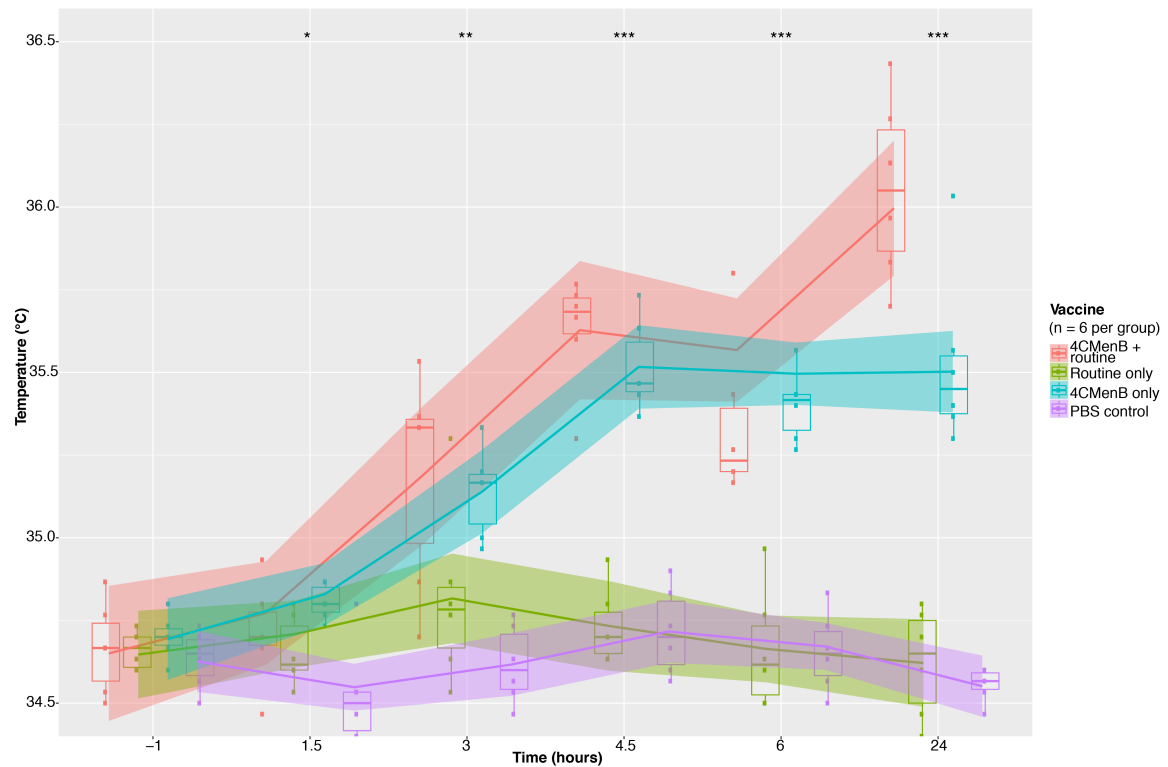


Figure 4.4. Changes in mouse surface temperature associated with vaccine combinations, 24 hours after the second dose of vaccine. Overall trend in temperature from baseline to 24 hours after the second dose of vaccine for each group. Loess smoothing was applied to group temperature trend lines. Group medians were compared between vaccine groups at each timepoint using a Kruskal-Wallis test. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

4.3.2 Mouse transcriptional responses to 4CMenB administered concomitantly with routine immunisations correlate with those of human infants 24 hours after concomitant 4CMenB immunisation

RNA-seq was performed on RNA extracted from mouse whole blood 24 hours after the second dose of immunisations (Fig. 4.3). To determine whether changes in the mouse transcriptome associated with 4CMenB + routine immunisation were comparable with those seen at the same timepoint in the 4CMenB + routine group of the infant study, significant genes (FDR < 0.01) were

ranked by LFC relative to the PBS control group and converted to human orthologs where possible. Spearman rank correlation indicated moderate correlation ($\rho \sim 0.55$) between one-to-one orthologs, but the association was highly significant ($p < 2.2 \cdot 10^{-16}$). An agreement plot of differentially expressed orthologs from each genus 24 hours after second dose of immunisation is shown in Fig. 4.5, with common significantly DEGs highlighted in blue. The strong association between these two datasets and the recapitulation of the vaccine-induced fever in mice provided a sound basis for the use of the mouse immunisation model to explore gene expression changes associated with the reactogenicity of this vaccine combination.

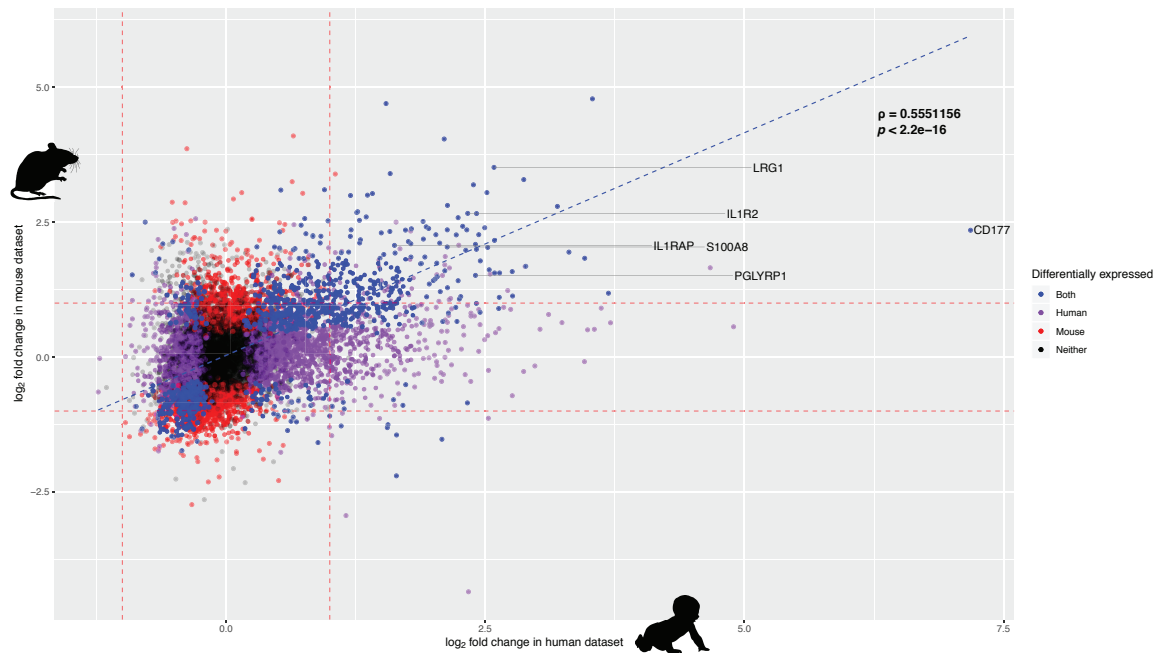


Figure 4.5. Agreement between mouse and infant datasets. Agreement plot of $\log_2$ fold-changes (LFCs) associated with all genes with one-to-one orthologs from the mouse 4CMenB + routine group (y-axis) and the infant 4CMenB + routine group (x-axis), 24 hours after the second dose. Spearman correlation rho value calculated between genes found to be significantly differentially expressed (FDR < 0.01) in both species (highlighted in blue) is shown beside the dotted blue trend line, above its associated p -value. The remaining genes are coloured purple, red, or black if they were significantly differentially expressed only in humans, mice, or neither, respectively. Several genes of interest, based on the downstream analysis in the infant study, are labelled and connected to their corresponding point.

4.3.3 4CMenB induces a greater magnitude of early pro-inflammatory gene expression when administered in combination with routine immunisations

Euclidean distances between each pair of samples were calculated through multidimensional scaling (MDS) analysis of the mouse dataset, and revealed a distinct association between samples from the 4CMenB and 4CMenB + routine groups. The routine only and PBS control groups were more closely related to each

other (Fig. 4.6). This suggests that gene expression changes induced by 4CMenB immunisation predominate over those induced by routine immunisations at this dose.

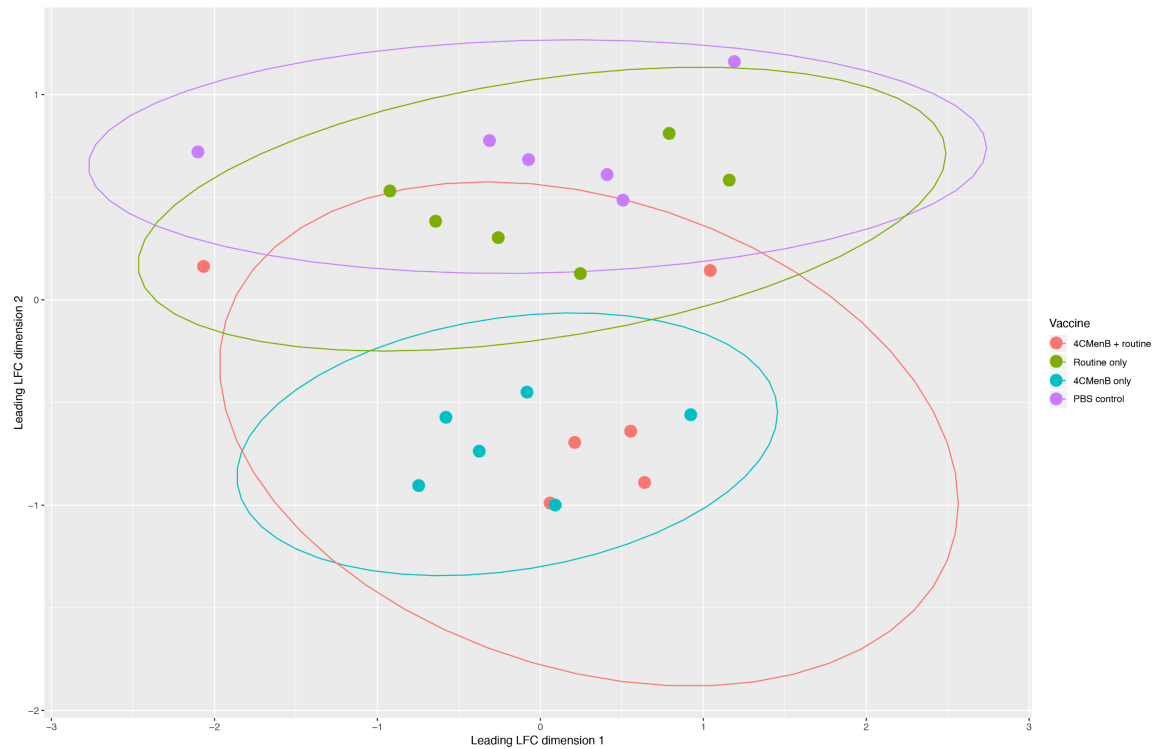


Figure 4.6. Multidimensional scaling plot of 4CMenB and routine immunisation groups. Initial clustering of samples based on pairwise root-mean-square deviations (Euclidean distances) calculated from gene expression values represented in two dimensions. Samples from groups immunised with 4CMenB associate along the two dimensions associated with the greatest $\log_2$ fold-changes (LFC), while the samples from groups that did not receive 4CMenB cluster separate along these dimensions.

The data were normalised by relative log expression and sample weights were applied before linear models were fitted and trended dispersions estimated to define sets of significantly DEGs ($FDR < 0.01$) for each experimental group relative to the PBS control group. 4CMenB + routine immunisation induced the greatest

perturbation of the transcriptome with 695 significant DEGs, the majority (576) of which were upregulated, while only a single gene was significantly upregulated in the routine only group, *Fcrl5* – encoding an Fc receptor-like immunoglobulin implicated in B cell immunoregulatory activity ³⁴². There was substantial overlap between the two 4CMenB-immunised groups with 253 genes commonly significantly DE (Fig. 4.7), and none of the uniquely significantly DEGs were significantly differentially regulated between these two combinations. This implies that, while there may be genes below the significance threshold that are differentially regulated between the two groups, the gene expression changes attributable specifically to the routine immunisations in the 4CMenB + routine group are not expressed to the same level as those implicated in the 4CMenB-induced response at this dose, but may instead lead to greater enrichment of 4CMenB-induced pathways.

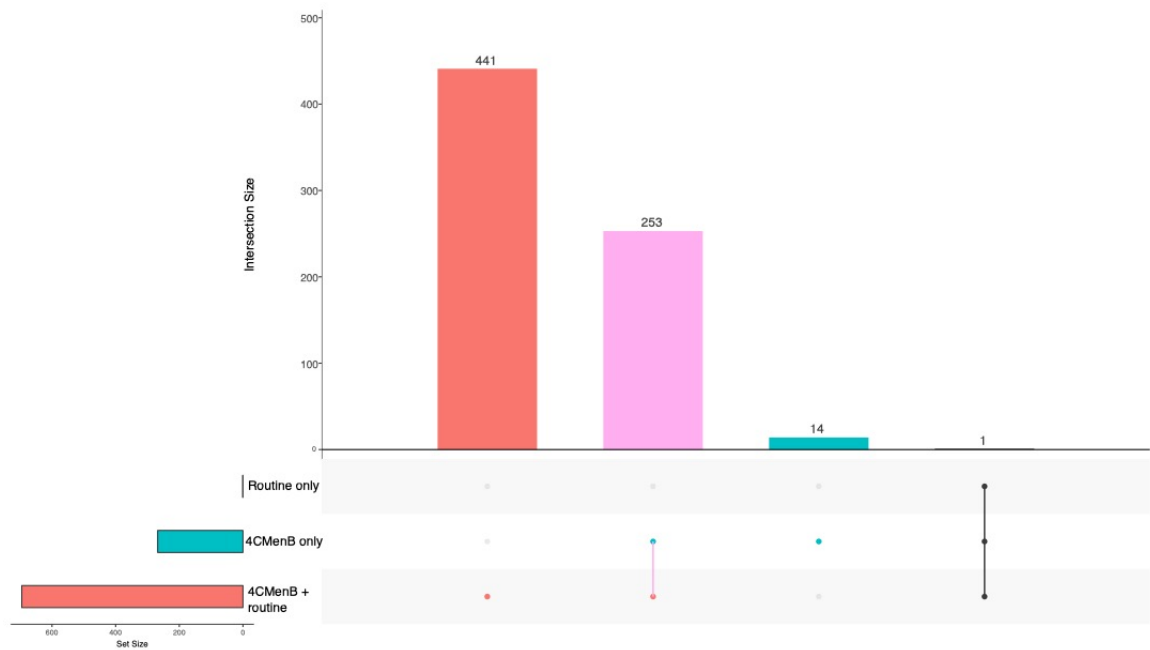


Figure 4.7. Overlap between significantly differentially expressed genes in the 4CMenB and routine immunisation groups. Upset plot depicting the proportion of significantly differentially expressed genes (DEGs, FDR < 0.01) unique or common to each of the three experimental groups. The total number of significantly DEGs are represented by the horizontal barplot to the left. The number of uniquely significantly DEGs are represented by the vertical bars above the individual dots corresponding to each group, while the number of commonly significantly DEGs are represented above the dots joined by a vertical line.

To establish the impact of 4CMenB + routine versus routine only immunisation on pathway enrichment, an over-representation analysis (ORA) was performed. This was done by testing each list of significantly DEGs (FDR < 0.05) from each pair of group contrasts for enrichment of defined gene ontologies and pathways from publicly available databases – integrating network objects with hierarchies INOH ³⁴³ and Reactome ³⁴⁴ for cell signalling (Fig. 4.8) and immunological pathways (Fig. 4.9), respectively. IL-1, TLR, and janus kinase (JAK)-signal transducer and activator of transcription protein (STAT) signalling pathways were among the top significantly differentially enriched pathways for all

three experimental groups but were substantially more significantly enriched in the 4CMenB + routine group than either the routine only or 4CMenB only groups (Fig. 4.8, top panels). This is further evidenced by the degree of enrichment of these pathways when the analysis was performed on genes significantly DE between the 4CMenB + routine and routine only groups (Fig. 4.8, bottom left panel).

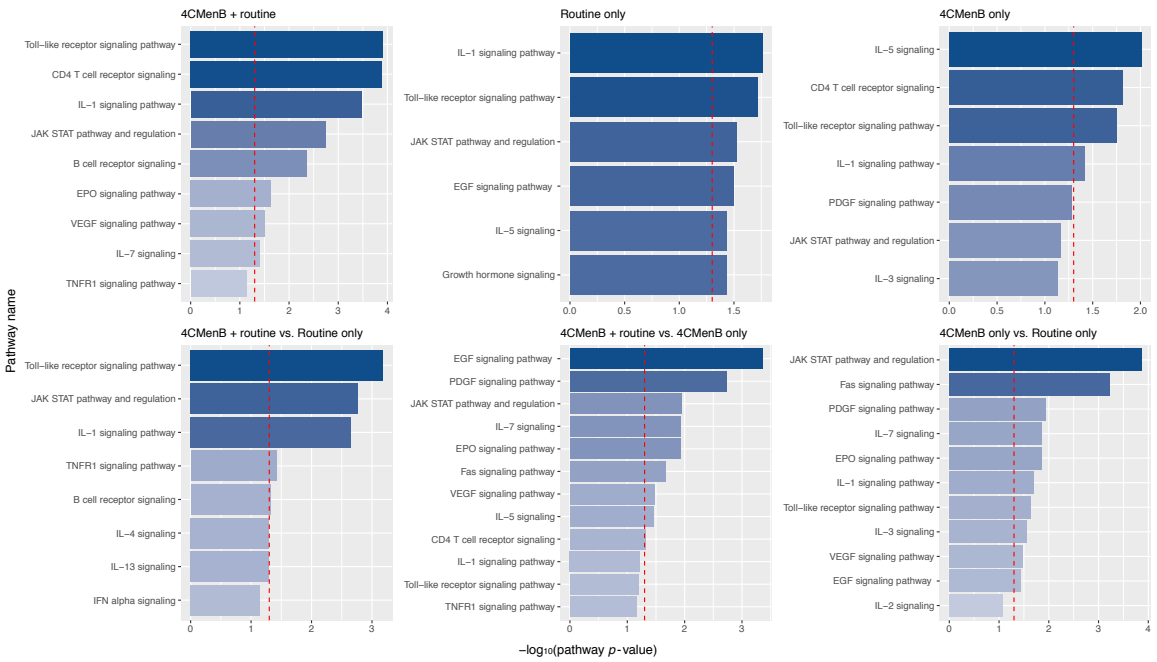


Figure 4.8. Cell signalling pathways significantly enriched 24 hours after the second dose of 4CMenB and routine immunisations. Significantly differentially expressed genes (FDR < 0.05) determined for each group/intergroup comparison were assessed for enrichment of genes pertaining to cell signalling pathway categories from the publicly available integrating network objects with hierarchies (INOH) database. The dashed red line corresponds to a negative $\log_{10}$ pathway adjusted p -value of 0.05.

Immunological pathway ORA revealed that the TLR, intracellular signalling, and pro-inflammatory pathways were all engaged following 4CMenB + routine immunisation (Fig. 4.9, left panel). Pathways corresponding to the activation of TLR2 alone and in complex with TLRs 1 and 6 indicate recognition of tri- or

diacylated lipoproteins, respectively ^{345,346} and TLR4 activation is stimulated in response to endotoxin; both TLR2 and TLR4 activation initiate MyD88 and MyD88-adaptor-like (Mal) signal transduction ³⁴⁷. The NOD1/2 pathway, known to be activated in response to peptidoglycan motifs present in Gram-positive and -negative bacteria ³⁴⁸, was also enriched in this group. The inflammasome and NLRP3 inflammasome pathways were significantly enriched and may be responsible for inducing the IL-1 signalling pathway. The endotoxin response, characterised by TLR4 signalling, “Transfer of LPS from LBP carrier to CD14”, and (NLRP3) inflammasome pathways, predominate when the significantly differentially enriched immunological pathways are compared between the 4CMenB + routine and routine only groups (Fig. 4.9, right panel). Taken together, the pathway ORA highlighted the role of bacterial PAMPs in triggering the inflammatory gene signatures that are characteristic of the response to 4CMenB, with or without routine immunisations, and indicated that these responses were amplified by the inclusion of routine immunisations.

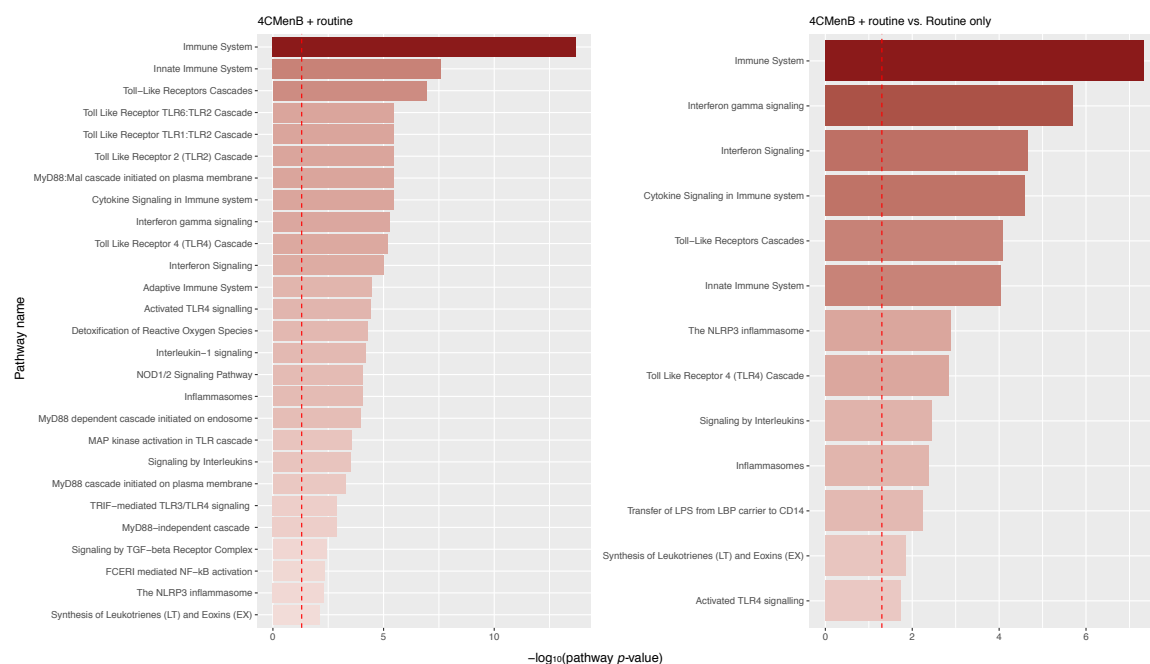


Figure 4.9. Comparison of significantly enriched immunological pathways 24 hours after the second dose of 4CMenB and routine immunisations. Significantly differentially expressed genes (FDR < 0.05) determined for the 4CMenB + routine group relative the PBS control group (left panel) and the routine only group (right panel) were assessed for enrichment of genes pertaining to immunological pathway categories from the publicly available Reactome database. The dashed red line corresponds to a negative $\log_{10}$ pathway adjusted p -value of 0.05.

Further to the elucidation of pathways associated with each vaccine combination, several of the genes implicated in these pathways were among the top significant genes differentially regulated between the 4CMenB-containing combinations and routine only/PBS control groups (Fig. 4.10). In addition to the TLRs mentioned previously, a TLR4 signal-amplifying receptor expressed on neutrophils and monocytes was highly upregulated in the 4CMenB and 4CMenB + routine groups. This receptor plays a role in endotoxin-induced shock, triggering receptor expressed on myeloid cells (TREM)^{1 349}. The engagement of TLRs is reflected in the expression of *Nlrp3* and subsequent expression of *Il1b*. The peptidoglycan recognition protein (PGLYRP1) was also significantly upregulated

in the 4CMenB-immunised groups, suggesting an immunostimulatory effect from residual peptidoglycan in the 4CMenB formulation. Overall, specific bacterial PAMPs contained within 4CMenB appeared to drive the gene expression changes that play an important role stimulating a pyrogenic cytokine pathway, which is characterised by production of IL-1 β following activation of the NLRP3 inflammasome via TLR-mediated signalling events.

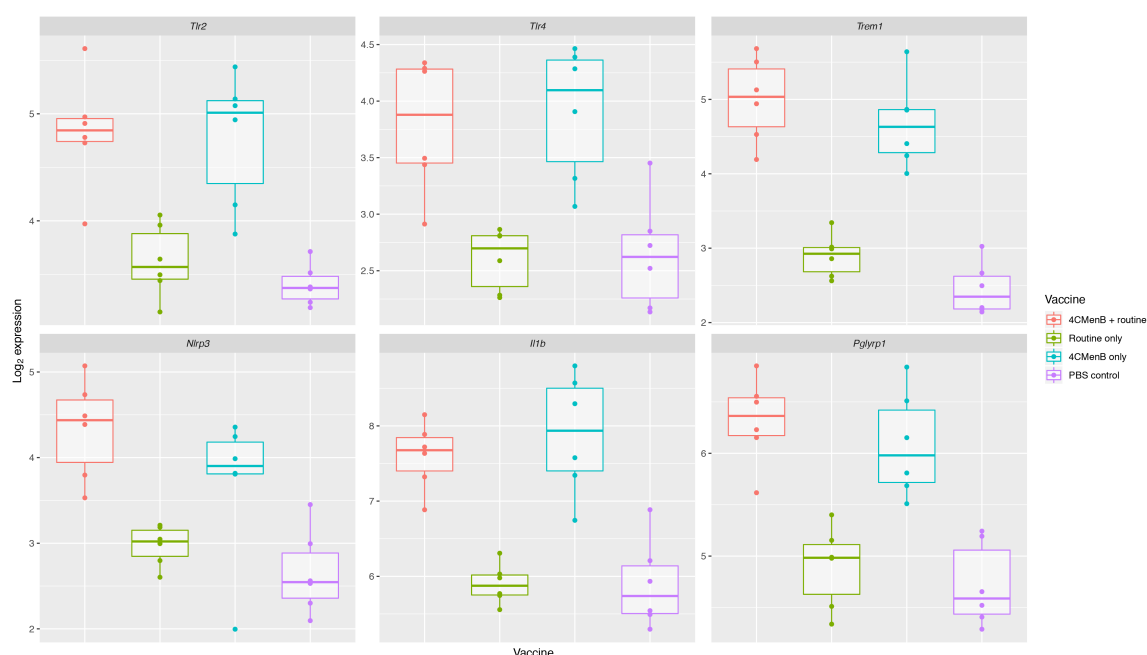


Figure 4.10. A selection of innate immune response genes that varied greatly between vaccine combinations with and without 4CMenB. Normalised log₂ expression values for genes encoding several PRRs and downstream innate immune responses, among the top genes identified as significantly differentially regulated between 4CMenB-containing combinations and routine only/PBS control groups.

4.3.4 Concomitant 4CMenB immunisation stimulates pro-inflammatory gene expression in a cluster of activated neutrophils

Having identified key receptors, signalling proteins, and cytokines involved in the differential responses to 4CMenB with, without, and compared with routine immunisations, a cell deconvolution analysis was conducted to attribute the gene signatures underlying these responses to the circulating immune cells. This involved statistical comparisons between log₂-transformed gene expression values for each experimental group and gene sets corresponding to the 207 murine immune cell populations defined on the Immunological Genome Project (ImmGen) database ³⁵⁰. Inferred relative quantities (represented as LFC) for each three-vaccine combination were calculated relative to the PBS control group (Fig. 4.11). A decrease in the proportion of most B cell phenotypes was observed in the 4CMenB + routine and 4CMenB only groups but this may indicate the presence of B cells in an organ other than whole blood. Granulocytes, with phenotype annotations corresponding specifically to neutrophils, had the greatest contrast between routine only- and 4CMenB-immunised groups, with positive enrichment of gene signatures associated with neutrophils from blood (Fig. 4.12). The enrichment of neutrophils in circulation 24 hours after immunisation with 4CMenB + routine immunisations is in agreement with what was observed *in silico* and *in vivo* (whole blood count) for this group in the infant study.

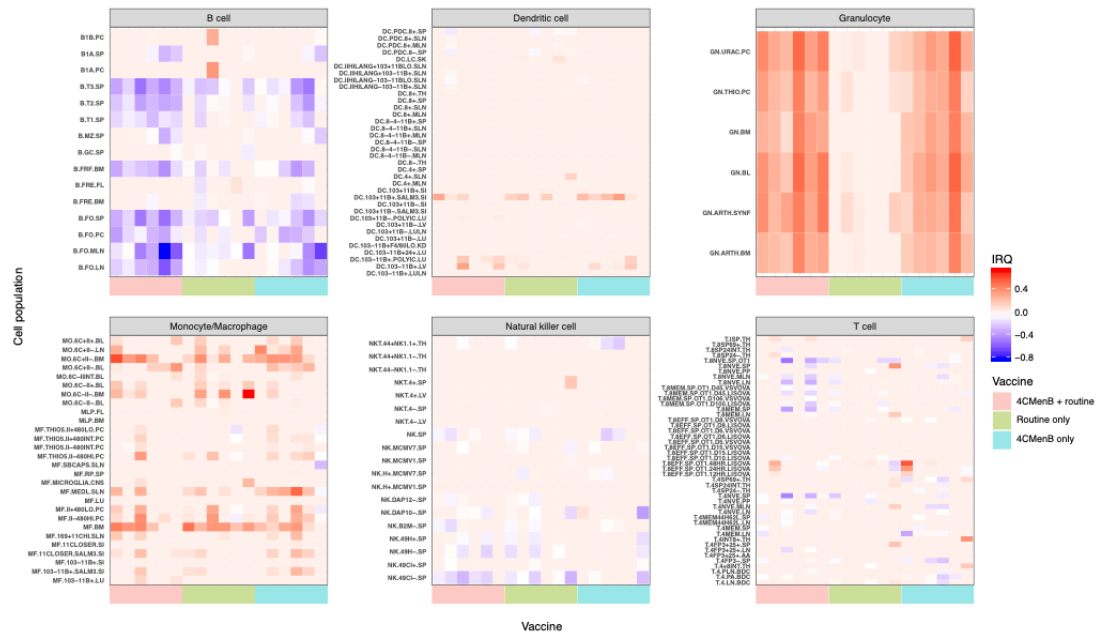


Figure 4.11. Heatmap of cell deconvolution analysis performed on whole blood gene signatures 24 hours after immunisation with 4CMenB and routine immunisations. Inferred relative quantity (IRQ) for each immune cell population (y-axis) are represented as $\log_2$ fold-change values corresponding to each tile of the heatmaps. Heatmaps are faceted by immune cell category.

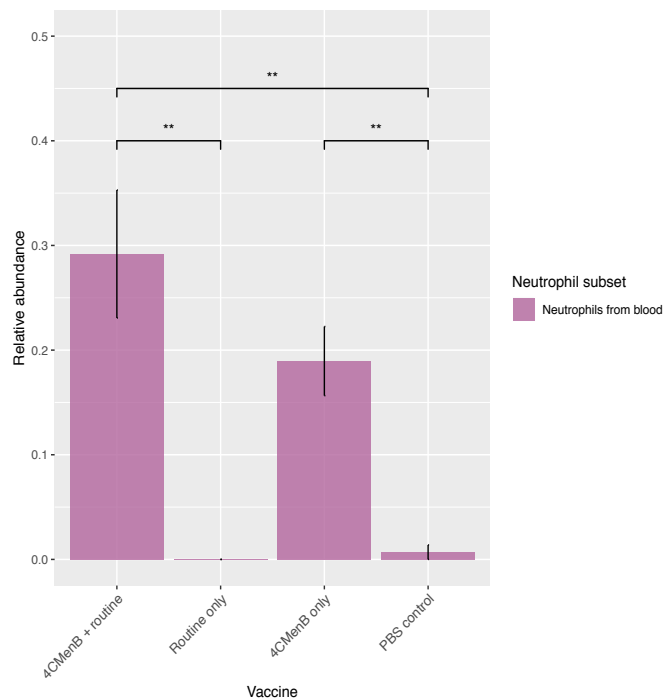


Figure 4.12. Enrichment of the neutrophils from blood subset of granulocyte fraction from the cell deconvolution analysis.

Relative abundance of neutrophils in whole blood inferred from $\log_2$ fold-changes of genes identified for this phenotype from the Immunological Genome Project (ImmGen) database. Significant differences between groups were determined using a student's t-test. ** < 0.01.

Given the number of neutrophil-specific genes found among the top significantly DEGs at 24 hours in the mouse 4CMenB-immunised groups, the enrichment of the neutrophils in blood fraction in the mouse and infant cell deconvolution analyses, and the increase in neutrophils observed 24 hours after immunisation in the infant study, scRNA-seq was performed on neutrophils isolated from mice immunised with routine vaccines with or without 4CMenB 24 hours after the second dose (Fig. 4.13), to determine whether concomitant immunisation led to differential transcriptional activity of neutrophils between these vaccine combinations.

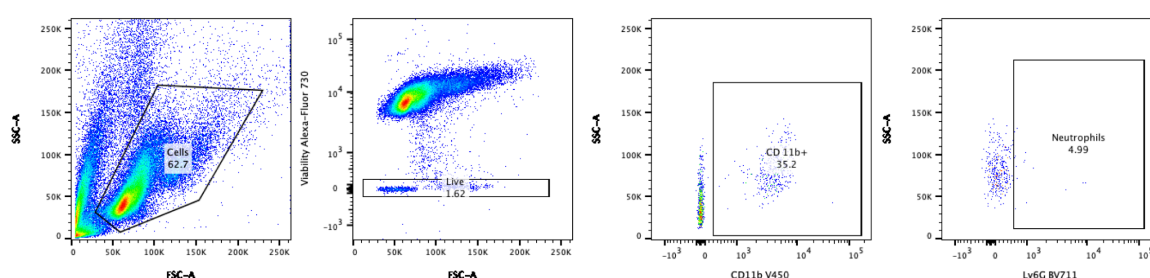


Figure 4.13. Gating strategy for isolating neutrophils from mouse whole blood. Neutrophils were identified as Ly6C⁺CD11b⁺Ly6G⁺ live cells in whole blood. Details of the antibodies used are provided in section 2.1.4.

Neutrophils isolated from each mouse were pooled to obtain one sample for each experimental group (4CMenB + routine and routine only) and then sequenced by scRNA-seq. The resultant data underwent canonical correlation analysis (CCA) to identify variation between conditions and facilitate an integrated analysis of all cells. A t-distributed stochastic neighbour embedding (tSNE) clustering analysis was performed to highlight differences and similarities between cells in low dimensional space; it showed a high degree of overlap between clusters from each condition. Comparative analysis of DEGs between conditions identified the innate

immune receptor genes *Il1r2*, *Cxcr2*, and *Csf3r* to be distinctly upregulated in the concomitant group, while the lysozyme-encoding *Lyz2* and complement genes *C1qc* and *C4b* underwent greater upregulation in the routine only group (Fig. 4.14). Also among the top DEGs in the concomitant group were *Plek* and *Lcp1*, two genes that will be discussed in section 4.2.6.

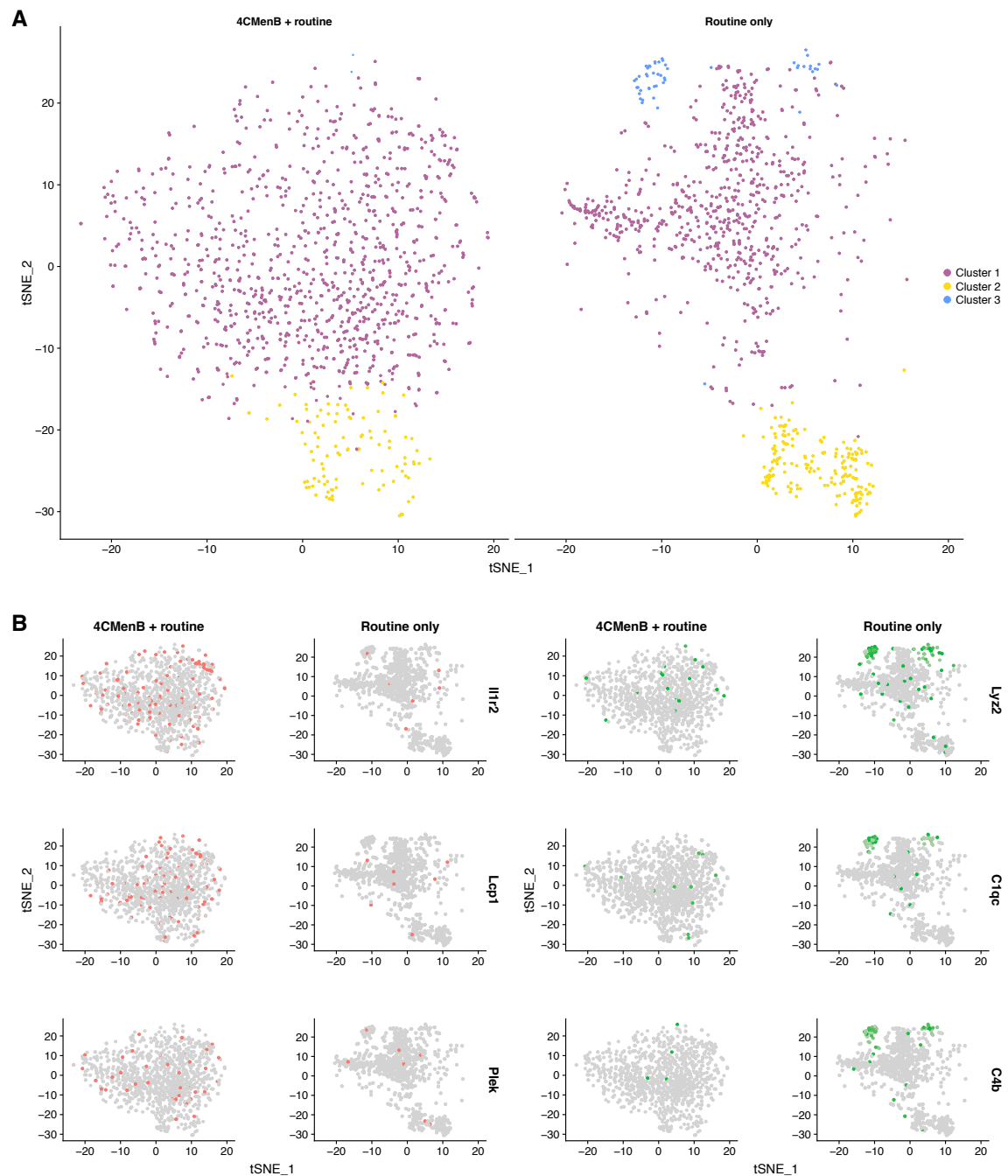


Figure 4.14. T-distributed stochastic neighbour embedding plots generated from canonical correlation analysis of neutrophils isolated from mouse whole blood, 24 hours after immunisation. A. Clustering of canonical correlation analysis (CCA)-aligned neutrophils, isolated and sequenced from pooled whole blood of mice taken 24 hours after the second dose of 4CMenB + routine and routine only immunisations. Neutrophil clusters were determined by t-distributed stochastic neighbour embedding (tSNE) dimensionality reduction during integrated analysis of CCA-aligned cells from both conditions. **B.** Feature plot maps of top genes identified by differential expression analysis for each

condition, with cells highlighted based on scaled expression values greater than one log₂ fold-change above the median across all cells. The cells highlighted in red correspond to those expressing the top genes, *Il1r2*, *Lcp1*, and *Plek*, identified as positively differentially expressed in the 4CMenB + routine group relative to the routine only group and those highlighted in green correspond to those expressing the top genes, *Lyz2*, *C1qc*, *C4b*, identified as positively differentially expressed in the routine only group relative to the 4CMenB + routine group.

4.3.5 4CMenB-induced fever is driven by the membrane-bound endotoxin present on its outer membrane vesicle component

To determine whether the specific component(s) driving the increase in temperature associated with 4CMenB immunisations groups of six mice were immunised with 1/5 of the human dose of 4CMenB, one of each of the four components of 4CMenB administered individually with alum, dOMVs from the H44/76 strain, lpxL1 nOMVs from the same strain, *E. coli* LPS in alum, and MVA – at day 0 and 21 (Fig. 4.15).

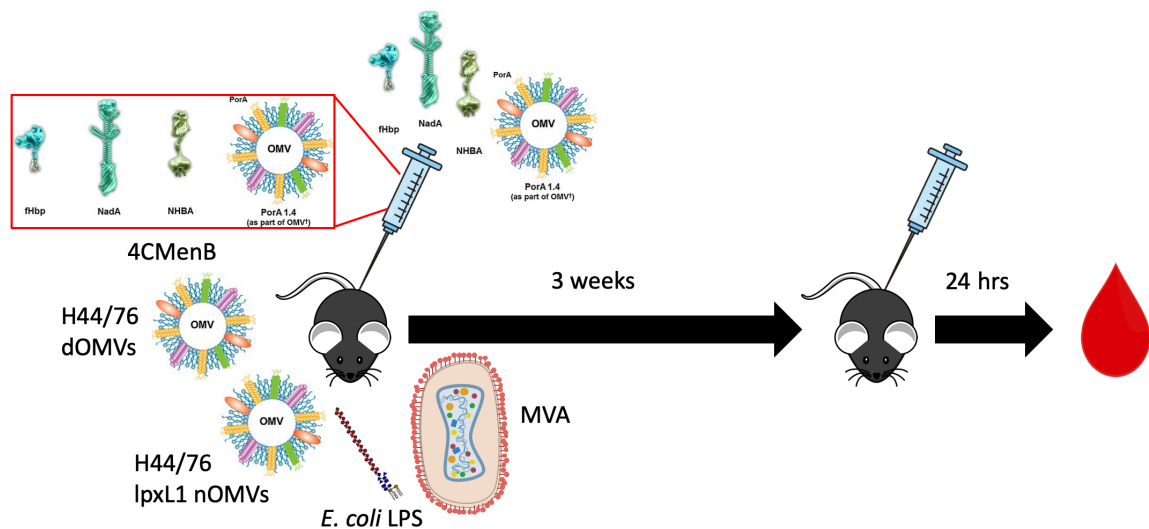


Figure 4.15. 4CMenB individual components comparison mouse immunisation strategy. Schematic of the mouse immunisation strategy for comparing the effects of each individual component of 4CMenB with those of the final formulation or one of several comparator groups. Groups of six C57BL/6 mice were immunised with 1/5 of the human dose of one of the vaccines/components displayed on the left (all adsorbed on alum with the exception of MVA and pre-adsorbed formulations) at day 0 and received a second shot of the same immunisation at day 21. Mice were bled 24 hours after this second dose.

The surface temperature of each mouse was measured to determine changes in temperature associated with the second dose of each of these vaccines/components. Temperature readings were taken at baseline (60 mins before second dose), two, six, and 24 hours after the second dose (Fig. 4.16). Only mice that received OMV components, specifically those containing dOMVs, displayed a statistically significant rise in temperature relative to the naïve control group. Both dOMVs, but not the lpxL1 nOMVs, induced a statistically significant increase in temperature relative to pre-immunisation. This demonstrates that attenuation of membrane-bound endotoxin abrogates fever in these mice and that the main driver of 4CMenB-induced fever is the residual bacterial LOS found in its constituent dOMVs.

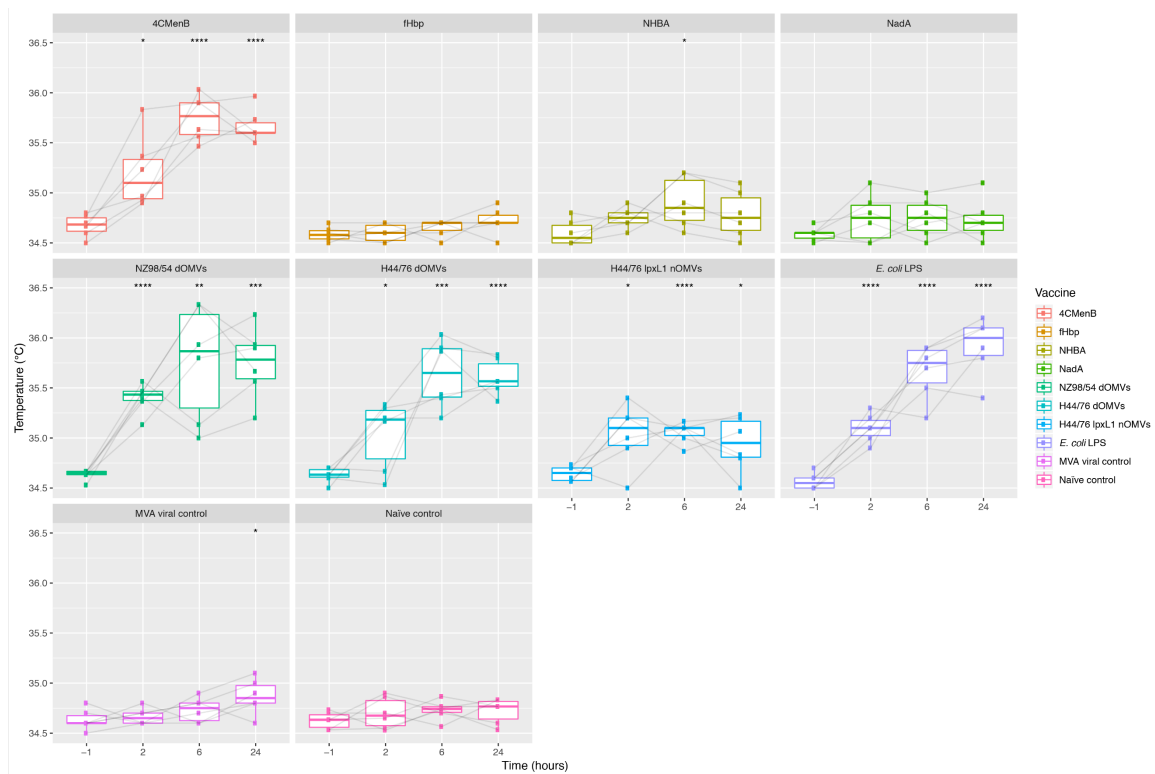


Figure 4.16. Changes in mouse surface temperature associated with 4CMenB, its individual components, and comparator immunisations, 24 hours after the second dose of vaccine. Boxplots depicting the change in temperature from baseline to 24 hours after the second dose of vaccine/component for each individual group. Vaccine group medians at each timepoint were compared with baseline for that vaccine using a Wilcoxon signed-rank test. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

4.3.6 Mouse whole blood gene signatures cluster according to the antigenic nature of the stimulus

Having defined and compared gene signatures between vaccine combinations that did and did not induce fever in mice and then further attributing 4CMenB-induced fever to a specific component of the vaccine, the next aim was to characterise gene signatures associated with the specific vaccines/antigens tested

in section 4.3.5. The aim of this study was to elucidate immunological pathways that may underlie the differential propensities of the vaccine components to induce fever in mice in a similar manner. To this end, a second RNA-seq experiment was performed on RNA extracted from mouse whole blood 24 hours after the second dose of immunisations (Fig. 4.15). Initial assessment of the global changes in gene expression associated with 4CMenB, the vaccine's components, and comparator test groups identified a large number of DEGs associated with each group (Fig. 4.17). NZ98/254 dOMV immunisation was associated with the greatest number of significantly (FDR-adjusted p -value < 0.01) DEGs and was also the component of 4CMenB with the greatest number of uniquely DEGs at 24 hours after the second dose (Fig. 4.17A). A greater overlap between significant DEGs was observed between 4CMenB, OMV, and *E. coli* LPS groups (Fig. 4.17B) than was observed for 4CMenB and its constituent antigens. Of the significantly DEGs associated with 4CMenB and its components, only 191 DEGs were common to the four components and the final formulation, whereas 409 DEGs were common to 4CMenB, the three OMV groups, and *E. coli* LPS.

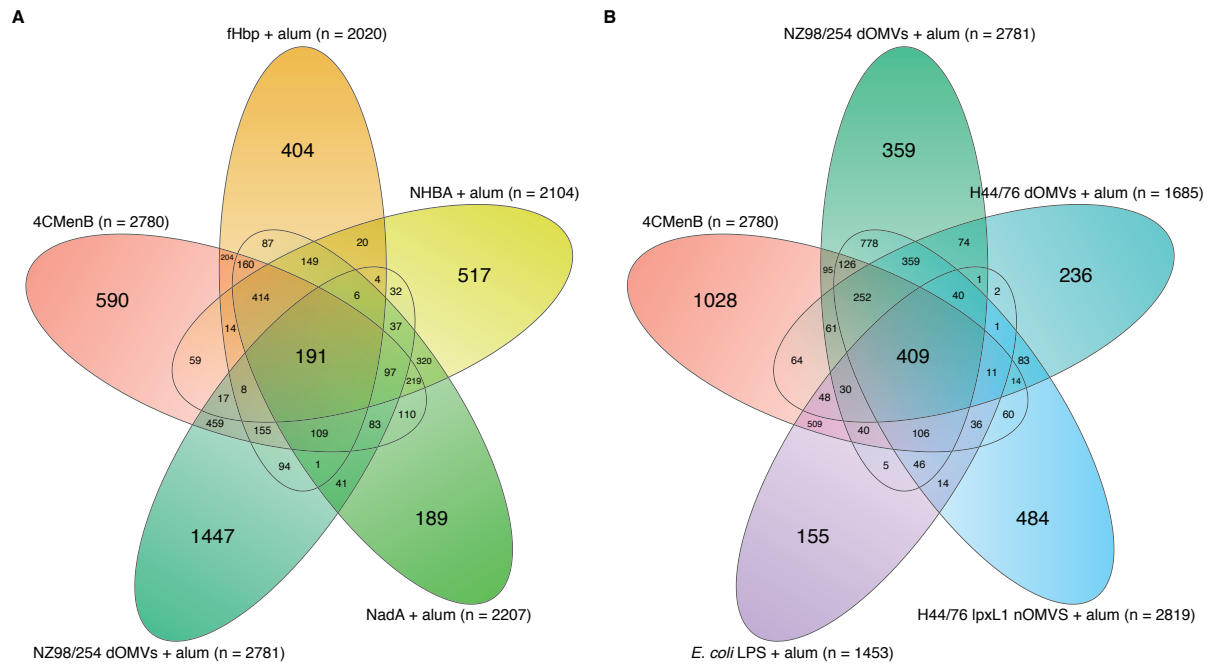


Figure 4.17. Global changes in gene expression at 24 hours after immunisation with 4CMenB, one of its four components, or one of several comparator immunisations.

Euler plots of the total number of significantly differentially expressed genes (DEGs, FDR-adjusted p -value < 0.01) associated with each test group 24 hours after the second dose of vaccines. **A.** Overlap between DEGs associated with 4CMenB, and each of its alum-adsorbed components. **B.** Overlap between 4CMenB, each of the alum-adsorbed deoxycholate and native outer membrane vesicles (dOMVs and nOMVs), and alum-adsorbed lipopolysaccharide (LPS) from *Escherichia coli*.

Applying principal component (PC) analysis (PCA) to the data demonstrated clustering of samples according to vaccine type, with the three OMV groups and the three protein groups forming distinct clusters along the first two PCs – accounting for 12.5% and 5.1% of the variation, respectively (Fig. 4.18). The majority of this clustering was driven by a small group of genes, with the histocompatibility antigen genes, *H2-Aa*, *H2-K1* and *Cd74*, the lymphocyte antigen gene *Ly6e*, and the immunoglobulin *Ighm* gene contributing most to the clustering (Fig. 4.18). This suggests that antigen-specific processing and presentation pathway genes account for the greatest divergence in early transcriptional response

patterns between the groups tested. The genes encoding a protein involved in lymphocyte interaction, *Lcp1*, and a degranulation protein, *Plek*, contribute most to the clustering of the three OMVs groups. These two genes were identified in the scRNA-seq analysis of neutrophils (section 4.3.4 and Fig. 4.14) among the top significantly DEGs between cells isolated from 4CMenB + routine- and routine only-immunised mice. This implies that the NZ98/254 dOMV component of 4CMenB may be driving a strong neutrophil response that contributes to the reactogenicity of the vaccine.

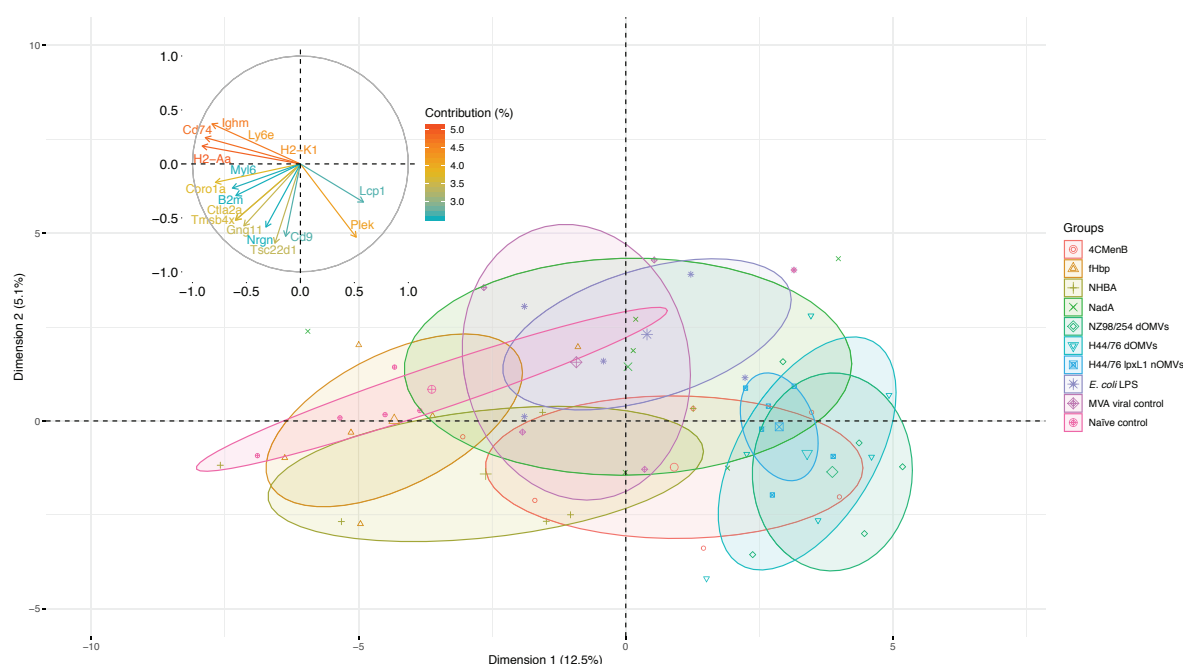


Figure 4.18. Early gene signatures induced by outer membrane vesicle-based and recombinant protein-based vaccines cluster according to vaccine type. Two-dimensional representation of principal components (PCs) one and two as determined by PC analysis of gene expression values from all groups. Ellipses correspond to 95% confidence intervals for each group. The contribution plot in the upper left quadrant depicts the percentage contribution of individual genes to the clustering observed along each dimension.

4.3.7 Transcriptional changes in innate immune response genes related to bacterial infection characterise the early gene expression changes induced by 4CMenB and outer membrane vesicle vaccines

Unsupervised hierarchical clustering of the most variable genes across all samples defined two divergent clusters defined by the recombinant protein and viral control groups on the one hand and the 4CMenB and the OMV groups on the other (Fig. 4.19). The classifying genes were broadly related to innate immune responses, such as PRR, antigen processing and presentation, and cell signalling pathway genes. Approximately one third of these genes were related to neutrophils – *Ngp*, *Lcn2*, *Lrg1*, *S100a8*, *Mmp8*, *Fpr1*, and *Ltb4r* – all of which have roles in neutrophil migration, anti-bacterial responses, and differentiation, further strengthening the hypothesis that OMV-based vaccines drive a strong pro-inflammatory neutrophil response.

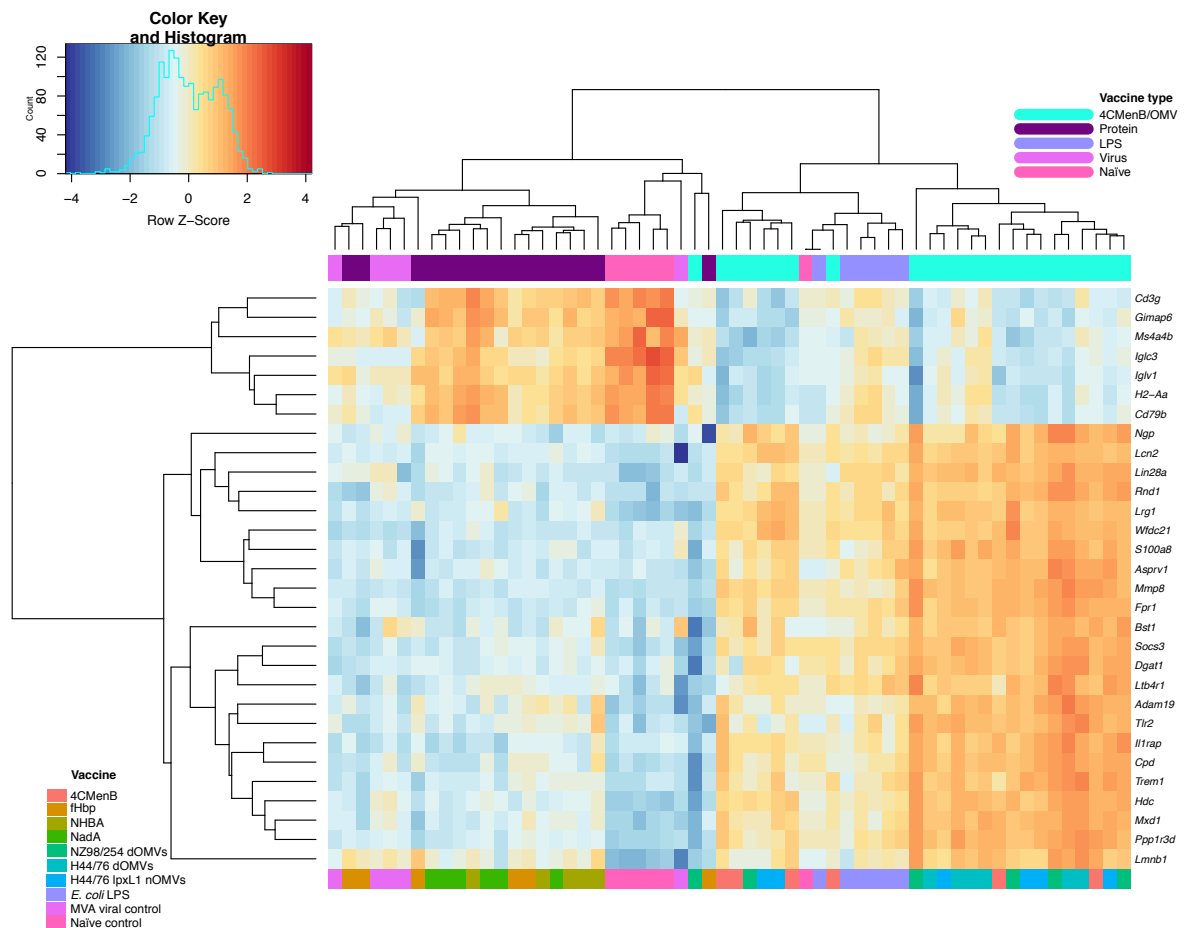


Figure 4.19. Top 30 most variable genes across all samples, 24 hours after immunisation with 4CMenB, one of its four components, or one of several comparator immunisations. Pairwise Euclidian distances between all samples were calculated from the normalised counts of genes with the maximum dispersion from the population mean and are represented by the column and row dendrograms. Heatmap tiles correspond to row z scores, centred and scaled upon the mean expression value for that gene across the dataset, with orange and blue tiles indicating increased or decreased expression relative to the population mean, respectively.

Genes identified as characteristic of the 4CMenB and 4CMenB + routine immunisation transcriptional response in the first mouse dataset (section 4.3.3 and Fig. 4.10) and the top DEGs in this second dataset were compared. This revealed a selection of genes involved in PRR-mediated activation of the NLRP3 inflammasome and highlighted a gene expression pattern common to the OMV-

containing vaccines and the related *E. coli* LPS (Fig. 4.20). This implicates the constituent OMVs of 4CMenB in driving the bacterial infection-like transcriptional response that predominates following 4CMenB immunisation, with and without routine immunisations.

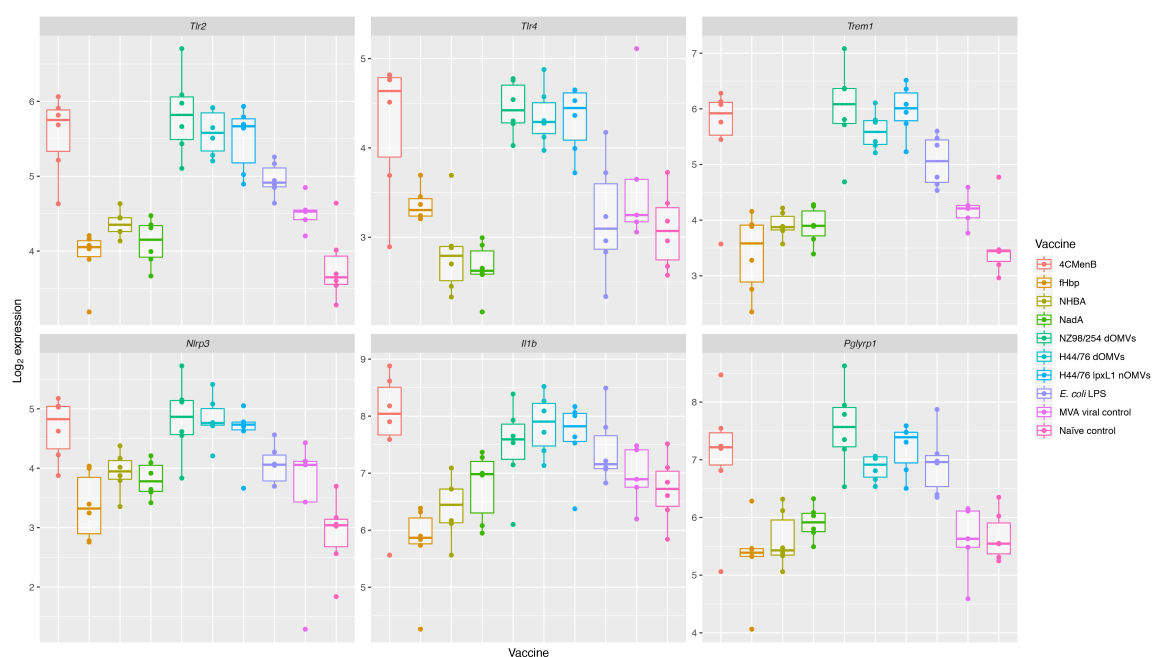


Figure 4.20. A selection of innate immune response genes that varied greatly between the 4CMenB, constituent component, and comparator groups. Normalised $\log_2$ expression values for genes encoding several PRRs and downstream innate immune responses, among the top genes identified as significantly differentially regulated across all samples.

4.3.8 Pathway enrichment analysis of significantly differentially expressed genes in whole blood

Taking all significantly DEGs identified for each group and conducting the same pathway ORA described in section 4.3.3, a common set of significantly enriched innate immune response pathways was identified for the OMV-containing vaccine groups, with the notable exception of the lpxL1 nOMV group,

and the related LPS group (Fig. 4.21) defined by TLR4 activation and (NLRP3) inflammasome activation.

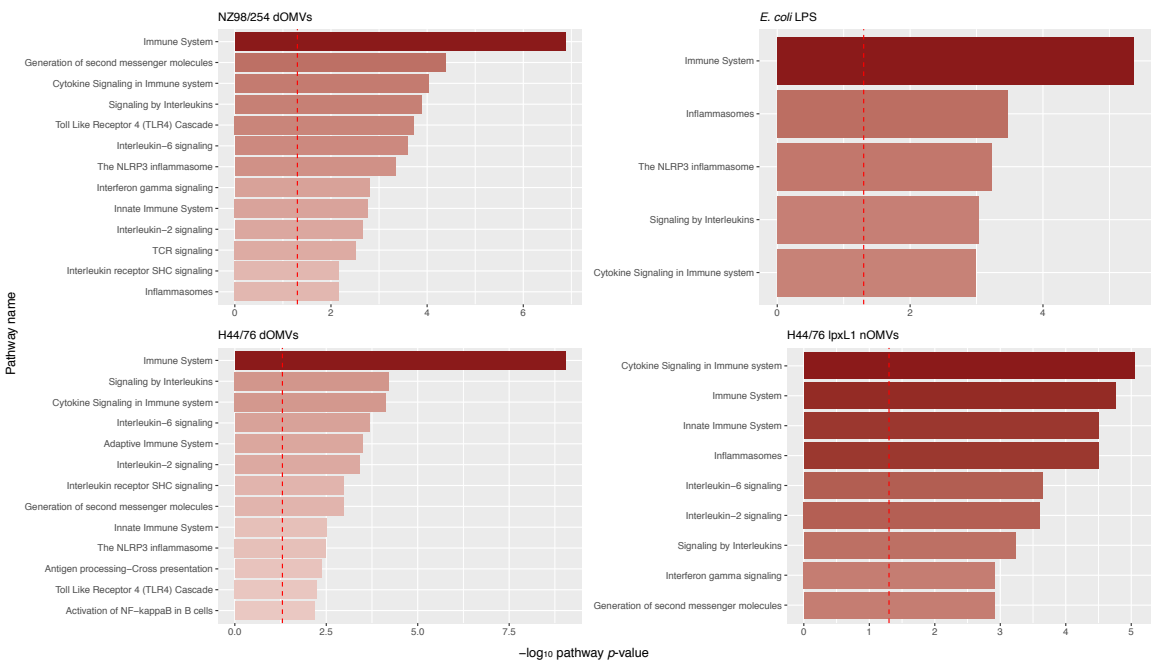


Figure 4.21. Comparison of significantly enriched immunological pathways 24 hours after the second dose of outer membrane vesicles or lipopolysaccharide. Significantly differentially expressed genes (FDR < 0.05) were determined relative the naïve control group for each experimental group and assessed for enrichment of genes pertaining to immunological pathway categories from the publicly available Reactome database. The NZ98/254 detergent-extracted (d)outer membrane vesicle (OMV, top left), *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS, top right), H44/76 dOMV (bottom right), H44/76 lpxL1 native (n)OMVs (bottom right) groups are presented here. The dashed red line corresponds to a negative log₁₀ pathway adjusted *p*-value of 0.05.

Cytokine/chemokine-specific ORA indicated that the IL-6 signalling pathway was significantly enriched for all groups, with the exception of the viral control (Fig. 4.22); additionally, several T_H2-type cytokine signalling pathways, including IL-4, IL-5, IL-6, and IL-13 were significantly enriched among the OMV groups. This may be partly explained by the fact that alum stimulates a T_H2 cytokine response ³⁵¹.

Significant enrichment of IL-1 was a shared feature of the 4CMenB and OMV groups, though it was notably below the significance threshold for the lpxL1 nOMV group.

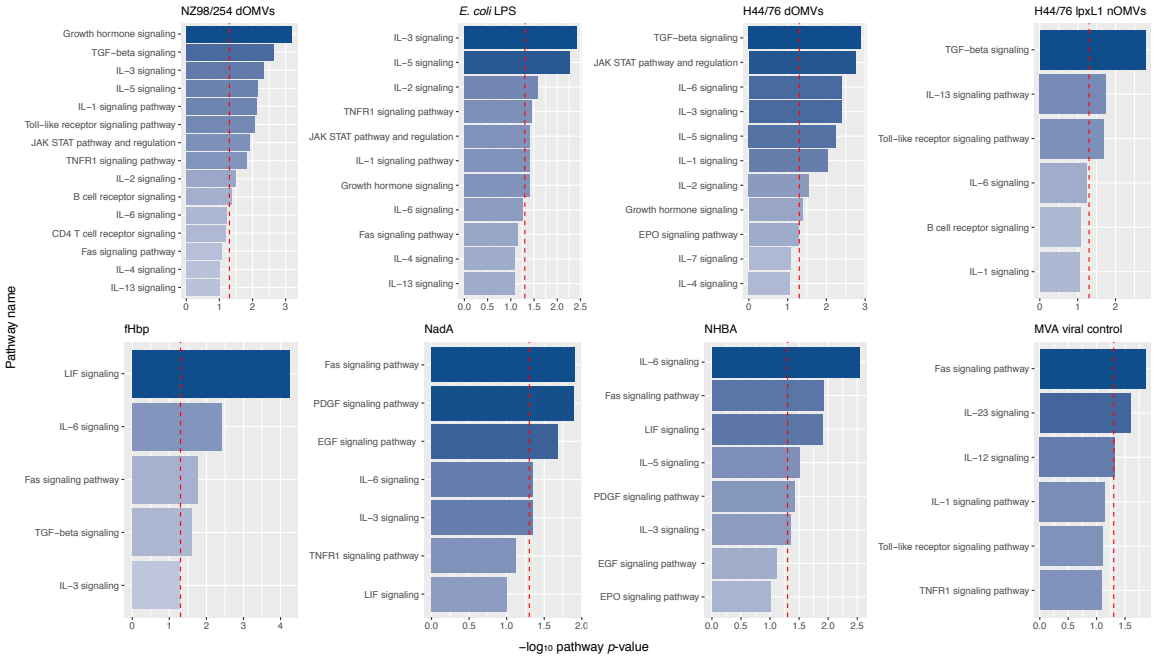


Figure 4.22. Cell signalling pathways significantly enriched 24 hours after the second dose of outer membrane vesicle vaccines or one of the components of 4CMenB. Significantly differentially expressed genes (FDR < 0.05) determined for each group comparison were assessed for enrichment of genes pertaining to cell signalling pathway categories from the publicly available integrating network objects with hierarchies (INOH) database. The dashed red line corresponds to a negative $\log_{10}$ pathway adjusted p -value of 0.05.

The cytochrome P450 (CYP) pathway gene ontology was significantly downregulated in the NHBA and NadA protein groups, but not the fHbp group. A clustering analysis of the genes associated with this family highlighted that these genes were highly and consistently downregulated only in the NHBA and NadA groups (Fig. 4.23). The CYP gene families play an important role in the metabolism of arachidonic acid, the precursor of PGE₂, encoding enzymes that convert it to eicosanoid metabolites³⁵². Their expression in hepatocytes is known to be

regulated by IL-6 during the inflammatory response, leading to their downregulation ³⁵³. Taken together, the results of the pathway ORA further detail the similar nature of the transcriptional changes underlying the pro-inflammatory responses induced by OMVs compared to those induced by the recombinant protein components of 4CMenB.

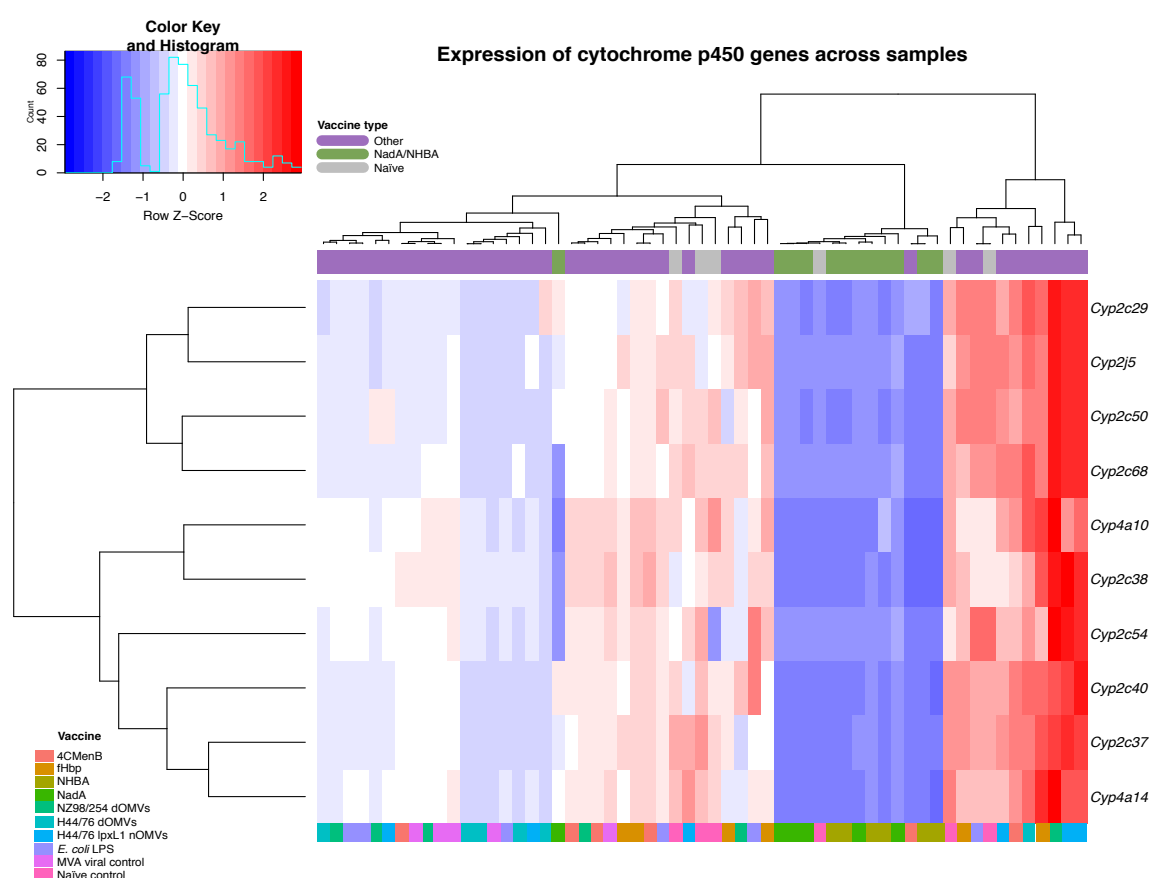


Figure 4.23. Hierarchical clustering based on the expression of genes encoding proteins of the cytochrome P450 family across all samples, 24 hours after immunisation with 4CMenB, one of its four components, or one of several comparator immunisations. Pairwise Euclidian distances between all samples were calculated from the normalised counts of genes with the maximum dispersion from the population mean and are represented by the column and row dendrograms. Heatmap tiles correspond to row z scores, centred and scaled upon the mean expression value for that gene across the dataset, with red and blue tiles indicating increased or decreased expression relative to the population mean, respectively.

4.3.9 IL-6 levels are significantly elevated in sera after 4CMenB immunisation

To determine the validity of the cytokine pathway ORA, a multiplex cytokine bead assay was performed on sera separated from whole blood 24 hours after the second dose of each vaccine/antigen (Fig. 4.24). IL-6 was significantly elevated for all groups, with the exception of the viral control, as suggested by the INOH pathway ORA. The highest levels of IL-6 were observed in the 4CMenB and OMV immunised groups. Significant quantities of TNF α were detected in the NZ98/254 OMV immunised group and a slight increase occurred in the LPS-immunised group, also suggested by the pathway ORA. While the changes in IL-1 β levels did not reach significance, there was an increase in the LPS-immunised group and several outliers in the 4CMenB and OMV immunised groups. A significant increase in IL-3 was detected in the fHbp immunised group, and a significant increase in IL-5 was detected for the 4CMenB, fHbp, and NadA immunised groups, with the highest levels seen in the fHbp immunised group. These cytokines, along with GM-CSF, make up a cytokine family that has roles in the differentiation, recruitment, and growth of immune cells, and are characteristic of a T_H2-type response³⁵⁴. The fact that alum is included in all vaccines/antigens, except MVA, indicates that this is an antigen-specific response.

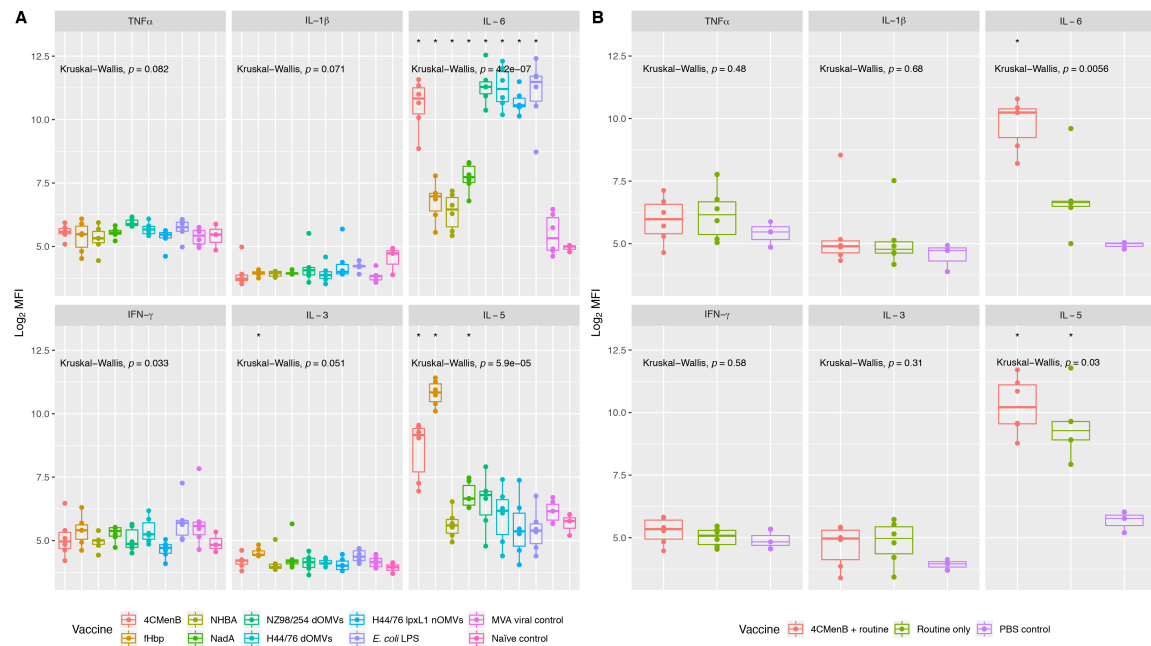


Figure 4.24. Detection of cytokines in mouse sera. Log₂ median fluorescence intensity (MFI) values for each of cytokine detected by a six-plex cytokine bead assay 24 hours after immunisation with **A.** 4CMenB, one of its constituent components, or one of several comparator antigens, or **B.** 4CMenB + routine, routine only, or PBS control immunisations. Kruskal-Wallis significance values are presented in the top left corner of each panel. Significance of group medians relative to naïve or PBS controls were calculated using Mann-Whitney rank-sum tests. * < 0.05.

The same multiplex assay was performed on sera from mice immunised with 4CMenB, 4CMenB + routine immunisation, and PBS control vaccines to determine whether any of these cytokine responses predominated in mice receiving concomitant 4CMenB (Fig. 4.24). IL-6 was the only significantly elevated cytokine in the test groups, with greater quantities detected in the concomitant group than the routine group. There was also an increase in the levels of IL-5 in both of these groups, relative to the PBS control. Several other cytokines were detected but did not present changes that reached significance.

4.3.10 Cytokine signal transducer and prostaglandin synthesising enzyme genes are significantly upregulated on brain tissue after 4CMenB and outer membrane vesicle immunisation

Among the top DEGs identified in the 4CMenB components dataset were several genes encoding a variety of innate immune cytokine receptors. A hierarchical clustering analysis of these receptors revealed their expression to be associated with 4CMenB and OMV immunisation (Fig. 4.25). Several of these receptor genes, such as *Il1rap*, *Il1r2*, *Tnfsfr1a*, *Tnfsfr1b*, and *Il6ra*, encode the cognate receptors for some of the reactogenic cytokines detected to varying degrees in sera (section 4.3.9).

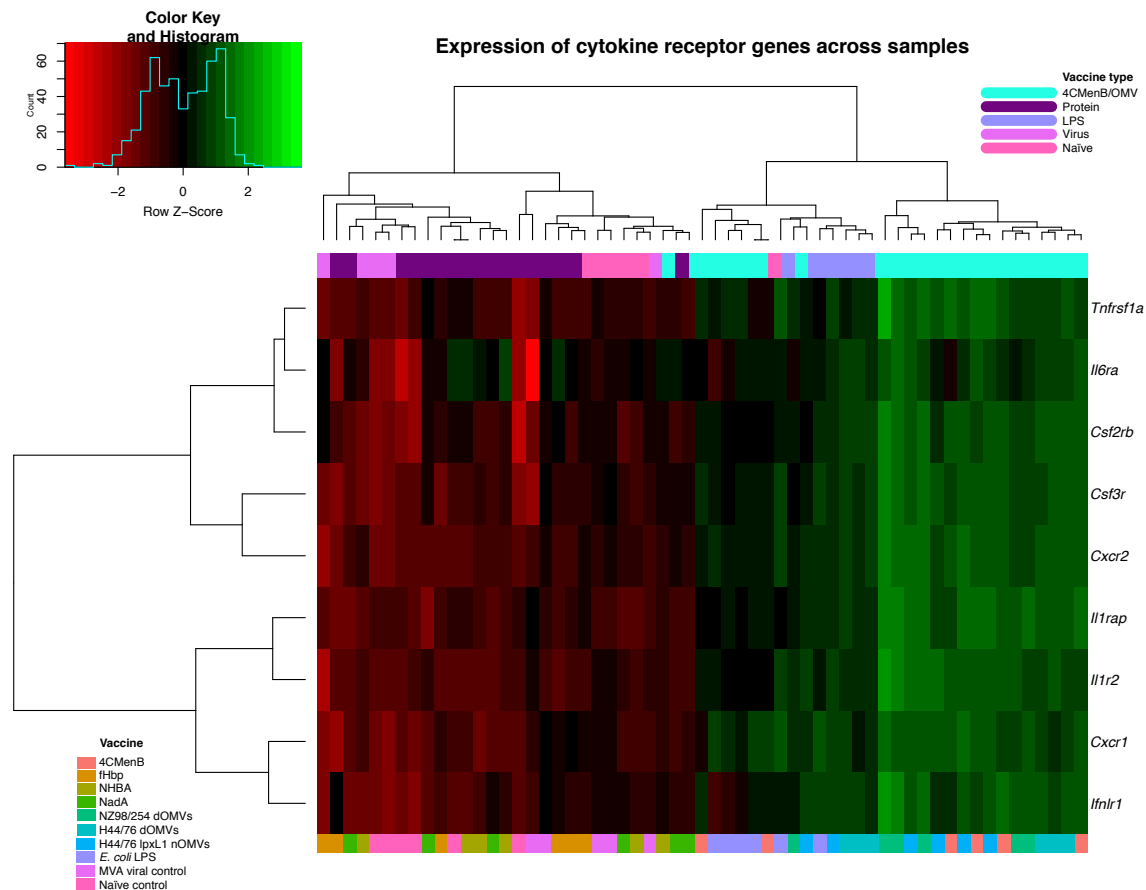


Figure 4.25. Hierarchical clustering based on the expression of a selection of cytokine and chemokine receptors across all samples, 24 hours after immunisation with 4CMenB, one of its four components, or one of several comparator immunisations.

Pairwise Euclidian distances between all samples were calculated from the normalised counts of genes with the maximum dispersion from the population mean and are represented by the column and row dendrograms. Heatmap tiles correspond to row z scores, centred and scaled upon the mean expression value for that gene across the dataset, with green and red tiles indicating increased or decreased expression relative to the population mean, respectively.

This finding prompted further exploration of the role of these cytokine receptors in the final mediation of fever at the blood-brain barrier, initiated by the binding of these cytokines to their cognate receptor on brain endothelial cells (BECs) to transduce inflammatory signals and subsequently induce PGE₂ synthesis. BECs

were isolated from the brains of mice immunised with 4CMenB or one of the vaccines/antigens of interest (Fig. 4.26).

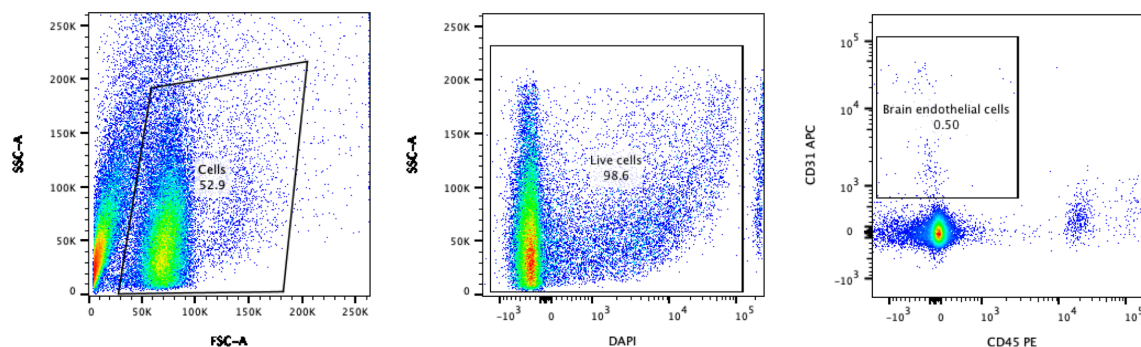


Figure 4.26. Gating strategy for isolating endothelial cells from mouse brain tissue.

Brain endothelial cells were identified as CD31⁺CD45⁻ live cells. Details of the antibodies used are provided in section 2.1.4.

RT-qPCR was then performed on RNA extracted from these cells. Figure 4.27 shows the negative ΔC_T values for each gene tested. Both of the TNF α receptor genes, *Tnfrsf1a* and *Tnfrsf1b*, were highly significantly downregulated in the 4CMenB, fHbp, and three OMV immunised groups. The IL-6 receptor gene, *Il6ra*, was significantly upregulated in the 4CMenB, LPS, and MVA immunised groups, but was significantly downregulated in the recombinant protein-immunised groups. IL1R1 was highly significantly upregulated in all groups except the fHbp and NHBA immunised groups, in which it was significantly downregulated. IL1RAP was only significantly upregulated in the H44/76 dOMV- and MVA-immunised groups and was significantly downregulated in the three protein-immunised groups. Two key prostaglandin synthesising proteins, mPGES-1 and COX-2, were highly significantly upregulated in the 4CMenB-, OMV-, and LPS-immunised groups.

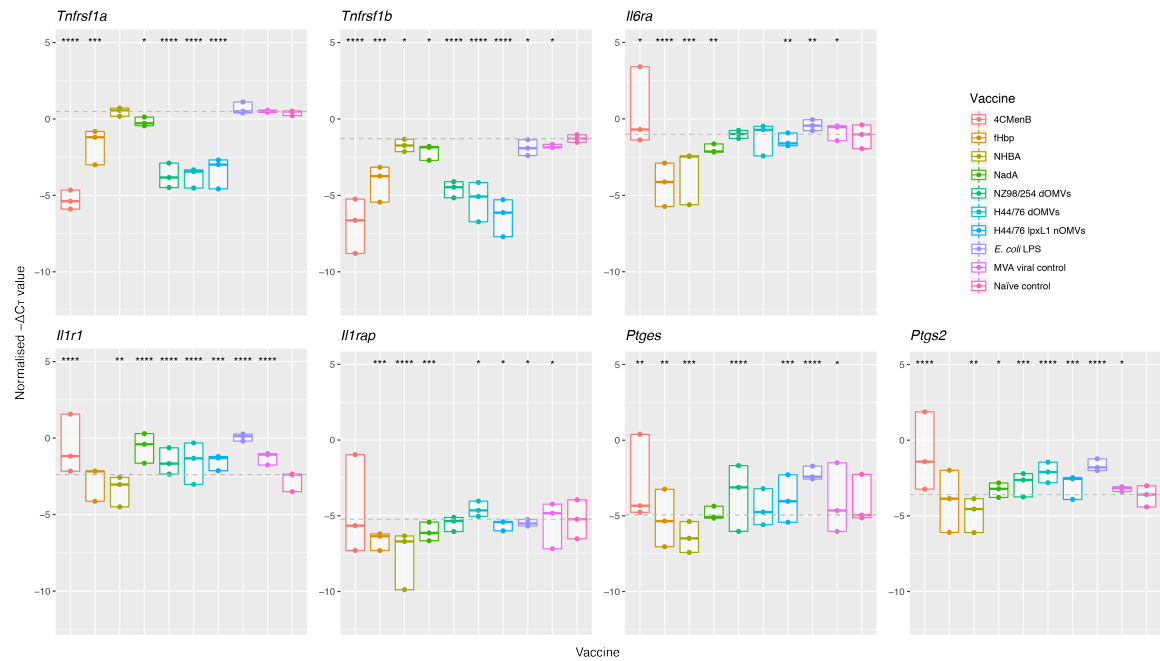


Figure 4.27. Expression of reactogenic cytokine receptor and prostaglandin enzyme genes on brain endothelial cells. Normalised (Δ) threshold cycle (C_T) values were calculated by subtracting the mean C_T value for the reference gene (*Pgk1*) from the target gene C_T values, averaged across triplicates. These values are plotted as negative ΔC_T to demonstrate their logical relation to the naïve control group. The horizontal line corresponds to the median of the naïve control group for each target gene, values above this line are considered upregulated and those below are considered downregulated, relative to the naïve control. Significance values were calculated between the test and naïve control groups using a Mann-Whitney U-test. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

4.3.11 Endotoxin drives the expression of select genes induced by 4CMenB, but other outer membrane antigens may contribute to expression changes in this pathway

Having identified endotoxin as a major driver of pro-inflammatory transcriptional changes and fever in mice, a low-throughput gene expression validation experiment was then performed to assess the regulation of a selection of genes identified as significantly DE following 4CMenB and OMV immunisation

(section 4.3.7 and Fig. 4.20). The aim was to determine the extent to which LPS preparations are capable of driving this response in isolation. To this end, groups of six C57BL/6 mice were immunised with the same dose of 4CMenB and the *E. coli* LPS in alum preparation used in the 4CMenB individual component experiment – 1/5 of the human dose or 1 µg, respectively – at days 0 and 21. To assess the effects of the purity of the LPS preparation, a group immunised with an ultrapure preparation of *E. coli* LPS adsorbed on alum was included, and to determine the effects of alum adsorption on LPS-induced gene expression, each form of LPS were administered to an additional group without alum and another group received alum on its own. As TLR2, TREM1, and PGLYRP1 are known receptors for peptidoglycan and OMV preparations may contain quantities of peptidoglycan in associated with OMPs, there may be a role for this ligand in stimulating or exacerbating the pro-inflammatory response to 4CMenB/OMV immunisation. Therefore, a group of mice were immunised with *E. coli* peptidoglycan adsorbed on alum and another were immunised with a combination of alum-adsorbed peptidoglycan and LPS, to determine whether a synergistic response could be induced. The surface temperature of each mouse was measured at baseline (60 mins before second dose), 90 mins, three hours, and 24 hours after the second dose of each antigen (Fig. 4.28). All *E. coli* LPS and peptidoglycan preparations/combinations induced a statistically significant rise in temperature as early as 90 mins after the second dose of vaccine, with no such rise observed in the alum only or naïve control groups. Temperature changes associated with alum-adsorbed peptidoglycan were more transient than those of the other antigens tested, with temperatures returning close to baseline by 24 hours. The highest temperatures were observed in the peptidoglycan + LPS + alum combination at 24 hours post-immunisation, but these temperature readings were not sensitive

enough to discern specific differences in the pyrogenicity of the antigens/antigen combinations tested.

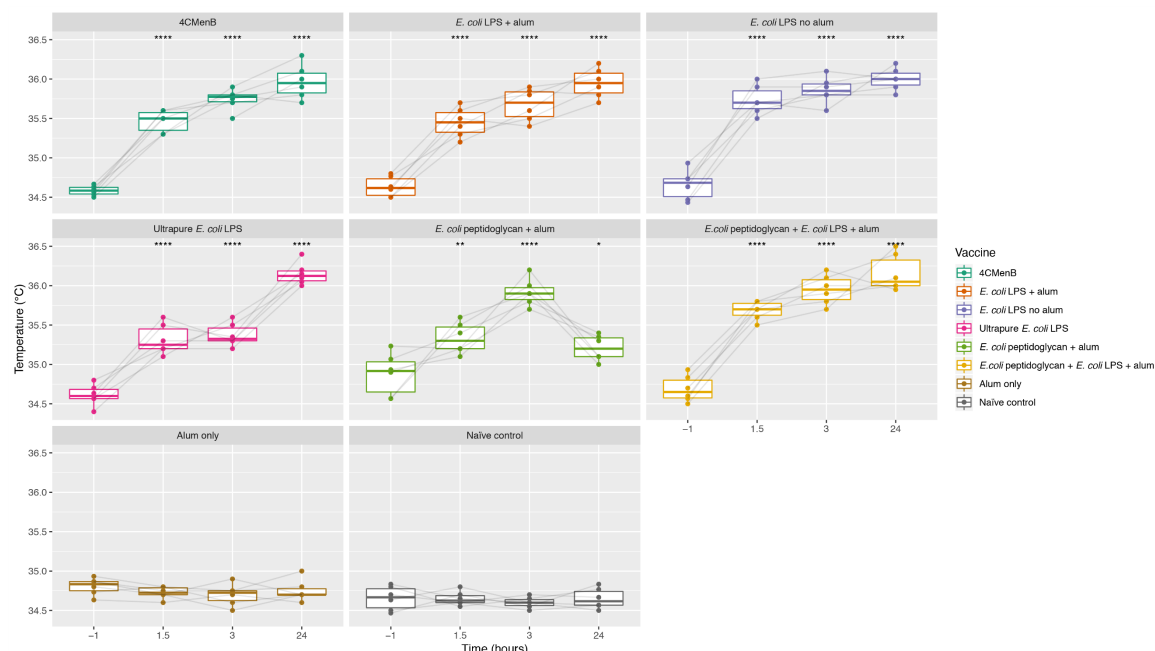


Figure 4.28. Changes in mouse surface temperature associated with a variety of preparations of Gram-negative bacterial outer membrane ligands. Boxplots depicting the change in temperature from baseline to 24 hours after the second 1 µg dose of each of a variety of *Escherichia coli* lipopolysaccharide (LPS) and peptidoglycan preparations, with and without alum, and combinations compared with 1/5 of the human dose of 4CMenB. Vaccine group medians at each timepoint were compared with baseline for that vaccine using a Wilcoxon signed-rank test. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

Blood samples were taken 24 hours after the second dose of antigen and RNA was extracted for qPCR analysis. The expression of the selection of innate immune response genes found among the top significantly DEGs following 4CMenB immunisation were validated as DE relative to a naïve control group (Fig. 4.29). The expression levels of these genes induced by *E. coli* LPS were dampened by the adsorption of this antigen on alum. Despite this effect of alum on LPS, most of

these genes were more significantly DE in the alum-adsorbed ultrapure LPS-immunised group suggesting that non-endotoxin ligands in the original LPS preparation may be reduce the immunostimulatory capabilities of this endotoxin. It is surprising that the ultrapure LPS preparation also stimulates the expression of non-TLR4 PRRs, but this may be due to the activation of immune cells in whole blood subsequent to the initial induction of the TLR4 cascade. These expression changes cannot be explained by the inclusion of alum as only a relatively low level of transcriptional activity was observed for these genes in the alum only group. The most interesting observation from this experiment is the dramatic increase in *Nlrp3* and *Il1b* expression triggered by the combination of peptidoglycan and LPS. This suggests a synergistic effect of these bacterial cell wall components on the activation of the NLRP3 inflammasome to release IL-1 β . Taken together, the findings of the temperature readings and qPCR analysis indicate that, while endotoxin appears to be the main driver of the temperature increases observed in mice and has been demonstrated earlier in this chapter as the cause of 4CMenB- and OMV-induced fever, other bacterial PAMPs present on these preparations may further stimulate immune pathways that contribute to fever in mice.

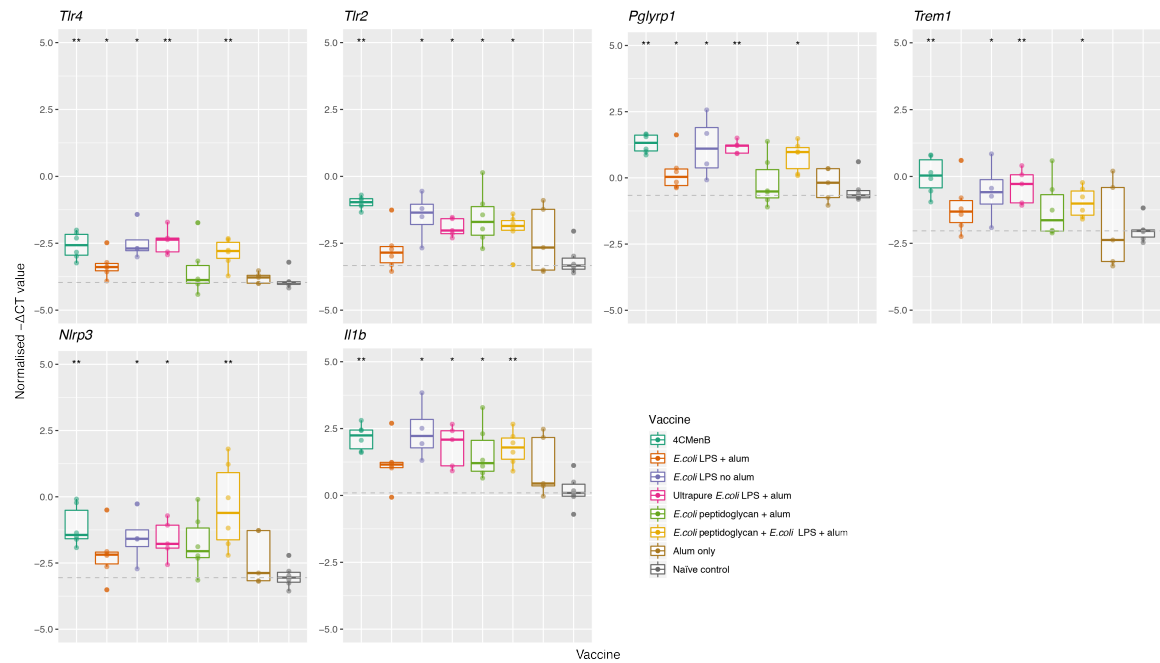


Figure 4.29. Expression of a selection of genes associated with the early transcriptional response to 4CMenB following a second dose of a variety of preparations of Gram-negative bacterial outer membrane ligands. Normalised (Δ) threshold cycle (C_T) values were calculated by subtracted the mean C_T value for the reference gene (*Pgk1*) from the target gene C_T values, averaged across triplicates. These values are plotted as negative ΔC_T to demonstrate their logical relation to the naïve control group. The horizontal line corresponds to the median of the naïve control group for each target gene, values above this line are considered upregulated and those below are considered downregulated, relative to the naïve control. Significance values were calculated between the test and naïve control groups using a Mann-Whitney U-test. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

4.4 Discussion

This section presents a comprehensive analysis of the temperature, cytokine, and gene expression changes observed in mice in response to 4CMenB, administered concomitantly and on its own, and in response to its constituent antigens or several comparator antigens. The temperature data demonstrate that the human observation of exacerbated fever resulting from concomitant administration of 4CMenB with routinely-administered vaccines can be recapitulated in a mouse immunisation model. The comparison of the individual 4CMenB components, and the inclusion of OMV and LPS comparator groups provide further evidence that the endotoxin-containing OMV component of the vaccine is the causative agent of febrile responses. 4CMenB elicits a substantial perturbation in the mouse blood transcriptome 24 hours after the second dose of vaccine. Furthermore, similar changes in the expression of genes involved in immune regulation occurred in mice receiving OMVs or LPS indicate that endotoxin is the likely driver of inflammation-induced responses, with noticeable enrichment of neutrophil-related genes, subsequently characterised through single-cell analysis. Finally, the systemic responses to 4CMenB and OMVs manifest in gene expression changes in brain tissue indicating the mechanism by which 4CMenB generates the febrile response. Taken together, these data attribute 4CMenB reactogenicity to the outer membrane composition of its constituent OMVs, providing evidence at each step of fever generation and supported by longitudinal temperature measurements.

The increased rate of fever associated with concomitant 4CMenB immunisation compared with routine vaccines or 4CMenB administered on its

own has been reported in several studies ^{202,203}. The fever data from the EUCLIDS study is also consistent with these findings, with ~70% of infants experiencing a temperature of $\geq 38^{\circ}\text{C}$ during the first 24 hours after the second dose of vaccine compared with ~38% of those receiving routine immunisations only (unpublished observations). A significant rise in surface temperature, beginning at approximately three hours after the second dose of immunisation and rising by close to 2°C by 24 hours, occurred in mice receiving 4CMenB concomitant with routine immunisations. No significant rise in temperature was associated with routine immunisations only, in contrast with an increase reported in some infants ²⁰². This may be a result of the reduced dose (section 4.3.1) used for mouse immunisations. The rise in temperature in mice receiving 4CMenB could be attributed to the endotoxin present in the OMV component of the vaccine. A similar rise in temperature was observed in mice immunised with other OMV-containing vaccines, though to a lesser degree in H44/76 lpxL1 nOMV-immunised mice. In its hexa-acylated lipid A form, endotoxin is a potent TLR4 ligand. Inactivation of the *lpxL1* gene results in the biosynthesis of an attenuated penta-acylated endotoxin that is known to substantially reduce pyrogenicity ^{355,356}, though this form of endotoxin still activates TLR4 in mice ¹⁷². The rise in temperature associated with the dOMV vaccines was on par with that of the *E. coli* LPS positive control group, while the protein in alum and MVA viral control vaccines induced only a mild rise in temperature in mice relative to baseline. The attenuated endotoxin contained in the lpxL1 nOMVs does not induce a significant rise in temperature. These data suggest that the febrile response to 4CMenB and OMV vaccines is indeed driven by the hexa-acylated lipid A motif found in the endotoxin of wild-type OMV-derived preparations.

There has been much debate as to the validity of comparing the genomic responses of mouse models of infection with those of humans in response to various immune stimuli based on correlation analyses performed on microarray data ^{357–360}. Valid points were raised on both sides of the argument, emphasising the relative merits and caveats of the use of such models to understand human responses ³⁶¹. An ImmGen consortium study of mouse and human cell lineages found widespread conservation between the gene expression patterns of most orthologs and, to a lesser degree, the transcriptional regulatory mechanisms ³⁶². More recently, the evolutionary conservation of innate immune responses at the gene expression and regulatory level have been compared between humans, mice, and other species, shedding light on the transcriptional convergence of the mouse and human LPS response at the bulk and single-cell level ³⁶³. Consistent with studies comparing the adult immune response to similar stimuli with those of mice at the same timepoint and using the Spearman correlation of LFCs between expressed orthologs, we observed a strong association between the mouse and human infant whole blood gene expression changes induced by concomitant 4CMenB ^{358,361,363}, demonstrating that mice undergo a similar transcriptional response to immunisation and providing further rationale for our use of a mouse immunisation model. Further exploration of the 4CMenB transcriptional response in mice and a comparative analysis of its components and other related vaccines/antigens revealed a gene signature defined by innate signalling pathway and neutrophil-specific genes that distinguished the 4CMenB and OMV groups from the recombinant protein and viral control groups, suggesting that these components induce two very different types of responses at the transcriptional level. The presence of genes encoding receptors recognising bacterial PAMPs, *Tlr2*, *Pglyrp1*, *Tlr4*, *Tlr6*, and *Trem1*, among the most significantly upregulated genes in

response to 4CMenB, with and without routine immunisations, and OMV vaccines indicate that these vaccines/antigens stimulate activation of immune cells in a manner similar to bacteria. Surprisingly, *E. coli* LPS did not induce TLR4 expression to the same degree as endotoxin-containing vaccines and appeared to activate TLR2 and other PRRs. One of the purposes of the adsorption of OMV-containing vaccines on alum is to minimise the amount of free/non-membrane-bound endotoxin, so it follows that adsorbing LPS directly on alum may dampen TLR4 stimulation ¹⁵⁸. The activation of TLR2 and other PRRs by LPS is likely due to the fact that the commercial preparation used is known to contain contaminant PAMPs from the bacterial cell wall and outer membrane, including peptidoglycan ³⁶⁴. Interestingly, TLR2 and PGLYRP-1 are both activated by peptidoglycan, and in its peptidoglycan-bound form, PGLYRP-1 also activates TREM-1 ^{365,366}. TREM-1 has been identified as an amplifier of endotoxin-induced septic shock, suggesting a potential mechanism for the synergistic activation of pyrogenic pathways through endotoxin and peptidoglycan innate signalling pathways ³⁴⁹. The signalling events initiated by the binding of these bacterial PAMPs were further reflected by the expression of the gene encoding the NLRP3 inflammasome, *Nlrp3*, and the expression of *Il1b* which encodes the highly pro-inflammatory cytokine produced by inflammasome activation ³⁶⁷. While the NLRP3 inflammasome is also known to be triggered by alum, we did not see the same degree of upregulation in the protein-in-alum groups, indicating that it is mainly triggered by the OMVs ³⁶⁸. The expression of these genes following 4CMenB and *E. coli* LPS in alum was validated and compared with different preparations and combinations of LPS. The expression of *Tlr4* was indeed dampened by alum and possibly the association of LPS with other bacterial outer membrane components. Interestingly, the combination of LPS with peptidoglycan appears to induce a synergistic activation

of the NLRP3 inflammasome, which supports a hypothesis that other OMV contaminants may promote inflammation and fever.

The differences between mouse and human innate immune cell populations and their relative abundances are well characterised; mice possess a higher proportion of lymphocytes in peripheral blood, 75–90% compared with 30–50%, while in humans neutrophils make up a greater proportion of circulating cells, 50–70% compared with 10–25% ³⁶⁹. The high representation of neutrophil-specific genes among the top DEGs in the mouse whole blood transcriptomics analyses, and the consistency of this finding with what was observed in the human infant study despite the discrepancy between typical abundances of this cell type, encouraged further exploration of vaccine-specific responses in these cells. These genes included *Ngp*, *Lcn2*, *Lrg1*, *S100a8*, *Mmp8*, *Fpr1*, and *Ltb4r*, all of which have roles in neutrophil migration, anti-bacterial responses, and differentiation, were found to be among the top 30 most variable genes in the mouse data and were all upregulated in the 4CMenB and OMV groups. This cell type also constituted the most significantly enriched fraction of whole blood following the administration of 4CMenB with or without routine immunisations, based on *in silico* deconvolution of gene signatures related to mouse immune cell populations. As neutrophils are considered to be quite transcriptionally inactive, few studies have examined the gene expression changes in neutrophils as they are short-lived and differentiated cells ³⁷⁰. The studies that have been conducted document transcriptional responses of neutrophils to infection. These studies used neutrophils isolated from human donors and stimulated *ex vivo* by microorganisms and stimuli including *E. coli*, *Candida albicans*, LPS, TNF α , and granulocyte-macrophage colony-stimulating factor (GM-CSF) for subsequent microarray or RNA-seq transcriptional profiling ^{371–374}. An RNA-seq study of neutrophils isolated from zebrafish infected with

Mycobacterium marinum, used to model tuberculosis, found several genes involved in NLRP3 inflammasome activation and IL-1 β production to be significantly upregulated ³⁷⁵. Single-cell pipelines afford a higher resolution of cell population analysis than bulk sequencing methods. Neutrophils are underrepresented in scRNA-seq data as there are technical difficulties associated with their isolation, stability, and preservation that existing pipelines are not yet fully suited to handling ³⁷⁶. Despite this, neutrophils isolated from mice in the present study were sequenced using the 10x platform to assess neutrophil-specific transcriptional responses to immunisation. The neutrophils sequenced in the present study yield information about the neutrophil-specific response to immunisation and indicate transcriptional divergence at the single-cell level in mice who received concomitant administration of 4CMenB when compared with the routine group. It is interesting to note that the two genes responsible for the clustering of OMV groups in the whole blood transcriptomic PCA, *Lcp1* and *Plek*, were among the most expressed genes in the concomitant group neutrophils, indicating that the OMV component of 4CMenB may be responsible for this transcriptional activity.

The release of cytokines from stimulated human whole blood and peripheral blood mononuclear cells (PBMCs) have been compared for dOMVs and lpxL1 nOMVs as a means of measuring reactogenicity *in vitro* ³⁷⁷. The human whole blood assay was capable of differentiating between the hexa- and penta-acylated forms of the endotoxin in these preparations based on TNF α and IL-6 production, suggesting the relevance of other immune cells in the production of reactogenic cytokines ³⁷⁷. A more recent study evaluated the released of cytokines and PGE₂ by human adult and neonatal whole blood and a dendritic cell assay upon stimulation

with 4CMenB, wild-type OMVs, dOMVs, lpxL1 nOMVs, BCG, DTwP (whole cell)-HBV-Hib, and the Hib vaccine ³⁷⁸. They found lower levels of pro-inflammatory cytokines, including TNF α , IL-6, and IL-1 β produced in response to lpxL1 nOMVs than the paediatric vaccines or other OMVs, and also demonstrated reduced production of IL-1 β and PGE₂ from dendritic cells upon lpxL1 nOMV stimulation ³⁷⁸. While a significant increase in IL-6 was detected in the sera of mice immunised with 4CMenB in the present study, its constituent components, and the comparator OMVs relative to naïve control mice, this response could not distinguish between the lpxL1 nOMVs and the dOMVs tested. This observation can be explained by the species-specific effects of lpxL1 nOMVs – the attenuation of the pro-inflammatory cytokine response is known to be less pronounced for mouse cells than human cells ¹⁷². Higher levels of IL-6 were observed in the sera from the 4CMenB and OMV groups compared with the protein in alum groups. Furthermore, this was the only cytokine found to be significantly different between 4CMenB + routine, routine only, and PBS control groups, with higher levels detected in the concomitant group indicating a possible cumulative effect of this cytokine induced by vaccine combinations. Levels of TNF α and IL-1 β were also low in sera from all groups, with only the NZ98/254 group showing a statistically significant increase in the former. Significant levels of IL-3 and IL-5 were detected in the fHbp group. These cytokines, along with GM-CSF, make up a cytokine family that has roles in the differentiation, recruitment, and growth of immune cells, and are characteristic of a T_H2 response ³⁵⁴. High levels of IL-5 were also detected in the concomitant and routine groups, perhaps owing to the presence of fHbp in both of these combinations.

The stimulation of cognate receptors on BECs at the blood-brain-barrier by the circulating pro-inflammatory cytokines IL-6 and IL-1 β is known to induce prostaglandin synthesis and is the accepted model of inflammation-induced fever (summarised in Fig. 4.30) ⁵⁰. TNF α also contributes to inflammation-induced fever by stimulating the production of other cytokines ⁵³. The expression of the receptors for these pyrogenic cytokines and the key PGE₂-synthesising enzymes on BECs was quantified. Significant upregulation of mPGES-1 and COX-2 on BECs from 4CMenB- or OMV/LPS-immunised mice indicates that these antigens elicit the strongest indicators of fever at the neurological level. These results are consistent with a study examining the BEC transcriptional response to LPS-induced fever where the expression of the genes *Il1r1*, *Il6ra*, *Ptges*, and *Ptgs2* were significantly upregulated ³⁷⁹. Contrary to their findings however, we found the TNF α receptor genes *Tnfrsf1a* and *Tnfrsf1b* to be significantly downregulated in response to 4CMenB or OMV-immunisation, suggesting that this cytokine either induces downregulation of its receptors through a homeostatic response or plays less of a role in the mediation of the febrile responses to these vaccines that are driven mostly by IL-1 β and IL-6.

These data show that a mouse model is suitable for the study of reactogenicity of vaccines with comparable increases in fever with concomitant 4CMenB immunisation, as compared with routine immunisation, and corresponding transcriptomic changes. Furthermore, the rise in temperature associated with this vaccine can be attributed to the endotoxin-containing OMV component of the vaccine and not the recombinant proteins. High-throughput sequencing allowed for the identification of a distinct bacterial gene expression signature that distinguished the transcriptional response to OMVs from that of the

other 4CMenB constituents. This response is primarily elicited by the endotoxin found in these vaccines, but could also be exacerbated by other bacterial outer membrane antigens, and results in the upregulation of neutrophil-specific inflammatory markers. The systemic responses induced by 4CMenB and wild-type OMVs are transduced at the blood brain barrier, manifesting in the upregulation of PGE₂ synthesis genes that mediate the final stages of fever induction. These data demonstrate that mice immunised with OMV-containing vaccines exhibit the immunological hallmarks of fever and the presence of hexa-acylated endotoxin determines their pyrogenicity.

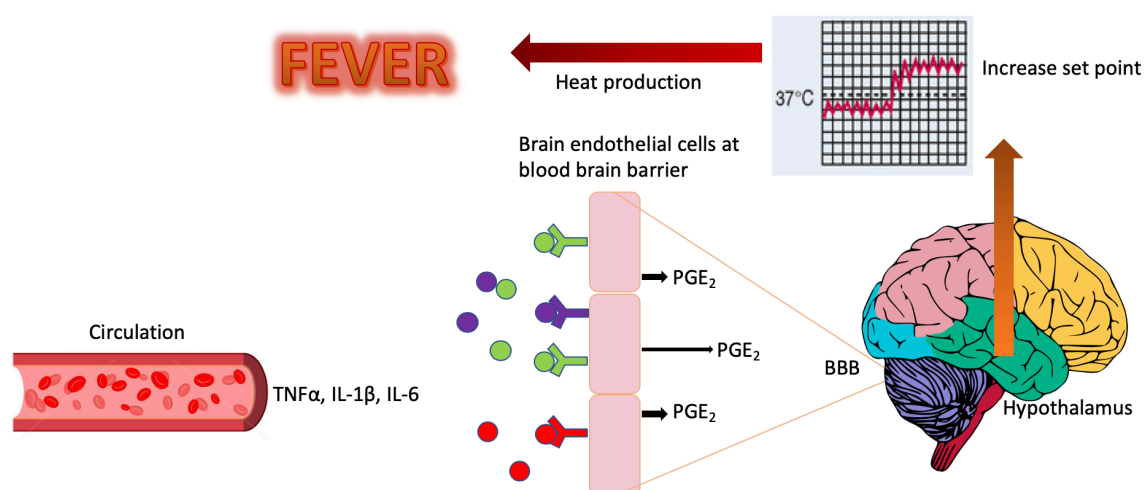


Figure 4.30. Schematic of the mechanism of vaccine-induced fever. Pro-inflammatory cytokines, or ‘endogenous pyrogens’, are released into the circulation from cells responding to vaccine components. These cytokines, including TNF α , IL-1 β and IL-6 reach the brain endothelial cells (BECs) at the blood-brain barrier (BBB) where they bind their cognate receptors. Receptor binding triggers prostaglandin E₂ (PGE₂) synthesis in these cells via microsomal prostaglandin E synthase-1 (mPGES-1) and cyclooxygenase-2 (COX2). PGE₂ then acts on the hypothalamus in the brain to increase the thermoregulatory set point leading to heat production and fever.

Chapter 5

Assessing the immune mechanisms underlying a novel viral vectored vaccine against the capsular group B meningococcus

5.1 Introduction

As discussed in section 1.2.3, efforts to develop a preventive MenB vaccine have focused primarily on the identification of immunogenic surface-exposed proteins, one of which, fHbp, is the target of immune response for two licensed vaccines, 4CMenB and MenB-fHbp. Both vaccines are associated with post-immunisation reactogenicity, particularly when administered to infants; 4CMenB is reactogenic when administered alone or concomitant with other routinely-administered vaccines, while the study of MenB-fHbp in infants was prematurely terminated due to the high incidence of fever in participants ¹⁶⁹. These vaccines also require multiple doses to achieve sufficient protection ³⁸⁰, rendering a 4CMenB immunisation campaign not cost-effective for the adolescent population in the UK ²⁰⁷, and there is some concern about the long-term persistence of SBA titres ^{381–383}. Efforts are being made to research and develop alternative MenB vaccines that are better tolerated and capable of inducing early and persistent protection across age groups and increase the breadth of protection. These factors are crucial to ensure that MenB vaccine uptake remains high, that MenB

immunisation campaigns become affordable enough for more countries to adopt them, and that herd immunity can be achieved, bringing disease eradication within closer reach.

fHbp as a MenB vaccine antigen was discussed in section 1.2.3C. It has the advantage of being surface-exposed on the bacterium and a variant is expressed by the vast majority of invasive strains. A non-lipidated variant from subfamily B (fHbp 1.1) was chosen for 4CMenB³⁸⁴, expressed in 17% and 30% of disease isolates from Europe and the US, respectively^{182,385}. Two lipidated variants, one from each subfamily, were chosen for MenB-fHbp with the aim of broadening the coverage of the vaccine³⁸⁶. The lipidation of the MenB-fHbp antigens is produced by cloning the fHbp gene such that the signal sequence of the non-typeable *Haemophilus influenzae* P4 lipoprotein is included ahead of the fHbp lipoprotein signal sequence on the recombinant plasmid construct¹⁷⁸. These lipidated variants demonstrated enhanced induction of bactericidal antibody compared with their non-lipidated counterparts¹⁷⁸, demonstrating the impact of lipidation on the immunogenicity of bacterial antigens.

A novel MenB vaccine has been developed at OVG that consists of a recombinant, replication deficient ChAdOx1 viral vector (introduced in section 1.3.2) encoding the immature form of the fHbp antigen that includes the complete original sequence, which comprises a bacterial lipobox domain (LTAC) at the C-terminus of a signal peptide (Fig. 5.1). The construct also contains a second signal sequence: a mammalian tissue plasminogen activator (tPA) signal peptide at the N-terminus of the fHbp signal sequence, to ensure addressing the resulting protein to the secretory pathway within the mammalian cells³⁸⁷. The fHbp sequence also contains a point mutation to decrease the affinity of the antigen to human factor H, as this was demonstrated to elicit higher titres of protective antibody in

macaques ³⁸⁸. In pre-clinical studies, the vaccine induced early, strong, and persistent protective SBA titres in mice shortly after a single dose of vaccine (unpublished observations). Based on extensive pre-clinical assessment of immunogenicity, the ChAdOx1-fHbp vectored vaccine candidate has entered a phase I trial to assess its safety and immunogenicity in humans and to compare it with the licensed 4CMenB and MenB-fHbp vaccines. In pre-clinical studies, this vectored vaccine also induced higher functional antibody titres compared with a vector encoding the mature form of the fHbp 1.1 antigen, without the signal sequence (unpublished observations). To determine the specific element of the bacterial signal sequence, from here on referred to as the “full-length (FL) signal sequence (SS)”, that contributes most to this stronger and more persistent functional antibody response, a panel of fHbp 1.1 N-terminal amino acid sequence variant plasmids with distinct deletions or mutations were designed, cloned, and incorporated into AdHu5 vectors for *in vitro* and *in vivo* analyses (Table 1). The FL SS fHbp construct contains the mammalian signal peptide NRTAFCCLSLTALI and the bacterial lipobox motif LTAC, so variants lacking one or both of these sequences as well as single amino acid variants of these sequences were assessed side-by-side to determine their relative contributions to the enhanced functional immune response.

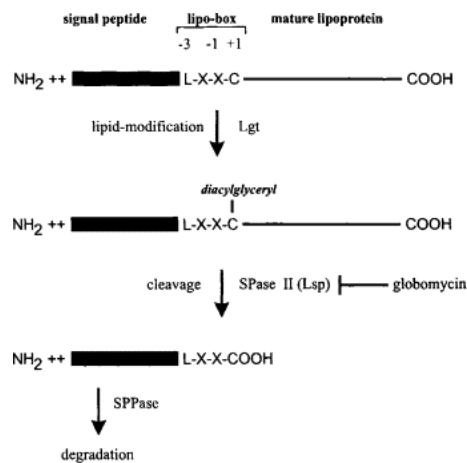


Figure 5.1. Stages of signal peptide cleavage from bacterial lipoproteins. The invariant cysteine (C) residue of the prelipoprotein lipobox (LXXC) is post-translationally modified by diacylglyceryl transferase (Lgt). This facilitates recognition of the prelipoprotein by signal peptidase (SPase) II. The cleaved signal peptide (SP) is broken down by SP peptidase (SPPase). Positively-charged amino acids are denoted with a '+'. NH₂, amino/N terminus; COOH, carboxyl/C terminus. From Tjalsma *et al.*, 2002 ³⁸⁹.

Construct name	N-terminal signal sequence
Full-length signal sequence – fHbp 1.1	NRTAFCCLSLTTALILTACSSG
Truncated signal sequence – fHbp 1.1	G
C to A mutation full-length signal sequence – fHbp 1.1	NRTAFCCLSLTTALILTAASSG
Lipobox KO signal sequence – fHbp 1.1	NRTAFCCLSLTTALI SSG
LTA KO signal sequence – fHbp 1.1	NRTAFCCLSLTTALI CSSG
Signal peptide (mammalian) KO signal sequence – fHbp 1.1	LTACSSG
Signal peptide (mammalian) and LTA KO signal sequence – fHbp 1.1	CSSG
Full-length signal sequence with methionine – fHbp 1.1	MNRTAFCCLSLTTALILTACSSG

Table 5.1. Panel of factor H binding protein signal sequence variants for comparative assessments of immunogenicity. N-terminal amino acid “signal sequences” for factor H binding protein (fHbp) 1.1 transgenes encoded by human adenovirus serotype 5 viral vector vaccines. The signal peptide portion of the sequence is highlighted in red, the LTA portion of the lipobox in green, the lipobox cysteine in blue, the N-terminal methionine in purple, and the SSG amino acids that remains following the natural cleavage of the signal peptide in black. KO, knockout.

The present chapter focuses on the comparative assessment of SS variants using a combination of *in vitro* expression assays and *in vivo* immunogenicity data from mice to determine the link between transgene expression and the enhanced functional antibody response associated with the candidate vaccine antigen. In addition, the potential application of this SS to other bacterial antigens is explored with a view to developing a generalisable sequence to boost the immunogenicity of viral vectored vaccines currently undergoing pre-clinical development.

5.2 Methods

5.2.1 Animal procedures

The procedures for mouse immunisations, bleeds, and spleen harvesting are detailed in sections 2.2.1, 2.2.2, and 2.2.6.

2.5 Molecular biology

The molecular biology techniques applied in this chapter include plasmid construct design, restriction digest, agarose gel electrophoresis, DNA ligation, transformation, DNA extraction, and cloning into adenovirus. The protocols for these methods can be found in sections 2.5.1–2.5.7.

2.6 *In vitro/ex vivo* assays

Protocols for the expression assays, indirect ELISAs, SBA and FluoroSpot assays can be found in sections 2.6.2–2.6.5.

2.7 Cell imaging

Details of the confocal microscopy and time-lapse imaging procedures are given in sections 2.7.1 and 2.7.2, respectively.

5.3 Results

5.3.1 Factor H binding protein N-terminal signal sequence variant transgenes exhibit differential expression levels at early timepoints *in vitro*

Expression of the variants at early timepoints was assessed by infecting 1×10^6 HeLa cells overnight with 5×10^8 infectious units of AdHu5 vaccines encoding one of each of the fHbp SS variants outlined in Table 1 and measuring fHbp expression by flow cytometry. The signal peptide (SP) knockout (KO) SS construct was expressed by a higher percentage of cells (25-30% of cells) after overnight infection, while the truncated SS fHbp was expressed by <10% of cells (Fig. 5.2). This implies that the lipobox (LTAC) portion of the SS is sufficient to induce the difference in early antigen expression levels observed between the FL and truncated SS fHbp constructs. The expression of the lipobox KO SS construct implies that the mammalian signal peptide does boost expression levels relative to the truncated SS fHbp, but not to the same degree as the lipobox itself. The LTA amino acids KO indicates that the C residue is important for this increase in expression, further evidenced by the low expression levels observed for the C to A mutation SS construct. However, knocking out both the signal peptide and the LTA portion of the lipobox results in a similarly low level of expression, highlighting the importance of an intact lipobox for boosting expression. The inclusion of a methionine (M) amino acid at the beginning of the bacterial native signal sequence, indicating a start codon at this position, also appears to negatively impact upon expression despite an otherwise unaltered SS.

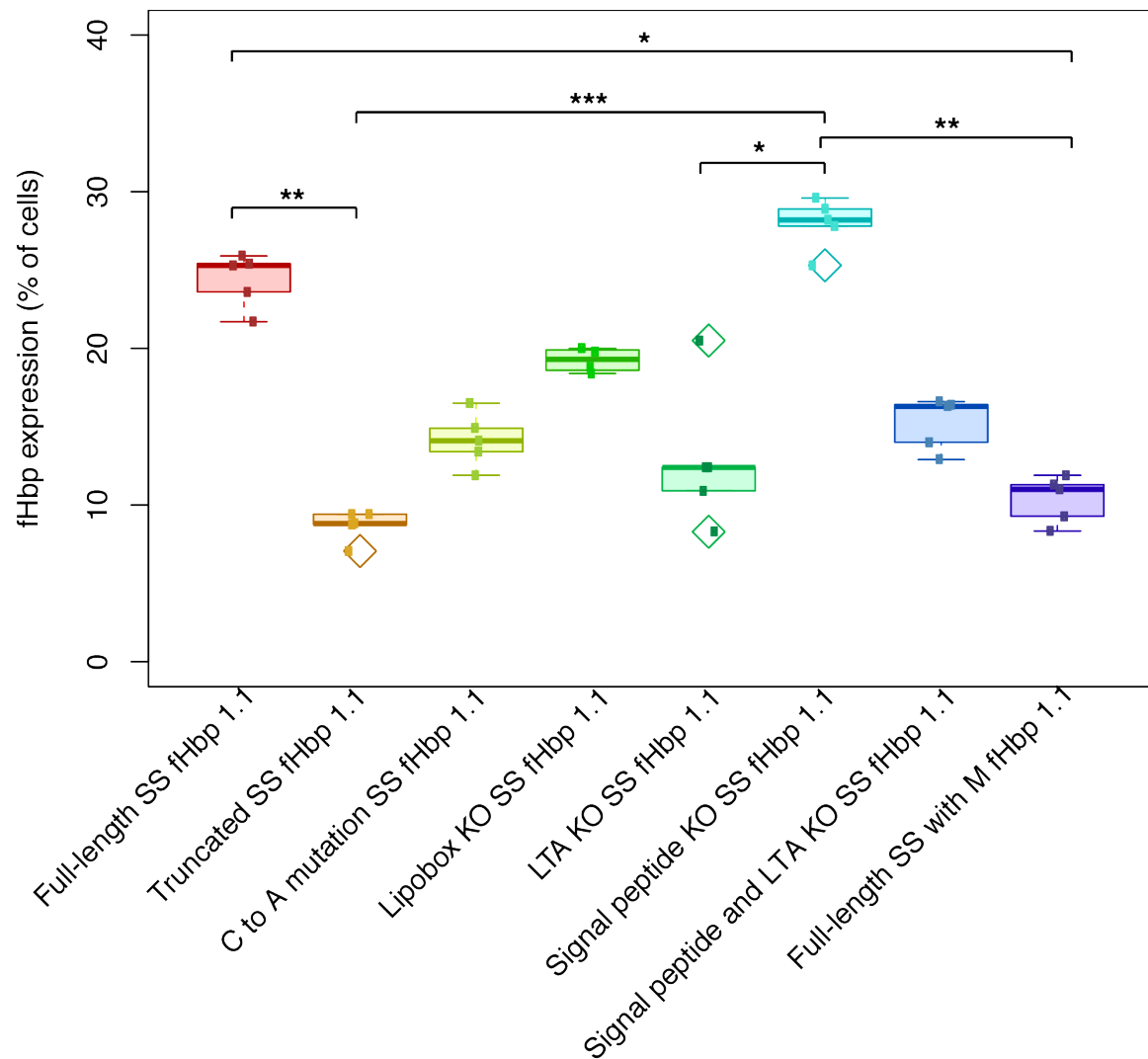


Figure 5.2. Overnight expression of human adenovirus serotype 5-encoded factor H binding protein N-terminal sequence variants from HeLa cells. HeLa cells (1×10^6 per sample) were infected overnight with 5×10^8 infectious units of one of a series of human adenovirus serotype 5 (AdHu5) constructs encoding an N-terminal sequence variant of the factor H binding protein (fHbp) and expression was quantified by flow cytometry after surface and intracellular staining of harvested cells with an anti-fHbp antibody (JAR5) and a fluorescently-tagged detection antibody. The y-axis corresponds to the percentage of total fluorescent (fHbp-expressing) HeLa cells after overnight infection. The amino acid composition of the N-terminal sequence impacts upon the expression of the transgene-encoded antigen within the first 24 hours of infection. Statistical comparisons were made using a Mann-Whitney U-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

By comparing the percentage of cells expressing fHbp after surface-only and intracellular-only staining, the intracellular expression of the antigen was shown to contribute to the majority of total antigen expression after overnight infection (Fig. 5.3).

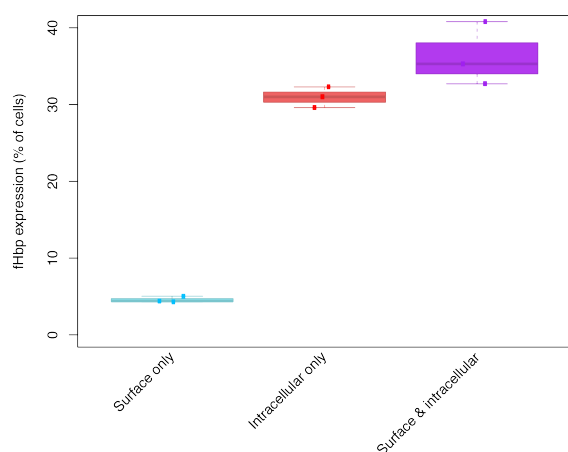


Figure 5.3. Intracellular and surface expression of human adenovirus serotype 5-encoded factor H binding protein with a full-length N terminal signal sequence on HeLa cells after overnight infection. HeLa cells (1×10^6 per sample) were infected overnight with 5×10^8 infectious units of a human adenovirus serotype 5 (AdHu5) construct

encoding the factor H binding protein (fHbp) with a full-length signal sequence and expression was quantified by flow cytometry after surface only, intracellular only, or both surface and intracellular staining of harvested cells with an anti-fHbp antibody (JAR5) and a fluorescently-tagged detection antibody. The y-axis corresponds to the percentage of total fluorescent (fHbp-expressing) HeLa cells after overnight infection. The vast majority of antigen is expressed within the cell at this timepoint.

To further examine the intracellular antigen expression levels at early timepoints, cells were treated with brefeldin A, an inhibitor that blocks protein transport to the Golgi apparatus causing protein to accumulate instead in the ER, and flow cytometry was performed hourly for 6 hours post-infection with a single AdHu5 construct (FL SS fHbp) to determine key timepoints for peak antigen expression (Fig. 5.4).

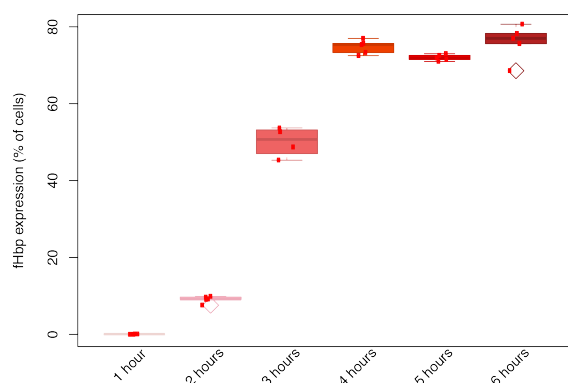


Figure 5.4. Time-course expression assay of human-adenovirus serotype 5-encoded factor H binding protein with a full-length N-terminal signal sequence on HeLa cells stimulated with brefeldin. A. HeLa cells (1×10^6 per sample) were stimulated with brefeldin A to stop protein transport within the cells and

subsequently infected with 5×10^8 infectious units of a human adenovirus serotype 5 (AdHu5) construct encoding the factor H binding protein (fHbp) with a full-length signal sequence and expression was quantified by flow cytometry after intracellular only staining of harvested cells with an anti-fHbp antibody (JAR5) and a fluorescently-tagged detection antibody. The y-axis corresponds to the percentage of total fluorescent (fHbp-expressing) HeLa cells after each hourly timepoint post-infection (x-axis) up to six hours. The antigen begins to be expressed at high levels at three hours post-infection and expression levels plateau around five hours.

Based on the expression levels measured, the three-hour timepoint appears to coincide with a window of increased antigen expression while expression appears to plateau at the five-hour timepoint for this construct. These timepoints were chosen for further comparisons of early antigen expression between the mutated constructs. HeLa cells were stimulated with brefeldin A, infected with each SS variant construct, and analysed by flow cytometry for intracellular expression of fHbp between three and five hours post-infection (Fig. 5.5). The FL SS fHbp construct was the only construct that expressed at high levels at both timepoints, while most constructs which expressed highly at three hours post-infection failed to maintain these expression levels at the five-hour timepoint (Fig. 5.5B). Several constructs which did not express at three hours post-infection showed modest levels of expression at the later timepoint. Surprisingly, the truncated SS fHbp construct expresses quite highly after three hours, but by five hours post-infection

its expression has declined. This suggests that the SS may play a role in promoting sustained expression of antigen within cells, or slows its degradation. Much like the truncated SS fHbp, the other variants that express highly at three hours post-infection decline over time. The LTA KO expressed at a consistently low level at each timepoint, indicating that the mammalian signal peptide portion of the SS does not appear to enhance the early expression of the antigen by itself in this mammalian cell line. The fact that the FL SS exhibits the most consistent levels of expression during these early timepoints, and the expression levels of each variant fluctuate between timepoints highlights variable contributions to the timing and persistency of antigen expression associated with each element of the entire SS, but strengthens the hypothesis that the inclusion of the entire SS may boost the immunogenicity of the fHbp antigen.

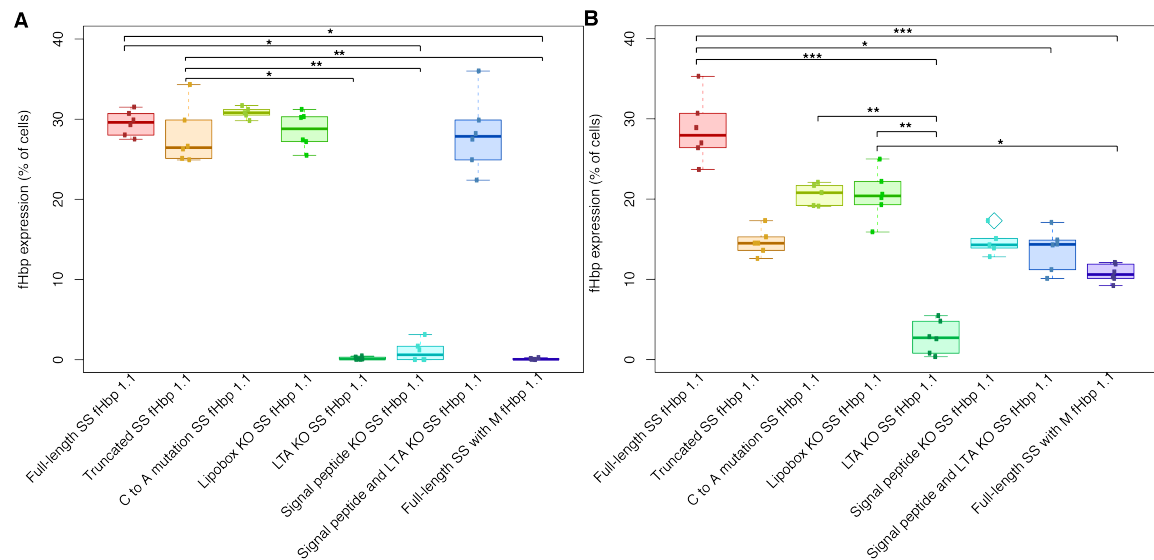


Figure 5.5. Intracellular expression levels of human adenovirus serotype 5-encoded factor H binding protein N-terminal sequence variants from HeLa cells at early timepoints post-infection. HeLa cells (1×10^6 per sample) were stimulated with brefeldin A to stop protein transport within the cells and subsequently infected with 5×10^8 infectious units of one of a series of human adenovirus serotype 5 (AdHu5) constructs encoding an N-terminal sequence variant of the factor H binding protein (fHbp). Cells were harvested at **A.** three-hour and **B.** five-hour timepoints post-infection to measure early expression levels by flow cytometry after intracellular only staining with an anti-fHbp antibody (JAR5) and a fluorescently-tagged detection antibody. The y-axis corresponds to the percentage of total fluorescent (fHbp-expressing) HeLa cells after infection. Each portion of the N-terminal signal sequence exhibits a variable contribution to the early expression of the fHbp antigen, but the full-length signal sequence induces early and sustained levels of expression. Statistical comparisons were made using a Mann-Whitney U-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

To assess whether the observed differential expression in HeLa cells was conserved in a cell line with more biological relevance to the *in vivo* situation, overnight infection was conducted in C2C12 mouse myoblasts, as muscle cells at the site of injection are the primary location of antigen expression following immunisation with adenovirus constructs. Most constructs expressed at much higher levels than in HeLa cells and the trend of differences in expression levels

was conserved across all constructs, with the notable exception of the FL SS, in C2C12 cells (Fig. 5.6). The FL SS fHbp construct had the lowest level of expression in C2C12 cells following overnight infection despite expressing at consistently high levels in HeLa infection assays. However, the percentage of cells expressing the FL SS fHbp antigen was relatively similar (~20% of cells) in C2C12 and HeLa cells, raising the possibility that the expression of this construct may be conserved between cell lines and species. This assay suggests that portions of the SS may exhibit cell- and/or species-specific differences in its ability to interact with the host cellular machinery to influence antigen expression. Taken together, the *in vitro* expression data indicate that slight variation on the amino acid sequence of the N-terminal SS can impact upon the cellular expression kinetics of the transgene-encoded antigen.

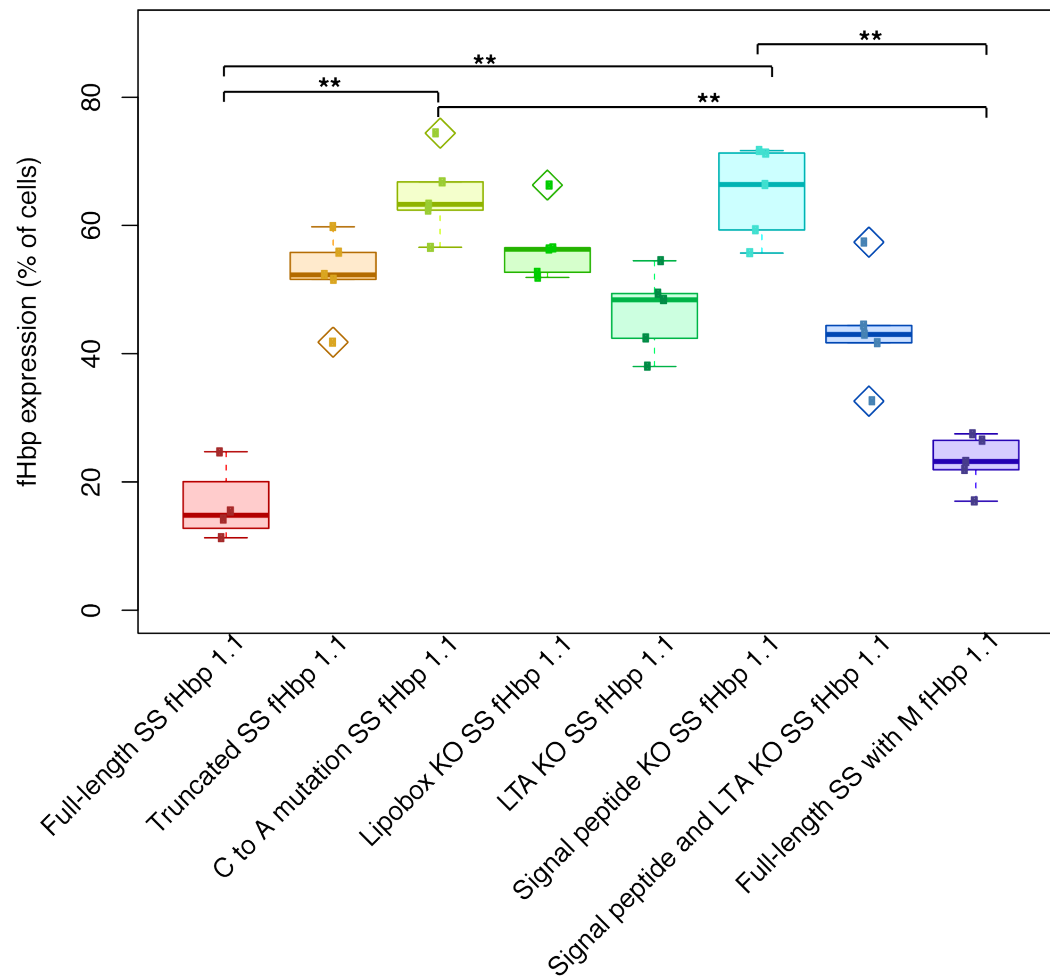


Figure 5.6. Overnight expression of human adenovirus serotype 5-encoded factor H binding protein N-terminal sequence variants from C2C12 mouse myoblast cells.

C2C12 cells (1×10^6 per sample) were infected overnight with a sub-optimal dose (1×10^7 infectious units) of one of a series of human adenovirus serotype 5 (AdHu5) constructs encoding an N-terminal sequence variant of the factor H binding protein (fHbp) and expression was quantified by flow cytometry after surface and intracellular staining of harvested cells with an anti-fHbp antibody (JAR5) and a fluorescently-tagged detection antibody. The y-axis corresponds to the percentage of total fluorescent (fHbp-expressing) C2C12 cells after overnight infection. The differences between the expression levels of all constructs is conserved between human HeLa and mouse C2C12 cell lines, with the exception of the full-length signal sequence construct, the trend for which is reversed. Statistical comparisons were made using a Mann-Whitney U-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

5.3.2 The amino acid composition of the N-terminal signal sequence influences the dynamics of antigen expression from HeLa cells by promoting early expression

As the *in vitro* expression assays alluded to differential contributions of distinct SS elements to the transgene expression kinetics, this phenomenon was further explored by selecting four SS variants – FL SS, SP KO SS, LTA KO SS, and SP + LTA KO SS – and employing microscopy techniques to visualise expression of these antigens in HeLa cells. To this end, plasmids encoding each of these antigen variants fused with eGFP via a flexible linker peptide were designed, cloned, and incorporated into AdHu5 vectors (section 2.5.7). To ensure accurate recapitulation of antigen expression, overnight expression from HeLa cells was compared between these eGFP fusion constructs and their corresponding original constructs (Fig. 5.7). The eGFP-expressing constructs were detected in a greater proportion of infected cells, possibly due to the greater natural fluorescence intensity of these antigens compared with the detection antibody-labelled antigens from the original constructs which may be less efficient. Overall, the trend in expression level differences was replicated for the eGFP constructs, confirming that the impact of the SS remains apparent even in the fusion antigens. The lower expression levels of the fHbp-eGFP fusion antigens compared with that of the eGFP only positive control confirms that the observed differences in expression between eGFP-containing constructs are due to the N-terminal fHbp SS variant antigens.

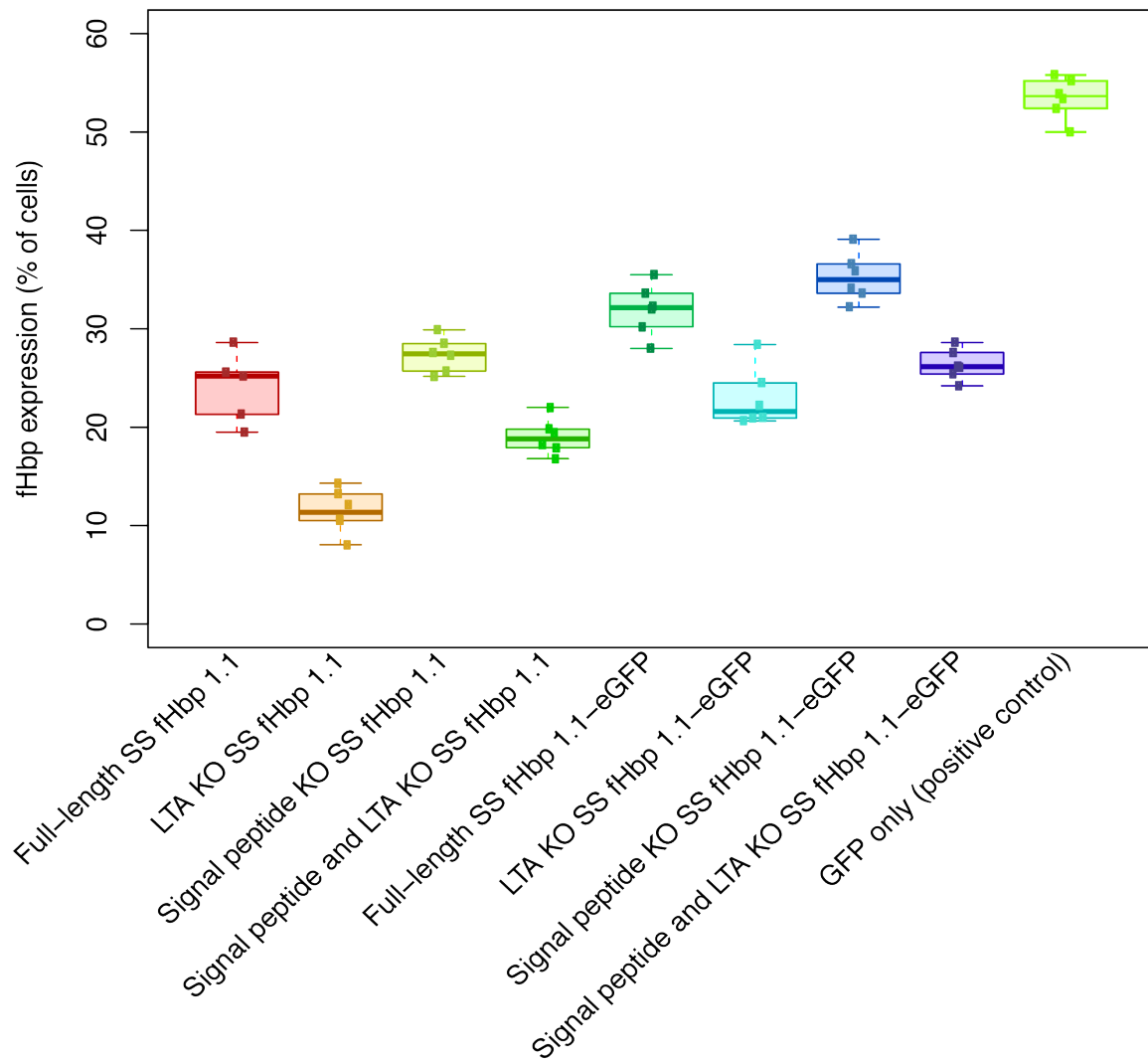


Figure 5.7. Side-by-side comparison of factor H binding protein N-terminal sequence variants, with and without enhanced green fluorescent protein tags, expressed from human adenovirus serotype 5 vectors after overnight infection HeLa cells. HeLa cells (1×10^6 per sample) were infected overnight with 5×10^8 infectious units of one of a series of human adenovirus serotype 5 (AdHu5) constructs encoding an N-terminal sequence variant of the factor H binding protein (fHbp), fHbp fused with enhanced green fluorescent protein (eGFP), or GFP only, and expression was quantified by flow cytometry after surface and intracellular staining of non-GFP-expressing cells with an anti-fHbp antibody (JAR5) and a fluorescently-tagged detection antibody. The y-axis corresponds to the percentage of total fluorescent (fHbp- and/or GFP-expressing) HeLa cells after overnight infection. The differences in expression levels between the antigen variants tested is conserved for the eGFP fusion antigens and lower for the eGFP fusion antigens than the eGFP only positive control, confirming that the differences in antigen expression levels are attributable to the fHbp N-terminal signal sequence.

Confocal microscopy was employed to visualise the expression of these fHbp-eGFP fusion antigens from AdHu5-infected HeLa cells. To relate the microscopy results to the flow cytometry data, 3×10^5 cells seeded on glass coverslips placed at the bottom of 6-well plates were first infected overnight with 1.5×10^8 IU (to obtain a multiplicity of infection (MOI) of 500) of each eGFP-expressing AdHu5 vaccine, and then fixed, DAPI-stained, and transferred to microscope slides for imaging using a Zeiss 780 inverted confocal microscope (section 2.7.1). In accordance with the relative differences in expression levels quantified for the four constructs as measured by flow cytometry, a similar pattern was observed in the confocal microscopy images (Fig. 5.8). All constructs displayed a greater intensity of eGFP expression relative to a negative control of uninfected HeLas (Fig. 5.8A), with the eGFP only construct expressing at the greatest intensity (Fig. 5.8B). The FL SS and SP KO SS constructs displayed similar levels of eGFP intensity (Fig. 5.8C and Fig. 5.8E, respectively), while both the LTA KO SS and SP + LTA KO SS constructs displayed similarly low levels of eGFP intensity (Fig. 5.8D and Fig. 5.8F, respectively). As accurate comparisons cannot be drawn by comparing individual fields from each sample between conditions, these results serve only as a visual confirmation that overnight expression of the fHbp-eGFP antigen could be detected by fluorescence microscopy.

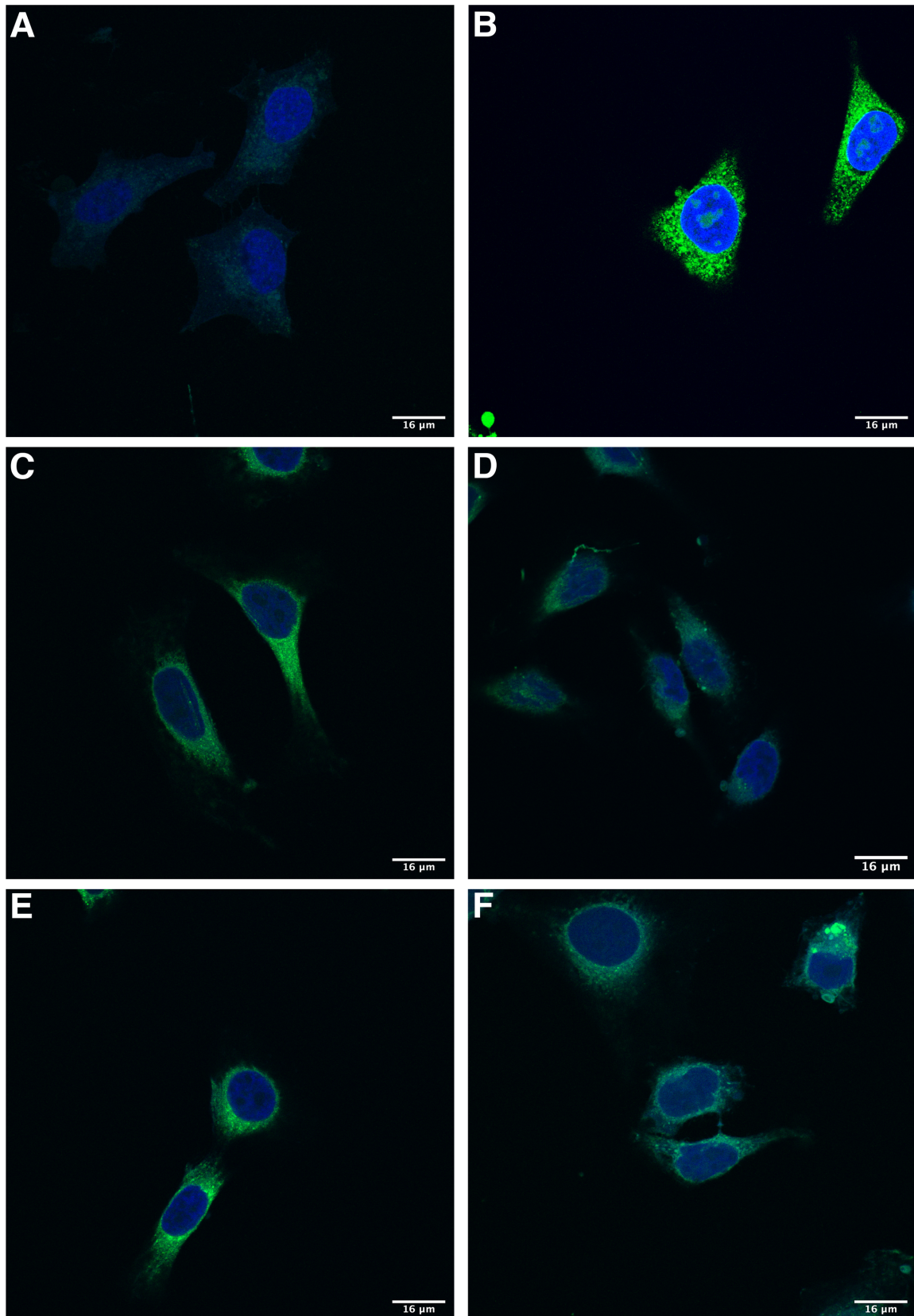


Figure 5.8. Confocal microscopy of HeLa cells infected overnight with human adenovirus serotype 5 expressing factor H binding protein N-terminal amino acid sequence variants fused with enhanced green fluorescent protein. HeLa cells were

grown overnight on glass coverslips placed in the wells of a six-well plate at a concentration of 3×10^5 cells per well. Cells were then infected overnight the following day with a proportional dose (1.5×10^8 infectious units) of human adenovirus serotype 5 (AdHu5) encoding one of the four factor H binding protein (fHbp) N-terminal signal sequence (SS) variants fused with enhanced green fluorescent protein (eGFP). Cells on the coverslips were fixed, 4',6-diamidino-2-phenylindole (DAPI)-stained and the coverslips were transferred to microscope slides for imaging on a Zeiss 780 inverted confocal microscope. Images were taken at multiple fields of vision and representative images for each construct are displayed here. **A.** Uninfected cells (negative control) **B.** eGFP only (positive control) **C.** Full-length SS fHbp 1.1-eGFP **D.** LTA knockout (KO) SS fHbp 1.1-eGFP **E.** Signal peptide (SP) KO SS fHbp 1.1-eGFP **F.** SP + LTA KO SS fHbp 1.1-eGFP. Blue fluorescence indicates DAPI-stained nuclei, green fluorescence indicates antigen-eGFP expression. 16 μm scale bars are shown in the bottom right corner of each image.

To compare the expression dynamics of these fluorescent antigen variants in a longitudinal manner, 1×10^5 HeLa cells were seeded overnight in each well of an eight-well chambered coverslip. The following day the cells were stained using far-red fluorogenic SiR-DNA, infected with 5×10^7 IU (to obtain an MOI of 500) of each eGFP-expressing AdHu5 vaccine, and imaged every ten mins over the course of 14 hours using a Zeiss Spinning Disc microscope (section 2.7.2). Time-lapse images for each infection were obtained across three separate fields for each well of AdHu5-infected cells (Fig. 5.9). Differences were observed between the timings of antigen expression between the constructs, particularly at early timepoints post-infection. The FL and SP KO SS variants displayed similar antigen expression kinetics. The SP + LTA KO SS variant was expressed at a marginally later time and at a lower level, while the LTA KO resulted in the lowest levels of antigen expression. These images confirm the *in vitro* antigen expression data and provide further evidence for the differential contribution of these specific SS elements to early antigen expression from mammalian cells.

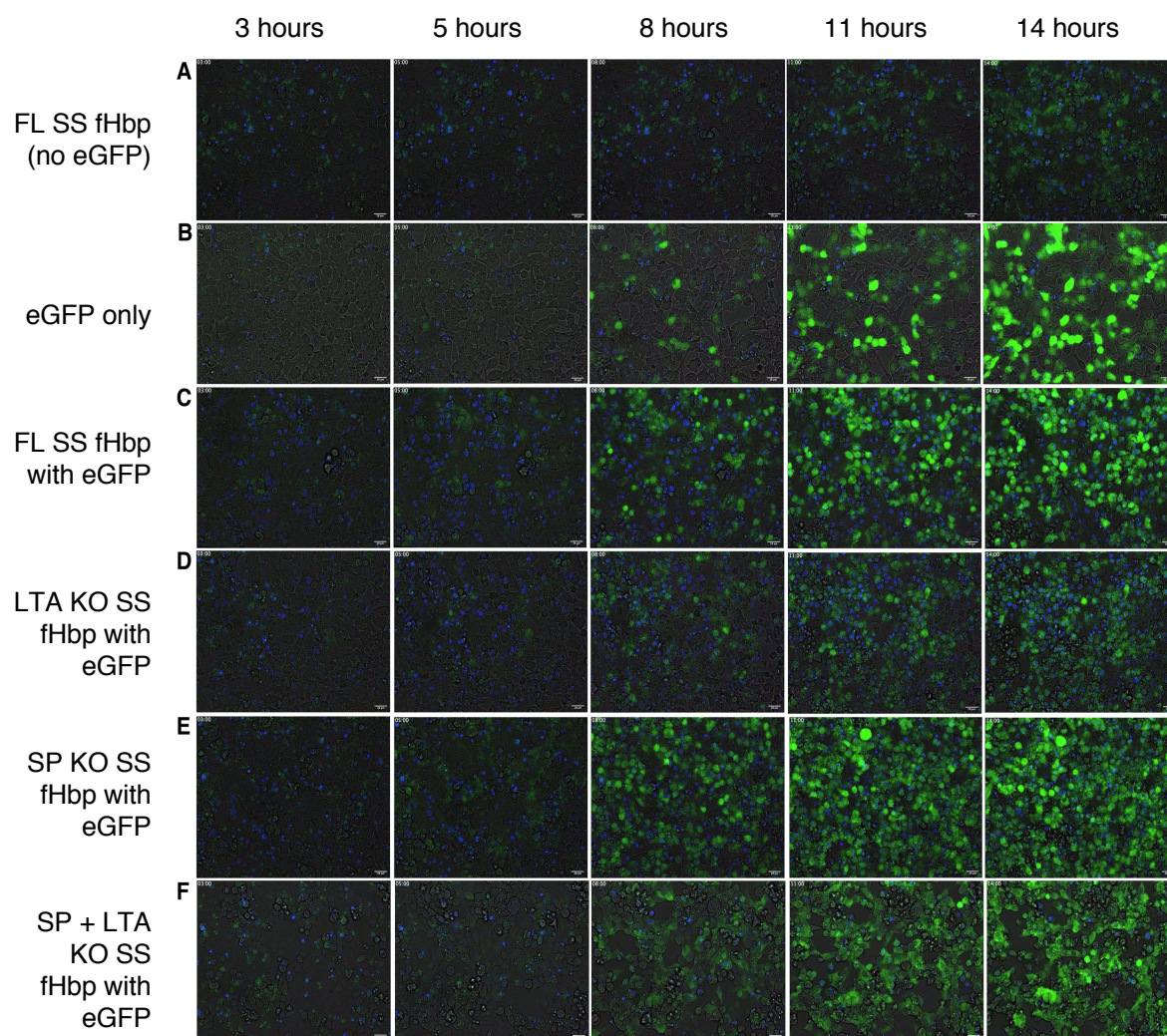


Figure 5.9. Time-lapse microscopy of HeLa cells over the course of a 14 hour infection with human adenovirus serotype 5 expressing factor H binding protein N-terminal amino acid sequence variants fused with enhanced green fluorescent protein. HeLa cells were grown overnight in an eight-well chambered coverslip at a concentration of 1×10^5 cells per well. Cells were then infected overnight the following day with a proportional dose (5×10^7 infectious units) of human adenovirus serotype 5 (AdHu5) encoding one of the four factor H binding protein (fHbp) N-terminal signal sequence (SS) variants fused with enhanced green fluorescent protein (eGFP). Cells were stained with far-red fluorogenic SiR-DNA and the coverslip chamber was transferred to a Zeiss Spinning Disc microscope. Images were taken at three fields of vision for each sample every ten mins over the course of 14 hours. **A.** Full-length SS fHbp 1.1 without eGFP (negative control) **B.** eGFP only (positive control) **C.** Full-length SS fHbp 1.1-eGFP **D.** LTA knockout (KO) SS fHbp 1.1-eGFP **E.** Signal peptide (SP) KO SS fHbp 1.1-eGFP **F.** SP + LTA KO SS fHbp 1.1-eGFP. Time points, from left to right for each sample, correspond to the three-, five-, eight-

, 11-, and 14-hour timepoints. Blue fluorescence indicates SiR-DNA-stained nuclei, green fluorescence indicates antigen-eGFP expression. 28 µm scale bars are shown in the bottom right corner of each image.

5.3.3 Factor H binding protein N-terminal amino acid sequence variants induce differential functional antibody titres at early timepoints in mice

The panel of adenovirus vaccines encoding the different fHbp with N-terminal SS variants were used to immunise BALB/c mice. A sub-optimal dose of 1×10^7 infectious units, rather than a standard dose of 1×10^9 , was used to observe greater differences between our variant constructs to better assess the effect of each element of the sequence. Sera collected at several timepoints were assessed for fHbp-specific IgG titres by indirect ELISA. Statistically significant differences in anti-fHbp antibody titres were observed between signal sequence variant groups at weeks two and four post-immunisation. The highest titres were induced by the FL SS and SP KO SS constructs; the lowest titres were induced by the truncated SS, lipobox KO and 4CMenB groups (see Fig. 5.10A-B). By weeks six and 14, the only statistically significant differences observed were between 4CMenB and several signal sequence variant groups, as the response had matured to reached plateau (see Fig. 5.10C-D).

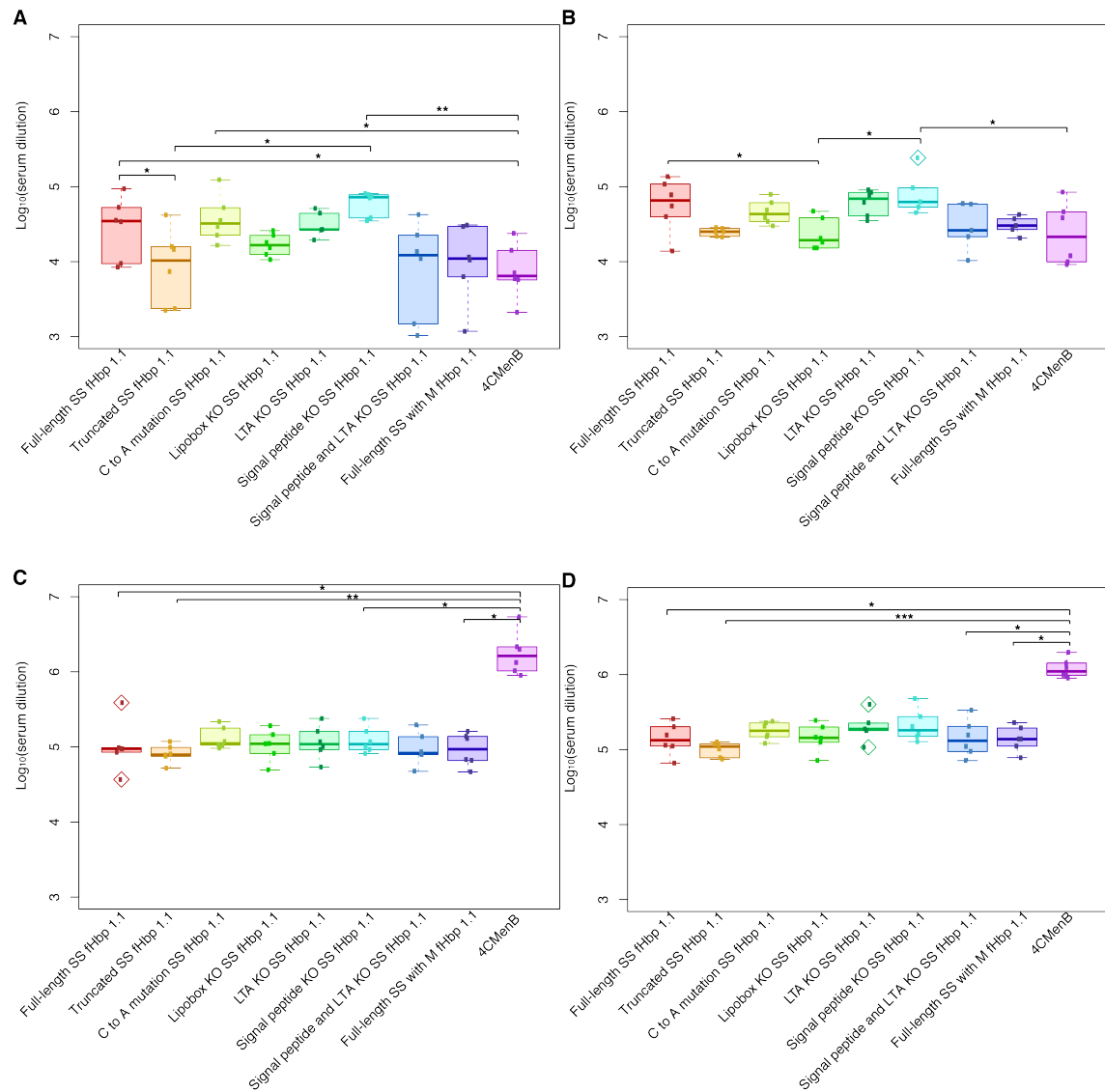
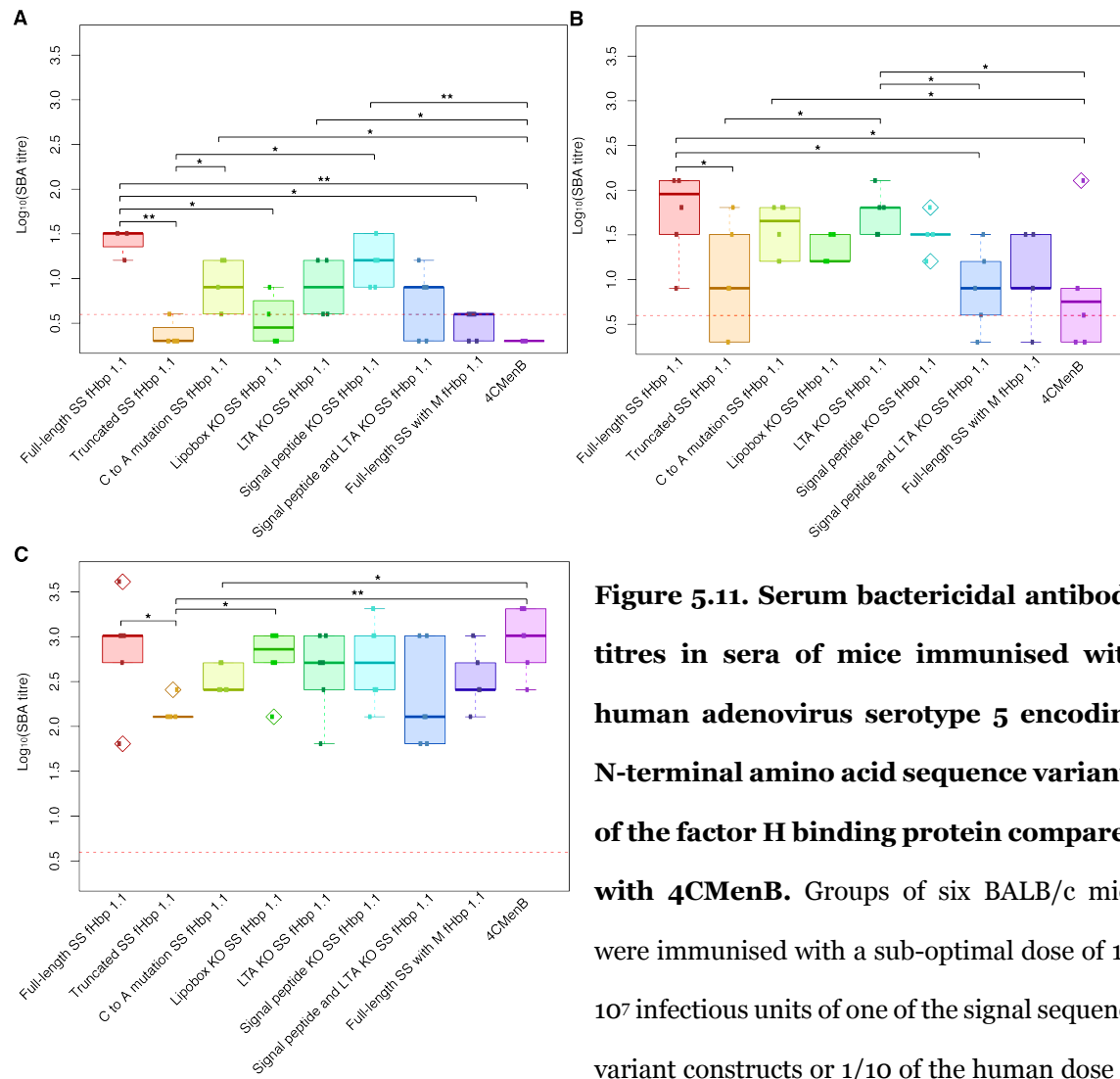


Figure 5.10. Anti-factor H binding protein IgG titres in sera from mice immunised with human adenovirus serotype 5 vectors encoding N-terminal amino acid sequence variants of the antigen. Groups of six BALB/c mice were immunised with a sub-optimal dose of 1×10^7 infectious units of one of the human adenovirus serotype 5 (AdHu5) vectors encoding factor H binding protein (fHbp) with N-terminal signal sequence variants or 1/10 of the human dose of 4CmenB as a comparator. Enzyme-linked immunosorbent assays were performed on serum samples taken at weeks **A**. two, **B**. four, **C**. six, and **D**. 14 post-immunisation to determine the titres of anti-fHbp IgG in sera. Statistical comparisons were made using a Mann-Whitney U-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The functionality of anti-fHbp antibodies was assessed by performing SBA assays against the H44/76-SL reference strain that naturally expresses fHbp 1.1 using the sera from weeks two, four, and six post-immunisation. Significant differences in SBA titre were measured at each timepoint, with greatest differences observed at weeks two and four post-immunisations (Fig. 5.11A-B). A single dose of AdHu5 expressing the FL SS fHbp antigen induced the highest titres of bactericidal antibody at week two, significantly higher ($p < 0.01$) than the truncated SS fHbp, while 4CMenB failed to induce protective titres after a single dose at this timepoint (Fig. 5.11A). The differences between the SBA titres associated with each construct closely resembled the differences in antigen expression levels observed after overnight infection of HeLa cells, with the exception of the lipobox KO SS which was highly expressed in this assay but induced lower SBA titres at this timepoint. This trend was still apparent by week four post-immunisation, with most constructs inducing SBA titres above the threshold for protection (Fig. 5.11B). Low titres were induced by 4CMenB, even one week after a second dose of this vaccine. By week six post-immunisation, all constructs had induced SBA titres of $> 1:4$, the putative threshold of protection for meningococcal vaccines (Fig. 5.11C). At this timepoint, the truncated SS fHbp was associated with the lowest titres, significantly lower than the FL SS and lipobox KO SS fHbp variants, while similar titres were obtained from the FL SS fHbp and 4CMenB groups.



Taken together, these results demonstrate that the N-terminal SS impacts on the functional antibody titres induced by expression of the fHbp transgene product. The pattern of bactericidal antibody response suggests that the early expression

associated with these SS variants influence their immunogenicity. Additionally, the FL fHbp SS is capable of inducing protective titres of SBA just two weeks after a single shot of a sub-optimal dose, while the licensed 4CMenB vaccine requires two doses and >four weeks to achieve such titres in mice.

5.3.4 Inclusion of the full-length signal sequence at the N-terminus of other adenovirus-encoded bacterial antigens boosts antigen-specific T cell responses to the transgene product

To determine whether the SS tested for fHbp could be applied to other bacterial antigens to increase immunogenicity, plasmids incorporating the *Y. pestis*, the causative organisms of the disease plague, F1 antigen with its native SS, lacking its native SS, and replacing the native SS with the FL fHbp SS were designed, cloned and incorporated into an AdHu5 vector (section 2.2.7). Groups of 12 BALB/c mice were immunised with a sub-optimal dose (1×10^7 IU) of one of each of the AdHu5-plague vaccines. The total anti-F1 IgG titres were measured from serum samples taken at weeks two and four post-immunisation by an indirect ELISA. The inclusion of the heterologous FL SS appeared to significantly increase anti-F1 IgG titres compared with the AdHu5-F1 construct at week four post-immunisation, though the titres were still significantly lower than those associated with the F1 antigen with native SS (Fig. 5.12). These data suggest that, for certain bacterial antigens, the native SS may perform better than the incorporated meningococcus fHbp SS.

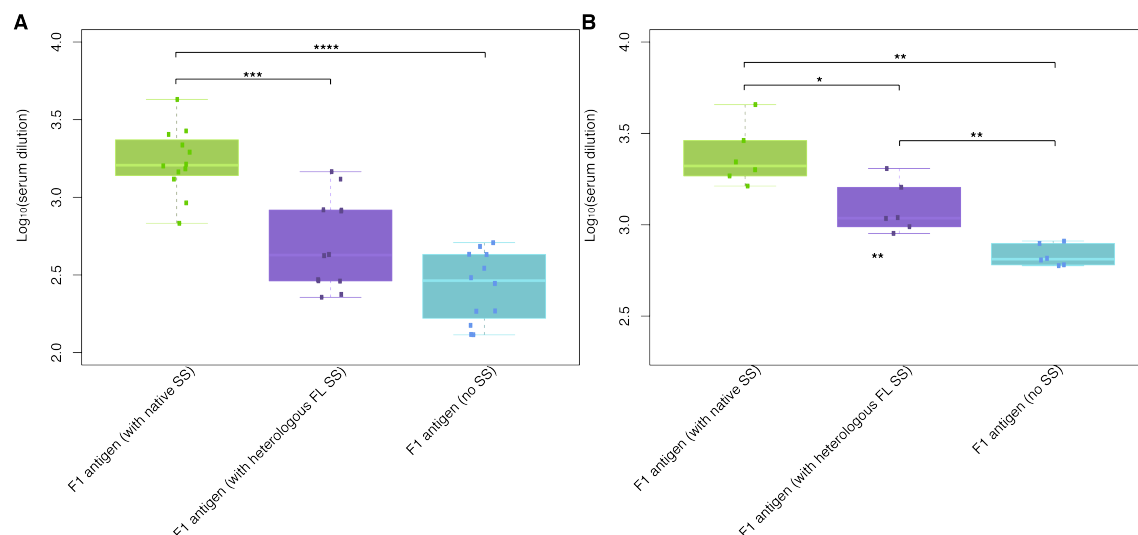


Figure 5.12. Anti-F1 antigen IgG titres in sera from mice immunised with human adenovirus serotype 5 vectors encoding the full-length F1 antigen, with or without the N-terminal amino acid signal sequence, or the truncated form of the antigen. Groups of 12 BALB/c mice were immunised with a sub-optimal dose of 1×10^7 infectious units of one of the human adenovirus serotype 5 (AdHu5) vectors encoding the *Yersinia pestis* F1 antigen with native signal sequence (SS), a heterologous N-terminal full-length (FL) SS, or a truncated form of the F1 antigen lacking any SS. Enzyme-linked immunosorbent assays were performed on serum samples taken at weeks **A.** two and **B.** four post-immunisation to determine the titres of anti-F1 antigen IgG in sera. The humoral response induced by the F1 antigen with native SS was superior to that induced by the incorporation of the heterologous FL SS to this antigen or its truncated form at both timepoints. Statistical comparisons were made using a Mann-Whitney U-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

To determine whether a more robust cellular response could be induced against the F1 antigen, fHbp, or a series of other antigens that utilise a signal peptide for expression in their native state, antigen-specific T cell responses were compared between these two antigens and several *Salmonella* antigens – CdtB, SipD, and SseB. Plasmids were designed to include the SS ahead of these *Salmonella* Paratyphi antigens, cloned, and incorporated into AdHu5 vectors (section 2.5.7). Groups of six BALB/c mice were immunised with a sub-optimal

dose (1×10^7 IU) of each AdHu5 vaccine encoding each of the bacterial antigens with or without the N-terminal SS. Spleens were harvested from these mice two weeks post-immunisation to assess antigen-specific IFN- γ - and IL-17A-producing T cell responses in this tissue by fluorospot. Inclusion of the N-terminal SS boosted the antigen-specific IFN- γ -producing T cell responses to most of the antigens tested, particularly for SipD and SseB resulting in a statistically significant increase in this cellular response (Fig. 5.13A), and even led to a significant increase in SseB-specific IL-17A-producing T cells which were otherwise mostly undetectable against the native counterparts of each antigen (Fig. 5.13B). These data highlight the additional attribute of the N-terminal SS in boosting antigen-specific T cell responses against heterologous bacterial antigens and demonstrate the utility of the SS as a broadly-applicable immune-enhancing peptide.

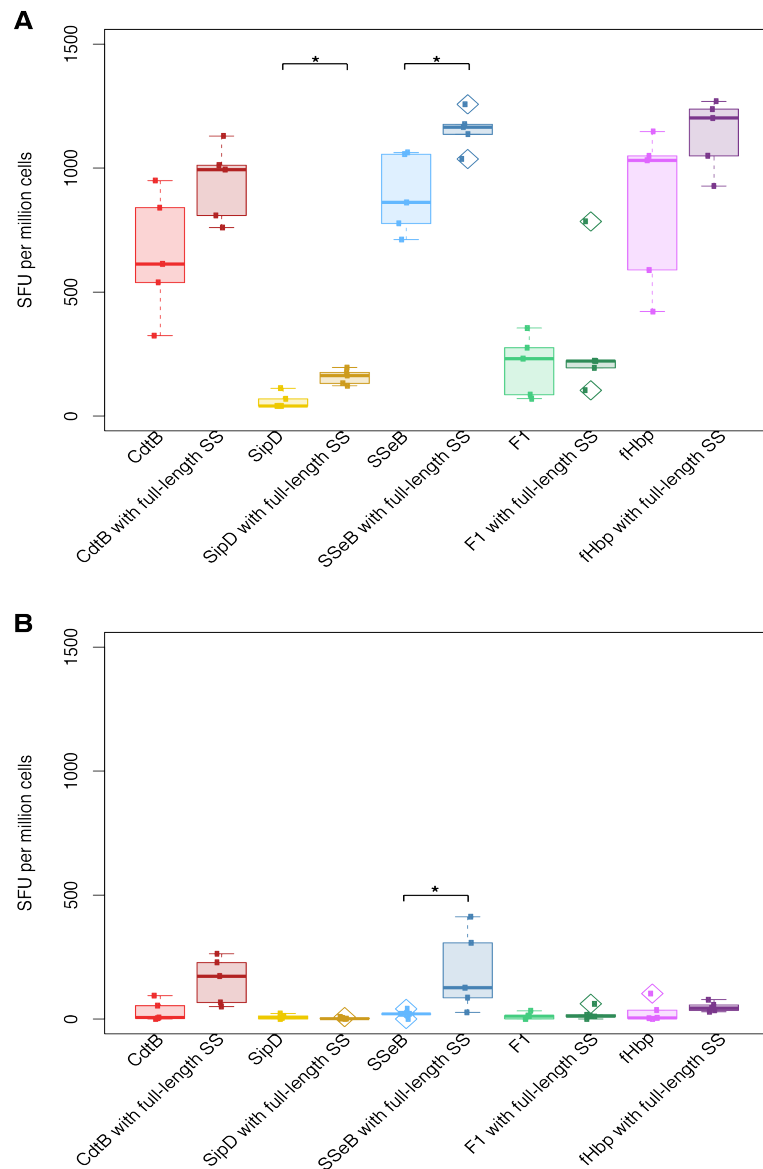


Figure 5.13. Antigen-specific T cell responses induced in mice two weeks after immunisation with human adenovirus serotype 5 vectors encoding bacterial antigens with or without an N-terminal signal sequence. Groups of six BALB/c mice were immunised with human adenovirus serotype 5 (AdHu5) vaccines expressing one of a series of bacterial antigens, with or without an N-terminal signal sequence (SS). Spleens were harvested two weeks post-immunisation, processed, and stimulated at a concentration of 1:100 with the relevant peptide pool. An interferon (IFN)- γ /interleukin (IL)-17A dual colour fluorospot was performed to assess antigen-specific T cell responses associated with these cytokines. **A.** IFN- γ and **B.** IL-17A spot-forming units (SFU) per million cells were quantified for each antigen. Inclusion of an N-terminal SS was found to boost both types of antigen-specific T cell responses. Statistical comparisons were made using a Mann-Whitney U-test. * $p < 0.05$.

5.4 Discussion

This study provides a functional exploration of the distinct elements of a novel signal sequence included ahead of a MenB lipoprotein expressed from a viral vector-encoded transgene. The relative contribution of each of these elements was assessed through *in vitro* expression assays in a mammalian cell line and validated with the use of high-resolution microscopy. The elucidation of the transgene expression kinetics provides a potential link with differences observed between these constructs through comparative immunogenicity analyses. Furthermore, the inclusion of the SS ahead of several other transgenes encoding heterologous bacterial antigens demonstrates the utility of this sequence in promoting early antigen-specific cellular immune responses.

Transgene antigen expression kinetics play an important role in determining the magnitude and quality of the immune response to adenovirus-encoded antigens, as outlined in section 1.3.1. Several strategies have been applied to optimise transgene expression through modification of the transgene sequence, promoting the expression and cellular processing of the antigen. Most antigens that are destined for secretion or translocation to the membrane contain SPs after translation³⁹⁰ and their inclusion may promote favourable processing of vaccine antigens. For example, the cleavage of the HIV-1 envelope protein (Env) SP has been correlated with the rate of transport and folding of its constituent glycoprotein gp120 within the ER of the host cell³⁹¹. The inclusion of the SP alters the pattern of glycosylation of this antigen which in turn alters the addressing of the peptide to the secretory pathway and influences the antigenic properties of gp120, a phenomenon that may prove useful from the perspective of HIV vaccine

immunogen design ³⁹². Certain antigens have stronger SPs than others and therefore lend themselves to the enhancement of heterologous antigen processing. The inclusion of a SP from VSV has been shown to enhance the generation of Japanese encephalitis virus (JEV) envelope protein-based lentivirus vectors to a greater degree than the native JEV envelope SS ³⁹³. A plant-derived SS has been used to elicit stronger antibody and cellular responses directed at human papillomavirus (HPV) antigens delivered via DNA vaccines to mice ³⁹⁴. The use of host-optimised SPs has been met with some success; the mouse-derived IgG kappa light chain SP has been shown to significantly increase the immunogenicity of a simian adenovirus-encoded *Plasmodium vivax* antigen in mice ³⁹⁵. Leader sequences have also been incorporated into viral vectors and have demonstrated their ability to boost transgene antigen-specific humoral and cellular responses. A prime example is the human tissue plasminogen activator (tPA) sequence that boosts these immune responses when placed before the tuberculosis Ag85B-TB10.4 antigen encoded by an MVA vector ³⁸⁷. Modification of bacterial proteins with lipid moieties is known to facilitate the attachment of these proteins to lipid membranes, cellular transport, and folding outside of the cytoplasm ¹⁸⁰. The precursor of fHbp contains a lipobox element (in this case LTAC) at the end of the bacterial SP and is modified at the invariant C residue for subsequent cleavage of the SP by signal peptidases at this site, leaving C as the N-terminal amino acid (Fig. 5.1) ¹⁸¹. A mammalian SP was elected to promote expression of the lipoprotein within mammalian tissues. The results of the expression assays presented here confirm that the FL SS is superior to the truncated SS for the promotion of fHbp 1.1 antigen expression in mammalian cells. It has also been demonstrated in the present chapter that these SS elements were judiciously chosen for their combined influence on transgene expression and immunogenicity; the expression assay data

provides rationale for the inclusion of the FL SS within the vaccine antigen sequence as the FL sequence promotes the most consistent levels of early transgene expression across each of the chosen timepoints. The only notable exception to this was in the C2C12 cell line, where the FL SS appeared to reduce antigen expression compared with all other SS variants. This observation may be due to differences in the cell machinery that negatively affect the intracellular processing of the FL SS. However, this cell line may not accurately reflect expression from muscle tissue as these cells tend to remain as myoblasts rather in culture than developing into myocytes ³⁹⁶. Muscle tissue is notoriously difficult to culture and so an accurate comparison to the biological setting could not be made.

The application of confocal and time-lapse microscopy was more revealing as to the timing and intracellular localisation of antigen expression in a mammalian cell line and confirmed the findings of the *in vitro* expression assays. As was shown by immunofluorescence analysis of the *Phaseolus vulgaris* SS-HPV antigen expression from DNA vaccines in infected HEK293 cells, fHbp-eGFP transgene expression was predominantly located within the cytoplasm in apparent association with membrane structures and no fluorescence detected in the extracellular media ³⁹⁴. This is in accordance with the typical role of SSs in targeting antigens to the membranes of the ER and cell surface ³⁹⁷, and studies of secretory protein trafficking in yeast have provided evidence that slight variations in the amino acid composition of SSs can result in altered recognition of these proteins by ER translocons ³⁹⁸. Further to this, certain SSs can impact on the post-ER translocation of antigens; the processing of the HBV core preprotein SS results in accumulation of the cleaved, translocated product within the cytosol ³⁹⁹. This may explain the differential accumulation of the SS variants following their expression in HeLa cells.

Delivery of exogenous antigen via viral vectored vaccines has been explored for the prevention of a wide range of diseases, particularly those requiring strong CD8⁺ T cell responses. This vaccine platform also has strong potential to induce robust humoral responses, making them an attractive target for diseases requiring antibody-mediated protection. IMD is one such disease, and vaccines have been licensed on the basis of their ability to induce a human SBA titre of ≥ 4 , the putative correlate of protection against the disease ⁴⁰⁰. It has been demonstrated here that all fHbp 1.1 SS variants elicit protective titres of antibody in mice within four weeks of a sub-optimal dose, while the FL SS fHbp 1.1 induces protective titres by as early as week two post-immunisation. This in contrast to 4CMenB which, at 1/10 of the human dose, requires two doses and more than four weeks to induce similar titres in mice. The FL SS also demonstrates superiority to the truncated SS variant, demonstrating its ability to enhance the antigenicity of the fHbp antigen. The level of antigen expression in APCs has been highlighted as an important contributing factor to the formation of antibodies against the transgene product from adenovirus vectors ⁴⁰¹. The induction of antigen-specific humoral immunity is primarily mediated by the interaction of CD4⁺ T_H cells with MHC class II-presented peptides on the surface of these cells. The subset of CD4⁺ T_H cells can influence the nature of the humoral response; IFN- γ production is a hallmark of T_H1 cells that promote IgG2 and IgG3 production from B cells ⁴⁰². Both of these antibodies are important in the context of early childhood as this age group is known to be poor at inducing these antibody subclasses, with the former playing an important role in immunity to *N. meningitidis* ⁴⁰³. IL-17A production indicates T_H17 cell activity, the role of which is of growing interest to vaccinologists due to its association with vaccine-induced immune responses against a variety of bacterial pathogens, such as *Bacillus pertussis* and *Streptococcus pneumoniae* ⁴⁰⁴.

The ability of the FL SS to enhance these distinct CD4⁺ T cell responses against the neisserial fHbp, the plague F1 antigen, and the *Salmonella* antigens CdtB, SseB, and SipD was investigated and found to significantly increase some of these responses. The increase in T_H1-mediated antigen-specific responses against SipD and SseB were significant, as was the T_H17-mediated response against SseB. Both of these proteins are constituents of the *Salmonella* type III secretion system (T3SS) and their potential as candidate vaccine antigens has been highlighted by studies in mice and humans ^{405,406}. CD4⁺ T cells of the IFN- γ and IL-17-producing variety also contributed to the response induced by the live-attenuated *Salmonella* Typhi vaccine, Ty21a ⁴⁰⁷, in humans. The findings of the T cell fluorospot assay provide compelling evidence that the FL SS can be incorporated ahead of adenovirus-encoded heterologous bacterial antigen transgene sequences to boost antigen-specific immune responses. These bacterial antigens are also currently undergoing pre-clinical development with the aim of bringing candidate ChAdOx1-based vaccines to phase I clinical trials.

The phase I clinical trial to assess the safety and immunogenicity of the ChAdOx1-fHbp 1.1 is currently ongoing. Functional genomics will be performed in order to further elucidate the distinct mechanisms underlying the immunogenicity associated with this novel candidate MenB vaccine. These mechanisms are explored using a mouse immunisation model and a series of AdHu5- and MVA-vectored vaccines, expressing the candidate vaccine antigen, administered singly and in heterologous prime-boost combinations in the next chapter.

Chapter 6

Exploring the gene expression changes induced by immunisation with viral vectored vaccines encoding a capsular group B meningococcus candidate vaccine antigen

6.1 Introduction

One of the primary aims of functional genomics analyses in the context of vaccinology is the acquisition of an understanding of the mechanisms of action of specific vaccine platforms, at the gene expression level. The goal is to determine the immunological pathways that are engaged and whether these responses are desirable for the diseases they are proven or designed to protect against. The emerging field of ‘systems vaccinology’ was introduced in section 1.1.5; its foundational studies focused on the elucidation of early gene expression changes that preceded the robust humoral and cellular immune responses induced at later timepoints, by the live-attenuated yellow fever vaccine, YF-17D ^{66,67}. These studies provided proof-of-principle evidence that gene signatures detected in the blood within the first few days post-immunisation accurately map the innate immune responses that forecast the subsequent well-characterised adaptive immune responses to a vaccine. Several other studies have since employed functional genomics to explore the early transcriptional changes following immunisation with several experimental and established vaccines, such as the RTS,S malaria vaccine ⁴⁰⁸, influenza vaccines ^{409–411}, and conjugate vaccines ⁴¹². This approach has also

been extended into mice for the exploratory analysis of different vaccine platforms and components through whole blood transcriptomics, for example for the acellular pertussis vaccine and pertussis OMVs ^{413,414}, and a variety of novel vaccine adjuvants ⁴¹⁵.

In chapter 4, whole blood transcriptomics was applied to the comparative assessment of constituent components of the 4CMenB vaccine, contrasting the gene expression changes induced by recombinant proteins, OMVs, and endotoxin. This analysis defined distinct gene signatures and revealed differential activation of innate immune response pathways associated with these components. In the present chapter, a similar approach was taken to explore the gene signatures associated with viral vectored vaccines encoding the candidate MenB vaccine antigen introduced in chapter 5. There is a paucity of data related to the characterisation of transcriptional responses induced by viral vectored vaccines. The transcriptional response to a candidate rVSV-based Ebola vaccine, which utilises a replication-competent viral vector to induce transgene-encoded glycoprotein-specific antibodies, was analysed using RNA-seq on samples taken from human participants during a phase I trial, to establish an early innate immune signature as a potential correlate of protection ⁴¹⁶. Transcriptomic analyses have also been applied to the simian adenovirus, with one study involving the immunisation of humans with ChAd3- or AdC63-vectored HCV or HIV-1 immunogens, respectively ⁴¹⁷. Participants in this study were also boosted with an MVA vaccine, commonly used in prime-boost vaccine regimens, and transcriptional changes were assessed using microarray technology.

RNA-seq is utilised in the present chapter to determine the early gene expression changes (24 hours post-immunisation) in whole blood after the administration of AdHu5- and MVA-based pre-clinical vaccine constructs to mice.

The aim is to characterise the immunological pathways that underlie their respective mechanisms of action. To better understand how these responses can be augmented by the administration of a booster vaccine, pathway enrichment analyses were also conducted on samples taken 24 hours after the final dose of a heterologous prime-boost regimen. The immunisation strategy for these experiments is outlined in Fig. 6.1. The data presented in this chapter provide insight into the hitherto underexplored molecular mechanisms of human adenovirus and poxvirus vector-induced immune responses.

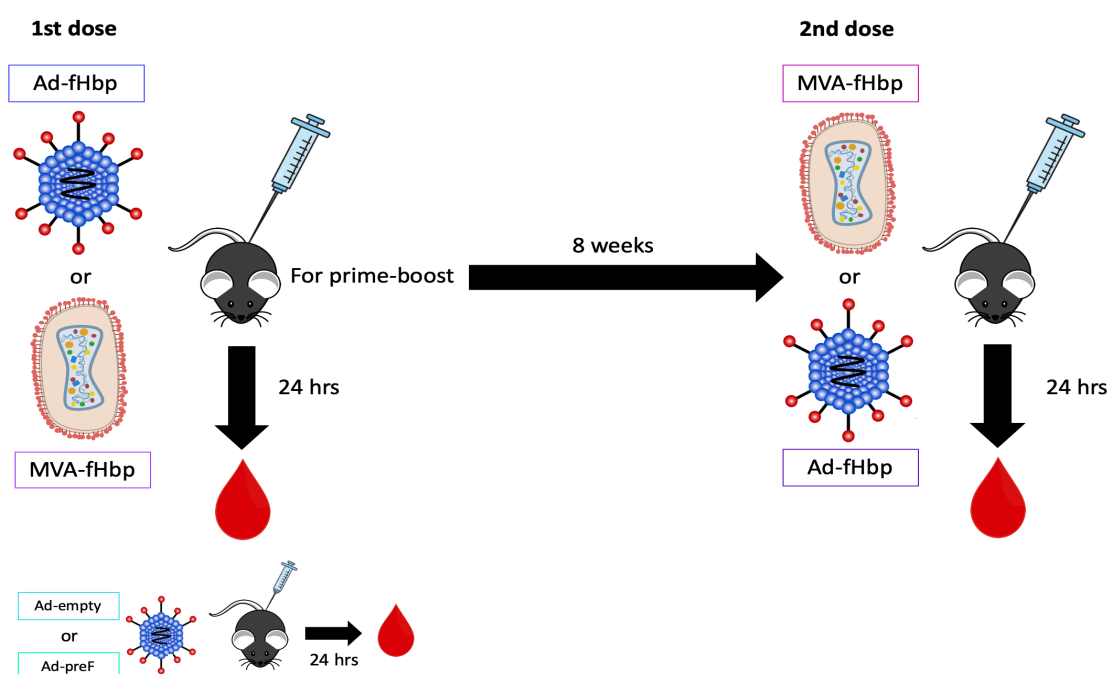


Figure 6.1. Adenovirus and MVA single dose or heterologous prime-boost vaccine regimen mouse immunisation strategy. Schematic of the mouse immunisation strategy for comparing the gene expression changes associated with human adenovirus serotype 5 or modified vaccinia Ankara vectors encoding the factor H binding protein, Ad-fHbp and MVA-fHbp. Groups of six C57BL/6 mice were immunised with 1×10^9 infectious units (IU) or adenovirus or 1×10^7 IU Single dose groups were bled 24 hours after this immunisation, while prime-boost groups received the opposite vaccine eight weeks later and were bled 24 hours after this boost dose. Mice were also immunised with a single dose of an empty adenovirus vector or one encoding the respiratory syncytial virus prefusion F (preF) antigen and were bled 24 hours later.

6.2 Methods

6.2.1 Mouse procedures

The mouse immunisations and bleeds procedures are outlined in sections 2.2.1–2.2.2, respectively. The vaccines and corresponding doses administered to the mice are outlined in Table 6.1.

Vaccine	Dose
Ad-fHbp	10 ⁷ viral particles (50 µL per leg)
Ad-empty	10 ⁷ viral particles (50 µL per leg)
Ad-prefusion F (preF)	10 ⁷ viral particles (50 µL per leg)
MVA-fHbp	10 ⁷ viral particles (50 µL per leg)

Table 6.1. Vaccines and corresponding doses for the MenB vaccine platform comparison experiment mouse immunisations.

4.2.2 RNA sequencing

The protocols for RNA extraction from whole blood, library preparation and bulk RNA-seq are detailed in sections 2.3.1 and 2.3.2, respectively.

4.2.3 Computational methods

The whole blood RNA-seq pre-processing steps are covered in sections 2.4.1–2.4.4. The DGE analysis pipeline for whole blood RNA-seq is outlined in section 2.4.5.

6.3 Results

6.3.1 Antigen-specific transcriptional changes are detectable in mouse whole blood 24 hours after intramuscular immunisation with an adenovirus vectored vaccine

The initial aim of this study was to elucidate the antigen-specific pathways associated with AdHu5-fHbp (Ad-fHbp) immunisation. This entailed making comparisons for DGE analysis between the Ad-fHbp-immunised mice and an AdHu5 vector lacking any transgene sequence and therefore expressing no exogenous antigen (Ad-empty). Firstly, significantly DEGs (FDR < 0.01) were defined for both of the groups individually by defining contrasts against the naïve control group. The overlap between these genes was determined, revealing an overlap of 101 significant genes and 372 uniquely DE, and therefore putatively transgene product-specific genes associated with Ad-fHbp immunisation (Fig. 6.2).

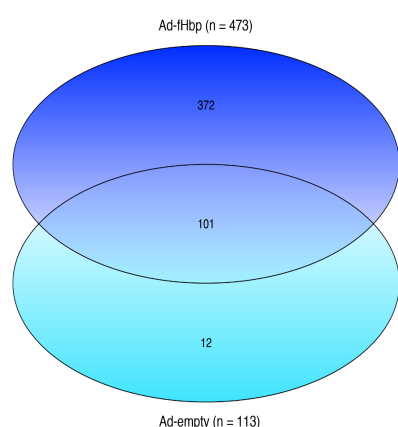


Figure 6.2. Overlap between significantly differentially expressed genes in mouse whole blood 24 hours after immunisation with human adenovirus serotype 5 with or without a factor H binding protein transgene. Significantly differentially expressed genes (DEGs) were defined as those with an FDR < 0.01. The intersection between the two ovals indicates common DEGs between the two conditions.

To discern the antigen-specific gene expression changes associated with the fHbp transgene product, the linear models fitted for the Ad-fHbp and Ad-empty groups were reoriented to make contrasts between the two conditions and then assessed for DE using eBayes to define significant DEGs. Gene ontology ORA was then performed on these genes to determine significantly differentially enriched ontologies (pathway p -value < 0.05) between each vaccine (Fig. 6.3). This analysis revealed three protein transport pathways among the top differentially enriched gene ontologies between the vaccines, indicating the induction of transgene product-specific cellular processing pathways. Additionally, several immune cell population ontologies were differentially enriched, including $\gamma\delta$ T cell differentiation and natural killer cell differentiation and chemotaxis pathways. This suggests that transgene expression also leads to immune cell engagement that may be specific to the fHbp antigen. Several epigenetic modification ontologies, including histone H4 acetylation and negative regulation of methylation of lysine (K)4 on histone H4.

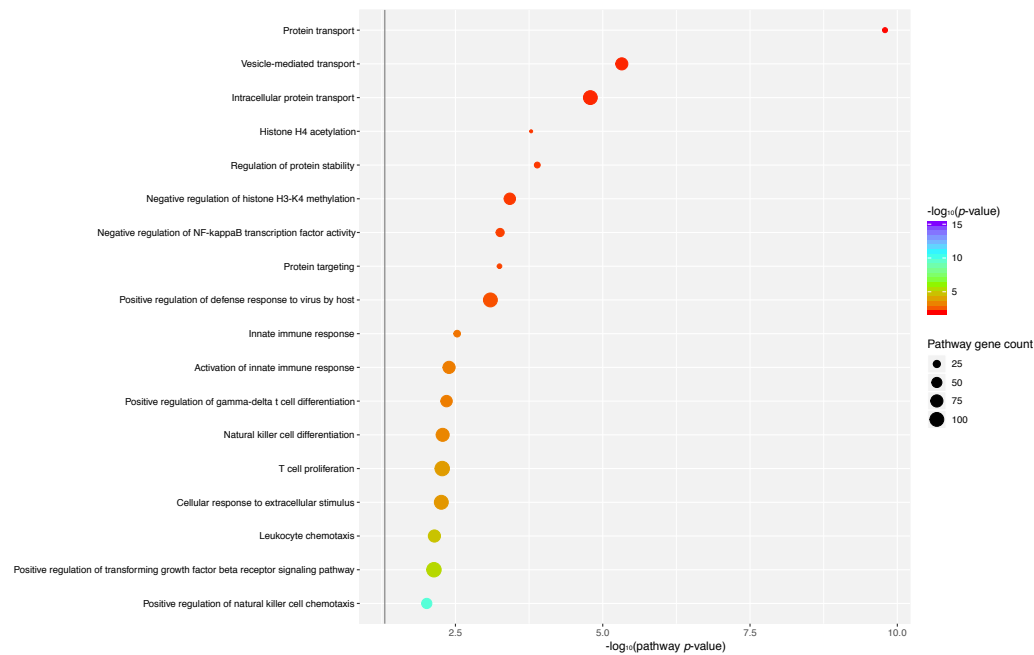


Figure 6.3. Significantly differentially enriched gene ontologies in mice associated with the expression of the factor H binding protein from a human adenovirus serotype 5 vector. Gene expression changes induced in mice 24 hours after immunisation with human adenovirus serotype 5, with or without the factor H binding protein transgene, were contrasted to determine significantly differentially expressed genes (DEGs) associated with each condition. Gene ontology over-representation analysis was performed on these DEGs. The colour and position of circles along the x-axis corresponds to the negative $\log_{10} p$ -value associated with a given gene ontology (y-axis), while the size of the circle corresponds to the number of genes defined for that ontology. The horizontal grey line indicates the threshold for significant (pathway p -value < 0.05).

Pathway ORA was also performed on significantly DEGs defined between Ad-empty and Ad-fHbp to elucidate the immunological and cytokine pathways specifically induced by fHbp when expressed from adenovirus. The former revealed the ‘gene expression’ pathway to be substantially enriched in the Ad-fHbp group relative to the Ad-empty group, likely due to the large quantities of transgene expressed from adenovirus vectors, as well as several pathways related to epigenetic regulation, from histone acetylation and deacetylation to DNA

methylation and demethylation (Fig. 6.4A). Further to this, the immunological pathway ORA also revealed the specific TLR pathways induced by fHbp produced from the adenovirus vector. These include stimulation of TLR2 and its complexes, TLR1:TLR2 and TLR6:TLR2, characteristic of an innate response to lipoprotein, as well as TLR4 engagement and the downstream MyD88:Mal cascade. Cytokine signalling pathway ORA revealed significant enrichment of CD4⁺ T cell signalling as well as IL-1 signalling, TNF receptor engagement, and TLR and BCR signalling (Fig. 6.4B). These pathway enrichment analyses demonstrate the high specificity and resolution of RNA-seq for defining transgene product-specific perturbations to the whole blood transcriptome.

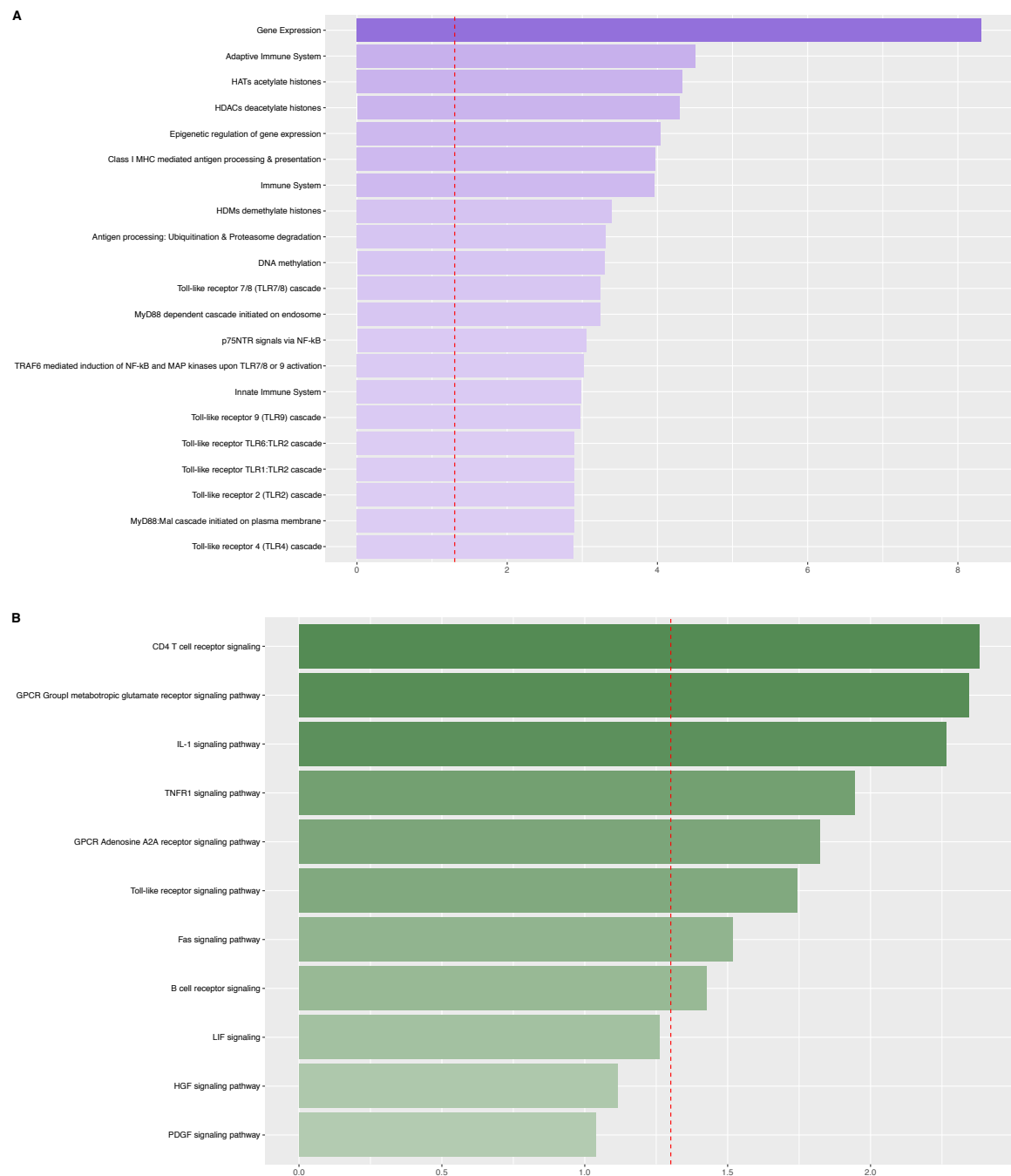


Figure 6.4. Significantly differentially immunological and cytokine signalling pathways in mice associated with the expression of the factor H binding protein from a human adenovirus serotype 5 vector. Gene expression changes induced in mice 24 hours after immunisation with human adenovirus serotype 5, with or without the factor H binding protein transgene, were contrasted to determine significantly differentially expressed genes (DEGs) associated with each condition. Pathway over-representation analysis was performed on these DEGs to determine the enrichment of genes pertaining to cell signalling pathway categories from the publicly available **A. Reactome** and **B. integrating network objects with hierarchies (INOH)**

databases. The x-axis corresponds to the negative $\log_{10}$ pathway adjusted p -value associated with each pathway (y-axis). The dashed red line corresponds to a negative $\log_{10}$ pathway adjusted p -value of 0.05.

6.3.2 The antigen-specific transcriptional response associated with two distinct adenovirus-encoded antigens can be distinguished through comparative assessment of whole blood gene signatures

To determine whether the gene expression changes and differentially enriched pathways between these two interventions pertained specifically to the expression of the fHbp transgene, the gene expression changes associated with Ad-fHbp were contrasted with those of another adenovirus vectored vaccine expressing the pre-fusion form of the F protein from RSV, Ad-preF. Prior to testing the Ad-preF vaccine against Ad-fHbp, a brief interspecies comparison was made between the gene expression values obtained for Ad-preF in mice and those from a human transcriptomics study in which participants were immunised with a simian adenovirus vaccine, PanAd-RSV, to determine whether the RSV vaccine-induced gene signature found to correlate with neutralising antibody titres was also induced by Ad-preF. To this end, a selection of the top significantly upregulated genes associated with a single dose of PanAd-RSV at the earliest timepoint, three days post-immunisation, were converted to their corresponding mouse orthologs and used to generate a heatmap based on the expression of these genes in the Ad-empty, Ad-preF, and naïve control groups (Fig. 6.5). This analysis demonstrated that the viral signature defined for PanAd-RSV was detected as early as 24 hours post-immunisation in mice, even with a different adenovirus vector and antigen combination, which suggests that this signature may not be antigen-specific and is

more likely to be a generic antiviral transcriptional response. This is further evidenced by the equivalent upregulation of these genes in mice immunised with Ad-empty. It is possible that antigen-specific gene expression changes occur within the first 24 hours post-immunisation and that the transcripts detected at day three in the PanAd-RSV data are persistent antiviral responses. However, the antigens encoded by the vaccine may also induce a distinctly antiviral response, making the antigen- and vector-specific signatures harder to discern.

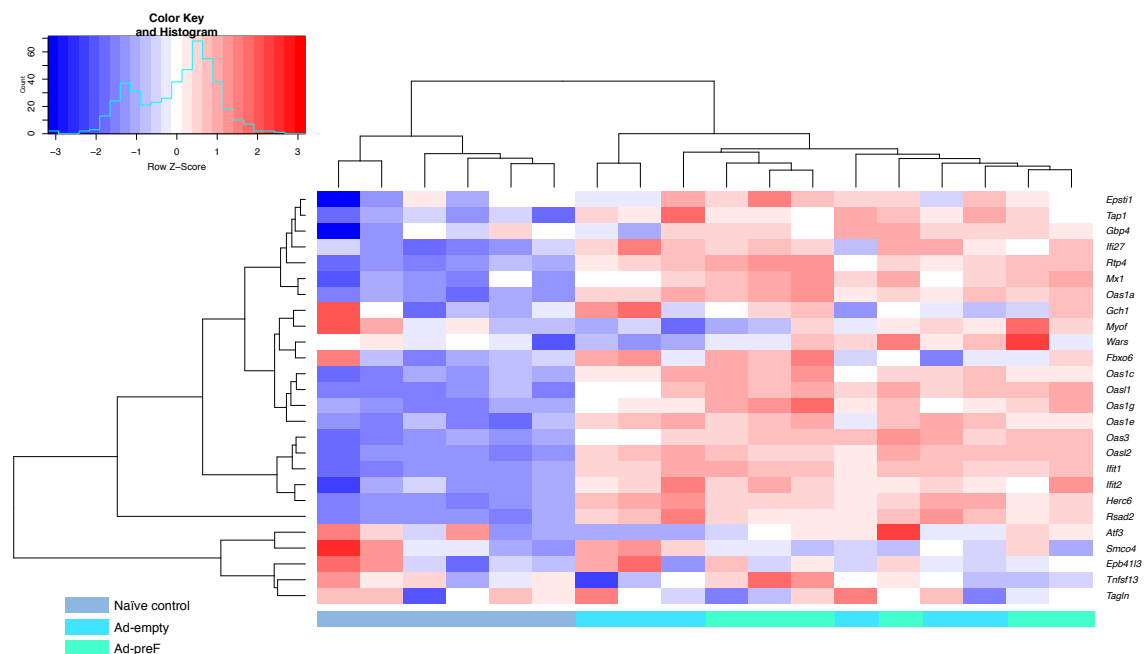


Figure 6.5. Hierarchical clustering based on the expression of mouse orthologs of a human respiratory syncytial virus vaccine-induced gene signature in mouse whole blood, 24 hours after immunisation with a human adenovirus serotype 5 vaccine, with or without a prefusion F transgene. Pairwise Euclidian distances between all samples were calculated from the normalised counts of genes with the maximum dispersion from the population mean and are represented by the column and row dendrograms. Heatmap tiles correspond to row z scores, centred and scaled upon the mean expression value for that gene across the dataset, with red and blue tiles indicating increased or decreased expression relative to the population mean, respectively.

Despite the overlap between the Ad-empty and Ad-preF groups in the expression of this RSV vaccine gene signature, further comparisons were made between the groups to attempt to discern mouse-specific ontologies or pathways induced by the expression of an RSV antigen from an adenovirus vector. Contrasts were defined between these groups in the same manner as the Ad-empty versus Ad-fHbp comparison. The subsequent gene ontology ORA revealed significant upregulation of viral innate immune response, antigen processing and presentation, and immune cell population ontologies in the Ad-preF group relative to the Ad-empty group (Fig. 6.6). Among the immune cell ontologies enriched after Ad-preF were those pertaining to B cell activation, neutrophil extravasation and enrichment of T cells of the T_H1 and T_H2 lineages. This analysis implies a transgene-encoded antigen-specific response associated with the preF that differs from that of fHbp in the nature of the innate immune response, viral compared with bacterial, and immune cell engagement, neutrophils and $\alpha\beta$ T cells rather than natural killer cells and $\gamma\delta$ T cells.

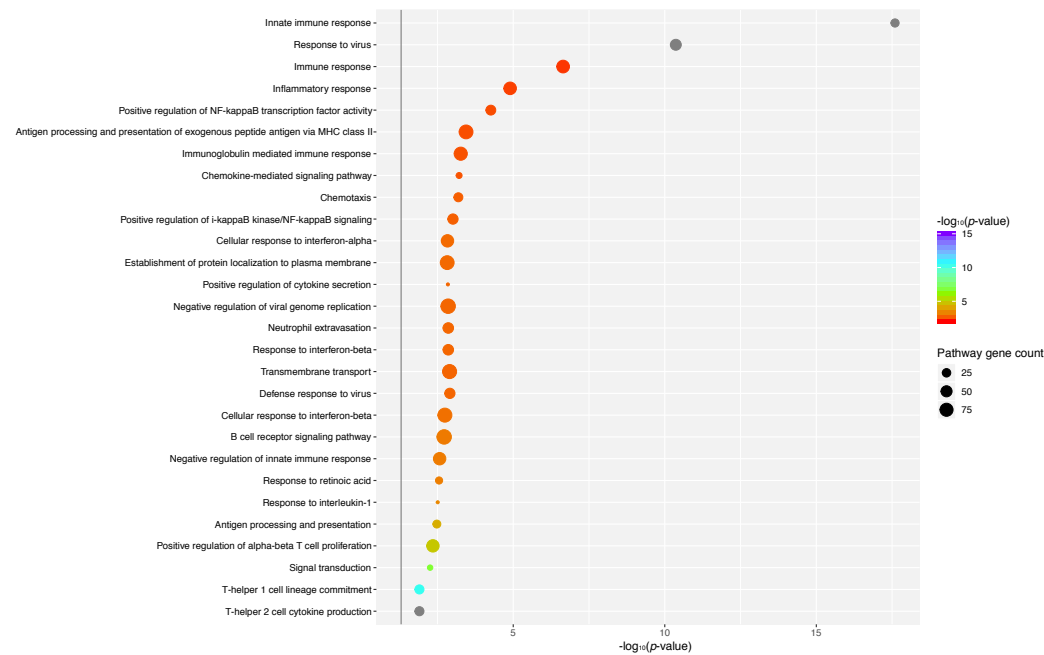


Figure 6.6. Significantly differentially enriched gene ontologies in mice associated with the expression of respiratory syncytial virus prefusion F antigen from a human adenovirus serotype 5 vector. Gene expression changes induced in mice 24 hours after immunisation with human adenovirus serotype 5, with or without the prefusion F transgene, were contrasted to determine significantly differentially expressed genes (DEGs) associated with each condition. Gene ontology over-representation analysis was performed on these DEGs. The colour and position of circles along the x-axis corresponds to the negative $\log_{10} p$ -value associated with a given gene ontology (y-axis), while the size of the circle corresponds to the number of genes defined for that ontology. The horizontal grey line indicates the threshold for significant (pathway p -value < 0.05).

Pathway ORA revealed no statistically significantly differentially enriched cytokine signalling pathways between the Ad-preF and Ad-empty groups. However, immunological pathway ORA further delineated aspects of the putative antigen-specific immune response, including enriched interferon signalling and engagement of TLRs 3, 7, 8, and 9 (Fig. 6.7). The TLR cascades initiated by the expression of the preF antigen are in contrast with those initiated by fHbp expression – TLR2, TLR1:2, and TLR6:2.

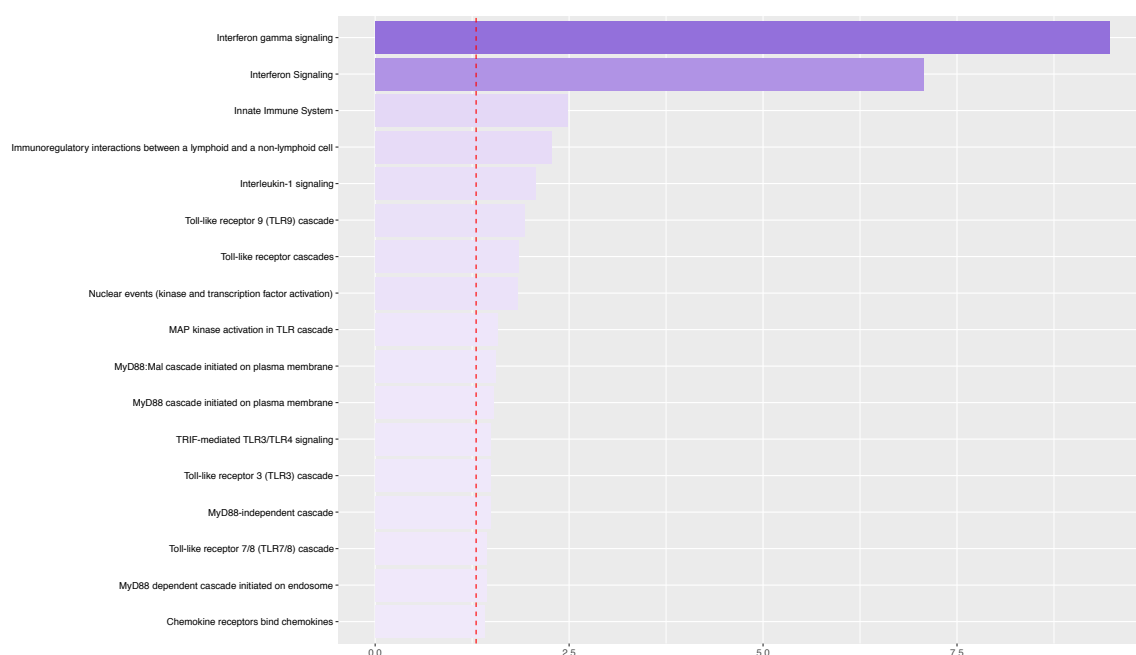


Figure 6.7. Significantly differentially enriched immunological pathways in mice associated with the expression of the respiratory syncytial virus prefusion F antigen from a human adenovirus serotype 5 vector. Gene expression changes induced in mice 24 hours after immunisation with human adenovirus serotype 5, with or without the prefusion F transgene, were contrasted to determine significantly differentially expressed genes (DEGs) associated with each condition. Pathway over-representation analysis was performed on these DEGs to determine the enrichment of genes pertaining to immunological pathway categories from the publicly available Reactome database. The x-axis corresponds to the negative log₁₀ pathway adjusted *p*-value associated with each pathway (y-axis). The dashed red line corresponds to a negative log₁₀ pathway adjusted *p*-value of 0.05.

Finally, the Ad-fHbp and Ad-preF groups were directly contrasted to highlight differences between their respective transgene product-induced immune response pathways. Given that the Ad-fHbp vaccine induced a greater magnitude of gene expression changes than the Ad-preF vaccine, it is not surprising that a greater proportion of ontologies and pathways were significantly enriched in the Ad-fHbp group relative to the Ad-preF group. The gene ontology ORA further

emphasised the enrichment of transgene product-specific immune responses defined previously (Fig. 6.8). IL-6 signalling following fHbp immunisation was demonstrated in section 4.3.9 and is perhaps a response to bacterial rather than viral antigens. Ad-fHbp immunisation appears to instigate substantial epigenetic changes that include the negative regulation of K4 and K9 methylation on histone H3 and acetylation of histone H3 and K5 and K8 on histone H4. Ad-fHbp also appears to induce differentiation of $\gamma\delta$ T cells while Ad-preF does not.

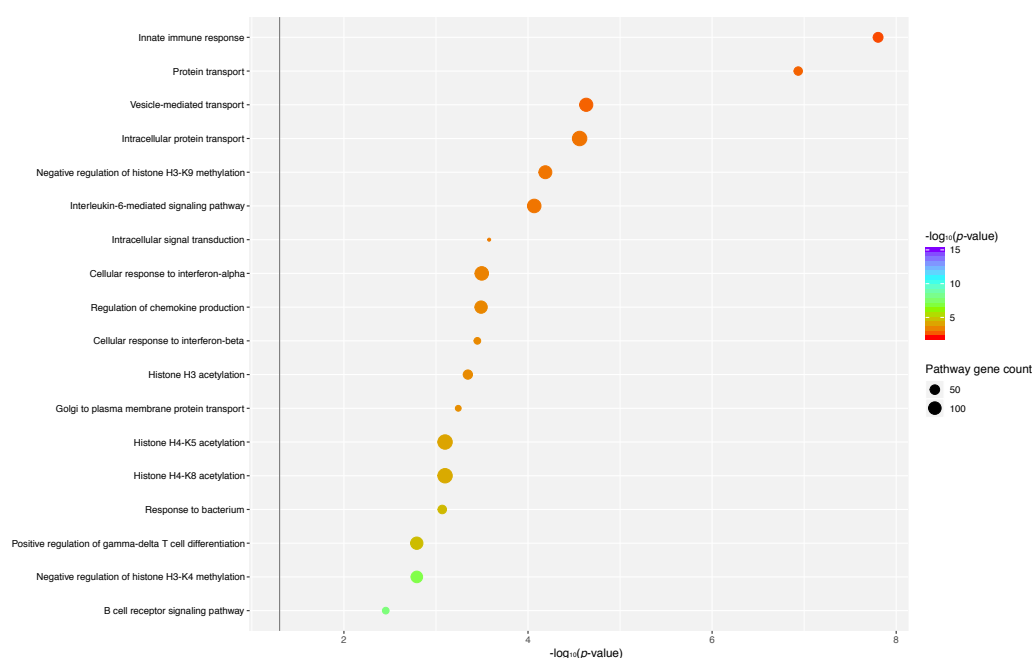


Figure 6.8. Significantly differentially enriched gene ontologies in mice enriched by the expression of the factor H binding protein from a human adenovirus serotype 5 vector compared with those associated with expression of the respiratory syncytial virus prefusion F antigen. Gene expression changes induced in mice 24 hours after immunisation with human adenovirus serotype 5, with the factor H binding protein or prefusion F transgene, were contrasted to determine significantly differentially expressed genes (DEGs) associated with each condition. Gene ontology over-representation analysis was performed on these DEGs. The colour and position of circles along the x-axis corresponds to the negative $\log_{10} p$ -value associated with a given gene ontology (y-axis), while the size of the circle corresponds to the number of genes defined for that ontology. The horizontal grey line indicates the threshold for significance (pathway p -value < 0.05).

6.3.3 Adenovirus and MVA viral vectors exhibit differential engagement of immunological pathways despite encoding the same transgene

Having characterised the response to Ad-fHbp, a similar analysis was performed for an MVA vector encoding the same antigen to determine vector-specific gene expression changes prior to exploring heterologous prime-boost combinations of these vaccines. Gene expression changes in whole blood were defined 24 hours after intramuscular immunisation with a single dose of MVA-fHbp. A greater number of significantly DEGs were obtained following Ad-fHbp immunisation than MVA-fHbp immunisation (Fig. 6.9) despite the larger size and repertoire of antigens associated with MVA vectors. This is likely due to the expression kinetics associated with adenovirus vectors which produce high levels of antigen expression soon after immunisation that persist over the course of several days compared with MVA vectors which exhibit a more transient spike of antigen expression that may not induce innate immune responses of the same magnitude ²²⁶. The common significant DEGs in the intersection between these two vaccine groups are related to the shared antiviral- and the fHbp-directed responses.

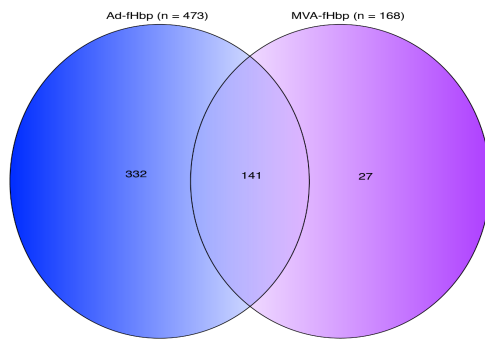
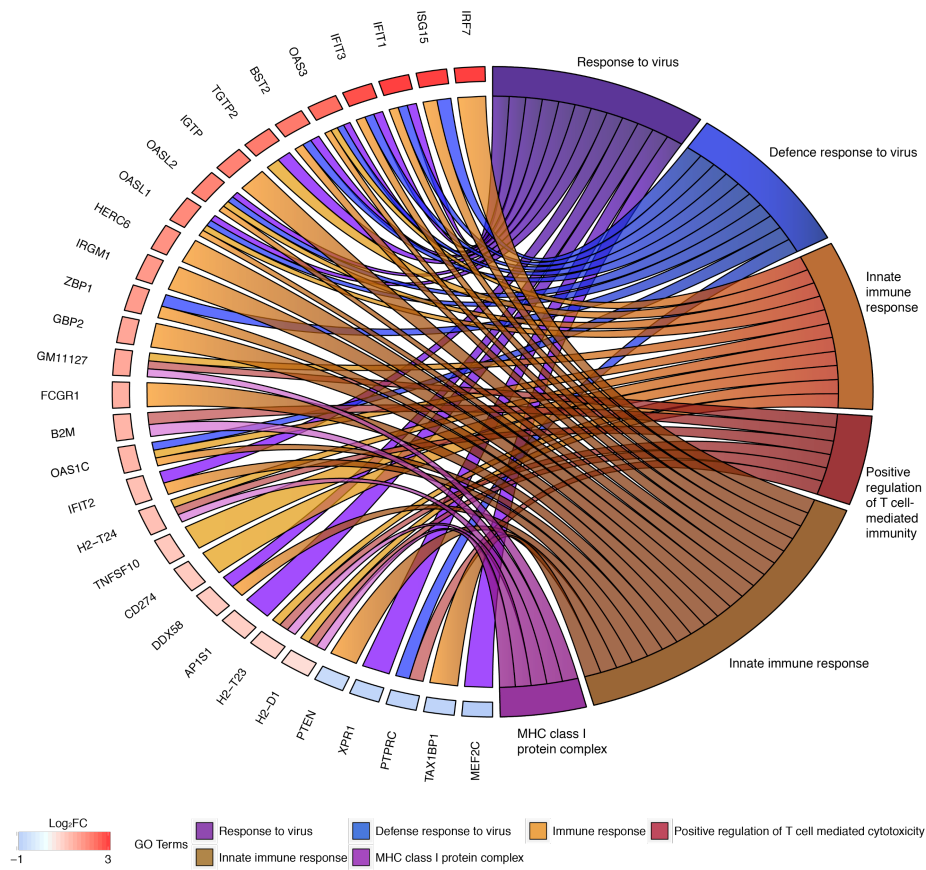


Figure 6.9. Overlap between significantly differentially expressed genes in mouse whole blood 24 hours after immunisation with a human adenovirus serotype 5 or modified vaccinia Ankara vector, both expressing a factor H binding protein transgene. Significantly

differentially expressed genes (DEGs) were defined as those with an FDR < 0.01. The intersection between the two ovals indicates common DEGs between the two conditions.

A side-by-side comparison of gene ontologies between the Ad-fHbp and MVA-fHbp vaccine groups highlighted a largely similar pattern of immunological pathway enrichment, with both vaccines eliciting a classical antiviral response characterised by induction of IFN regulatory factor (IRF), IFN-induced transcript (IFIT), and oligoadenylate synthase (OAS) family genes (Fig. 6.10). The MHC class I protein complex is the primary mediator of antigen presentation from these vectors, but Ad-fHbp immunisation appears to promote greater enrichment of T cell-mediated immunity (Fig. 6.10A). On the other hand, MVA-fHbp immunisation leads to greater induction of type I IFNs, IFN- α and IFN- β (Fig. 6.10B).

A



B

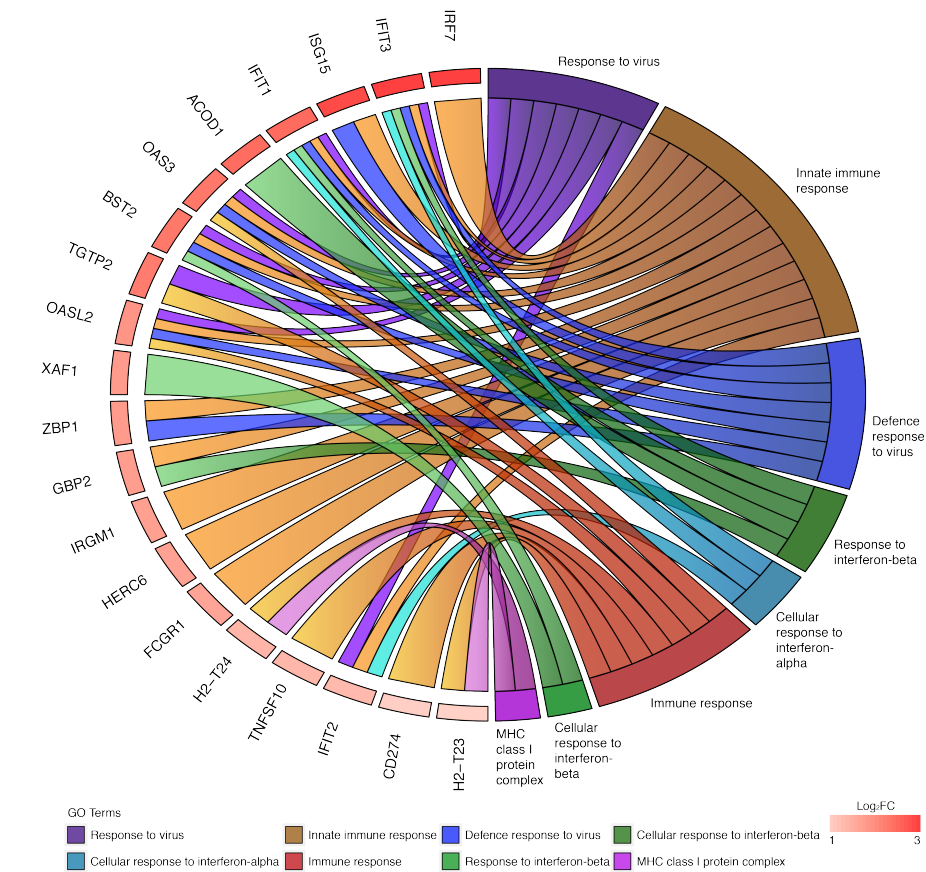


Figure 6.10. Chord plots of top significantly enriched gene ontologies associated with human adenovirus serotype 5 and modified vaccinia Ankara immunisation in mice.

Significantly differentially expressed genes (DEGs) (FDR < 0.05) associated with a single dose of human adenovirus serotype 5 or modified vaccinia Ankara encoding a factor H binding protein transgene (Ad-fHbp and MVA-fHbp, respectively), 24 hours after a single dose were analysed for enrichment of gene ontology (GO) categories. GO categories containing greater than three of the top significantly DEGs were included in the chord plots and their names are displayed along the right hemisphere of each plot. The names of the proteins encoded by the corresponding DEGs were ranked according to the gene's log₂ fold-change along the left hemisphere, from highest to lowest. **A.** Ad-fHbp. **B.** MVA-fHbp.

6.3.4 Differential engagement of immunological pathways following heterologous boost of viral vectored vaccine combinations may be influenced by epigenetic regulation

Significantly DEGs (FDR < 0.01) were determined for the two heterologous prime-boost vaccine combinations – MVA-fHbp prime, Ad-fHbp boost (MVA-fHbp/Ad-fHbp) and Ad-fHbp prime, MVA-fHbp boost (Ad-fHbp/MVA-fHbp) – 24 hours after the booster dose of vaccine, administered eight weeks after the prime. The MVA-fHbp/Ad-fHbp combination induced the greatest perturbation of the mouse whole blood transcriptome, with 842 significantly DEGs relative to the naïve control group, while the Ad-fHbp/MVA-fHbp induced even fewer significantly DEGs than a single dose of MVA-fHbp, with just 120 genes passing the significance threshold (Fig. 6.11). MVA-fHbp appears to induce relatively mild transcriptional perturbation, even when administered as a heterologous boost. Ad-fHbp, on the other hand, induces an even greater transcriptional response when administered as a heterologous boost.

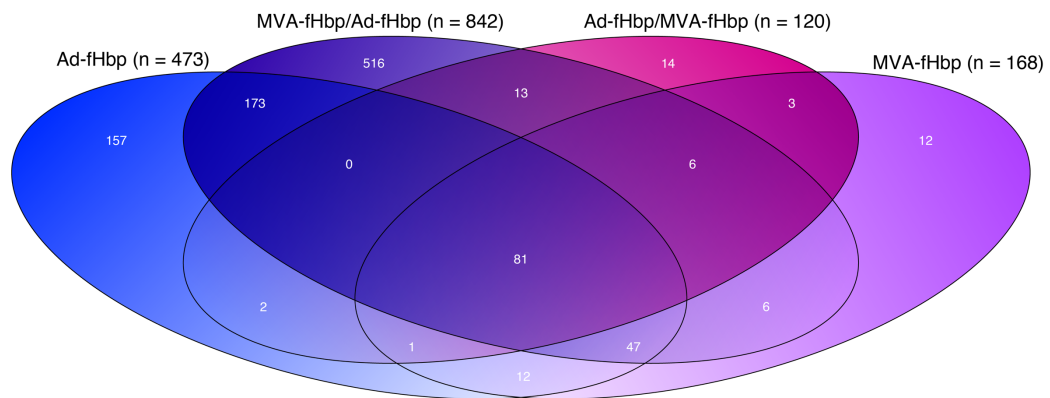


Figure 6.11. Overlap between significantly differentially expressed genes in mouse whole blood 24 hours after immunisation with one of a series of candidate viral vectored vaccines, administered as a single dose or heterologous prime-boost combination. Significantly differentially expressed genes (DEGs) were defined as those with an FDR < 0.01. The intersection between the two ovals indicates common DEGs between the two conditions.

Comparative assessment of the heterologous prime-boost vaccine combinations revealed substantial enrichment of immunological pathways related to epigenetic modification associated with the MVA-fHbp/Ad-fHbp combination; these pathways predominated among the top significantly enriched pathways based on contrasts drawn between these two groups (see Fig. 6.12). Additionally, the MVA-fHbp/Ad-fHbp group displays greater enrichment of antigen processing and presentation pathways related to both MHC class I and II molecule, indicating differential ability to express and present exogenous antigen at this timepoint.

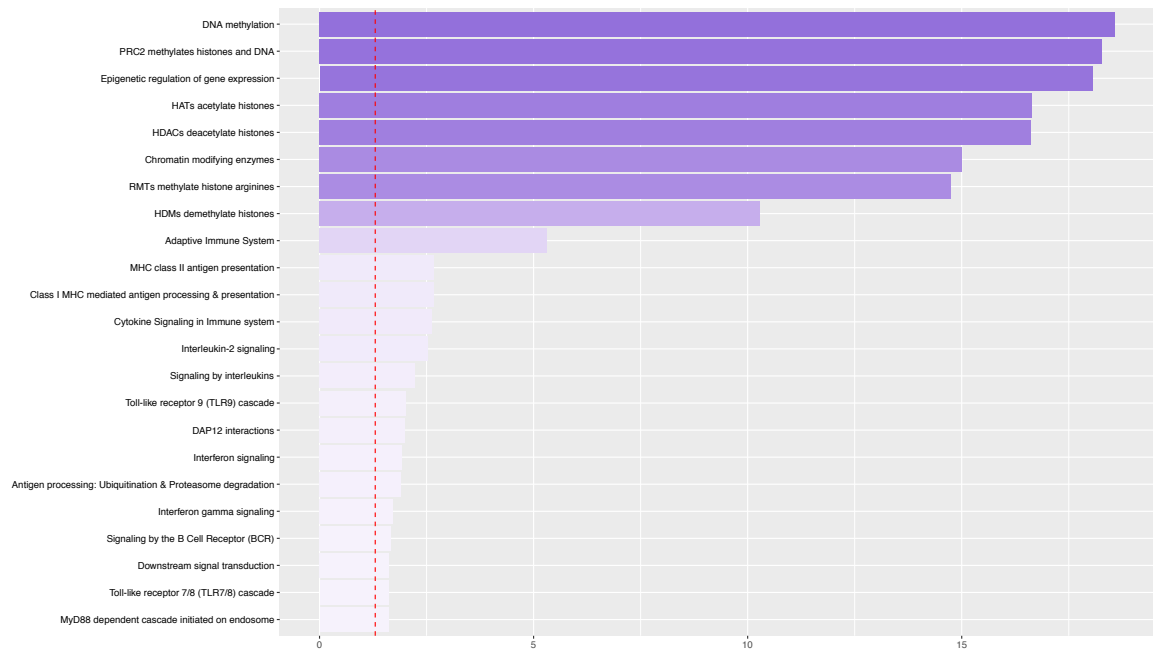


Figure 6.12. A comparative assessment of significantly enriched immunological pathways associated with the immunisation of mice with one of two heterologous viral vectored vaccine prime-boost combinations, 24 hours after the boost dose. Gene expression changes induced in mice 24 hours after the boost dose of either a human adenovirus serotype 5 vaccine encoding a factor H binding protein (fHbp) transgene (Ad-fHbp) prime, modified vaccinia Ankara (MVA)-fHbp boost, or an MVA-fHbp prime, Ad-fHbp boost heterologous vaccine combination were contrasted to determine significantly differentially expressed genes (DEGs) associated with each vaccine combination. Pathway over-representation analysis was performed on these DEGs to determine the enrichment of genes pertaining to immunological pathway categories from the publicly available Reactome database. The x-axis corresponds to the negative log₁₀ pathway adjusted *p*-value associated with each pathway (y-axis). The dashed red line corresponds to a negative log₁₀ pathway adjusted *p*-value of 0.05.

These results imply that the strong immune response observed following an adenovirus boost vaccine may be accompanied or preceded by alterations to the epigenetic landscape. To assess the impact that these alterations may have on the accessibility of transcription factor binding sites (TFBS), an ORA of TFBS was performed for each prime-boost combination group using the publicly available

CisRED regulatory elements database ⁴¹⁸ (6.13). Among the biologically relevant TFBS enriched specifically in the Ad-fHbp boost group were the following:

- PAX5, a transcription factor involved in B cell differentiation ⁴¹⁹ and known to undergo frequent methylation in the context of cancer ⁴²⁰.
- XBP1, a transcription factor that plays a key role in the secretory apparatus of plasma cells and DCs when chromatin is accessible ⁴²¹.
- HNF1A, another transcription factor of importance in B cell development ⁴²².

Of the TFBS that were uniquely identified for the MVA-fHbp boost group, two corresponded to transcription factors with particular relevance to the vaccine-induced response: ETV4 binds the promoter of the adenovirus E1A gene ⁴²³ and CEBPD enhances transcription of IL-6 ⁴²⁴. The differential usage of TFBS following these heterologous prime-boost vaccine combinations, particularly those with epigenetically regulated accessibility, implies that the prime vaccine may render particular portions of chromatin accessible in such a manner as to augment or restrict the gene expression changes underlying the immune response to the subsequent boost vaccine. The identification of several of the transcription factors with roles in B cell lymphopoiesis associated with the Ad-fHbp boost may implicate these pathways in the humoral response induced by this vaccine strategy.

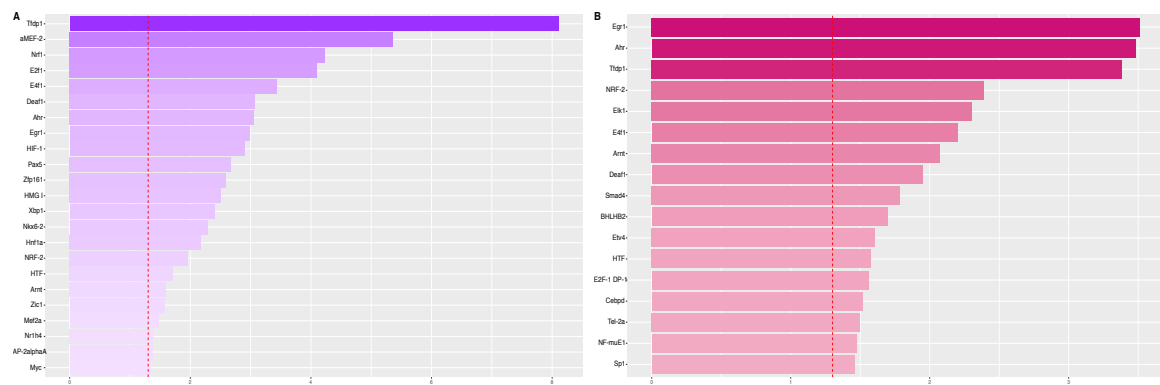


Figure 6.13. A side-by-side assessment of significantly over-represented transcription factor binding site pathways associated with the immunisation of mice with one of two heterologous viral vectored vaccine prime-boost combinations, 24 hours after the boost dose. Gene expression changes induced in mice 24 hours after the boost dose of either a human adenovirus serotype 5 vaccine encoding a factor H binding protein (fHbp) transgene (Ad-fHbp) prime, modified vaccinia Ankara (MVA)-fHbp boost, or an MVA-fHbp prime, Ad-fHbp boost heterologous vaccine combination were contrasted to determine significantly differentially expressed genes (DEGs) associated with each vaccine combination. Pathway over-representation analysis was performed on these DEGs to determine the enrichment of transcription factor binding site pathway categories from the publicly available CisRED database. **A.** MVA-fHbp prime, Ad-fHbp boost. **B.** Ad-fHbp prime, MVA-fHbp boost. The x-axis corresponds to the negative $\log_{10}$ pathway adjusted p -value associated with each transcription factor pathway (y-axis). The dashed red line corresponds to a negative $\log_{10}$ pathway adjusted p -value of 0.05.

6.3.5 Single doses and heterologous prime-boosts of adenovirus and MVA vaccines lead to the enrichment of specific immune cell populations in mouse whole blood within 24 hours

Finally, to compare the transcriptional activity of immune cell populations between the single dose and boost dose groups, a cell deconvolution analysis was performed using significantly DEGs associated with each vaccine/vaccine combination. Statistical comparisons were made between the $\log_2$ -transformed

gene expression values obtained for each vaccine group and the immune cell population phenotypes based on gene expression data from the ImmGen database³⁵⁰. Inferred relative quantities (represented as LFC) for each vaccine/vaccine combination were calculated relative to the naïve control group (Fig. 6.14). Both regimens with MVA-fHbp as the final dose led to an increase in the abundance of CD103⁺ DCs, a subset associated with a migratory phenotype and the ability to cross-present antigen to CD8⁺ T cells⁴²⁵. Ad-fHbp immunisation was associated with the greatest enrichment of bone marrow macrophages, enriched to some degree in all groups, while all vaccines induced macrophages in the mesenteric lymph nodes and non-classical MHC class II-presenting monocytes in the blood. Interestingly, both the Ad-fHbp and MVA-fHbp single dose immunisations led to an increase in splenic NK cells and genes associated with the induction of CD4⁺ and CD8⁺ T cells, the expression of which was not apparent for either prime-boost combination. Taken together, these data demonstrate that specific immune cell populations can be inferred from whole blood gene expression data, allowing further characterisation of the specific immune mechanisms that underlie the responses to commonly used viral vectored vaccine platforms.

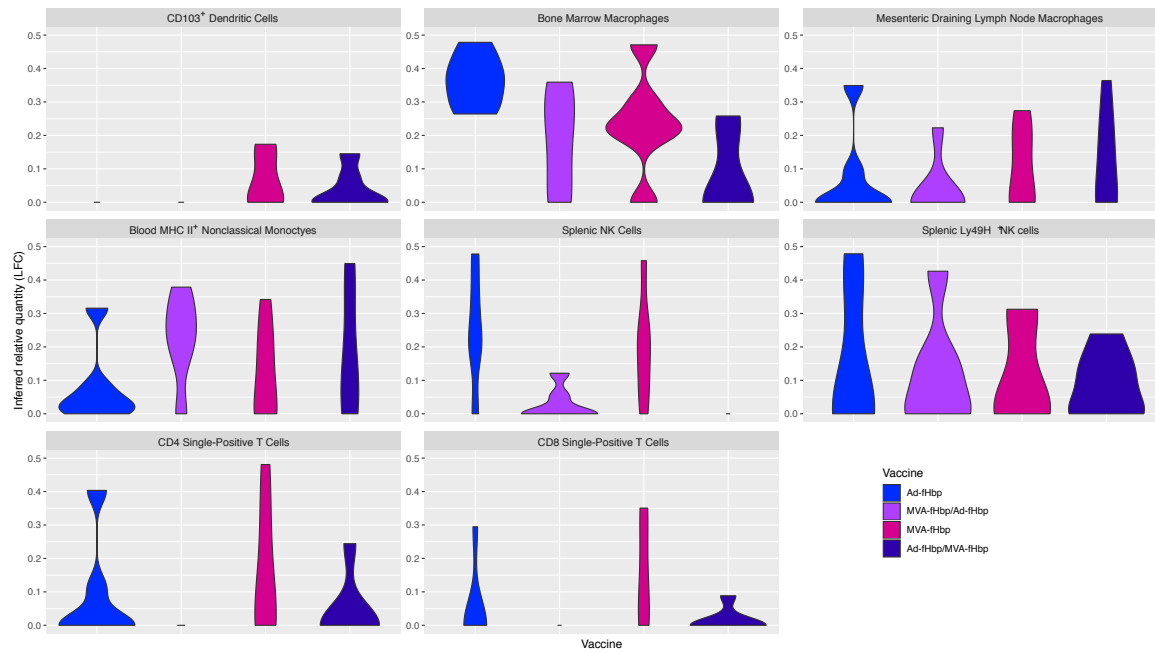


Figure 6.14. Violin plots of immune cell populations inferred from whole blood gene signatures 24 hours after immunisation with each viral vectored vaccine or heterologous prime-boost combination. Inferred relative quantity (IRQ) values, represented as $\log_2$ fold-change (LFC) values, for each immune cell population (y-axis) were calculated relative to the naïve control group (not shown). The most variable immune cell populations relative to this control group are displayed here.

6.4 Discussion

The results presented in this chapter fulfil its main objectives – to elucidate the distinct molecular pathways that underlie early immune responses to two distinct viral vectored vaccine platforms, administered as a single dose or as part of a heterologous prime-boost combination. Whole blood transcriptomics, performed on samples taken 24 hours after the final dose of vaccine, proved sensitive enough to detect changes in gene expression in response to two different transgene products expressed from the same AdHu5 vector. The side-by-side comparison of gene expression data from AdHu5- and MVA-immunised mice revealed differences in both the magnitude and the nature of the transcriptional perturbations induced by these vaccines following intramuscular immunisation. Finally, the comparative approach incorporating the data from mice 24 hours after a heterologous boost expanded on these results to demonstrate distinct patterns of epigenetic modification associated with Ad-fHbp immunisation and further characterised the immunological pathways and cell types engaged by these vaccines. These data contribute to the current understanding of the mechanisms of action of viral vectored vaccines, underexplored in the field of transcriptomics.

As mentioned in the introduction, knowledge of the molecular mechanisms underpinning the immune response to adenovirus vaccine vectors is lacking. However, the molecular response to adenovirus as an infectious agent and vector for gene therapy has been characterised at the cellular level. cDNA microarrays were used in one study of adenovirus type 2-infected HeLa cells and identified the downregulation of several cytokines, including IL-6, and upregulation of stress response genes as consequences of the viral infection ⁴²⁶. Another array-based

study found that gene expression and ontology changes triggered in baby rat kidney cells upon AdHu5 and AdHu12 infection were associated with upregulation of RelA and dysregulated type I collagen expression ⁴²⁷. In human primary lung fibroblasts, a variety of transcription factors related to cessation of growth and induction of inflammation were found to be significantly upregulated over the course of adenovirus infection ⁴²⁸. As for replication-defective AdHu5, microarray analysis of infected human mammary epithelial cells indicated significant upregulation of the genes encoding IL-8, IL-6, NF- κ B, and RELB at 24 hours ⁴²⁹. Primary fibroblasts derived from murine embryos revealed induction of TLR pathways and IFN-stimulated gene expression, subsequently validated in a human A549 knockout cell lines, when infected with the same replication-defective adenovirus ⁴³⁰. At the organ level, the liver has received the most attention as it is the main site of adenovirus sequestration when delivered intravenously (section 1.3.1). Transcriptional response to AdHu5 has been studied in the livers of Syrian golden hamsters, where the virus was found to substantially upregulate the expression of antiviral response genes ⁴³¹, and in the livers of C57BL/6 mice infected with replication-defective adenovirus, transcripts for STAT1, IFN- γ , and IRF1 were found to be significantly upregulated by cDNA array ⁴³². A similar study of the effects of a variety of human adenoviruses on gene expression from the livers of C57BL/6 and knockout mice implicated the MyD88 TLR signal transduction pathway in the immune response to these vectors ⁴³³.

The findings presented in this chapter expand upon the *in vivo* characterisation of gene expression in the context of replication-defective AdHu5 as a vaccine vector, focusing on changes to whole blood transcriptome. The stimulation of TLR pathways and the downstream MyD88:Mal cascade observed for the AdHu5 constructs tested here is in agreement with what was observed in

the aforementioned *in vivo* studies highlighting the central role of TLR signalling via the MyD88 adaptor in the adenovirus vector-triggered innate immune response ^{430,433}. Additionally, the pathway ORAs performed here reveal extensive enrichment of DNA methylation and acetylation pathways. This may be a host response to the curtailment of viral infection, as epigenetic modification has been studied in the context of DNA virus oncogenesis ⁴³⁴, but vaccine-induced changes to the epigenome of immune cells have gained attention in recent years as part of the emerging concept of ‘innate immune memory’ ⁴³⁵. Further to the characterisation of the vector-induced responses, transgene product-specific pathways were elucidated by contrasting the transgene-encoding adenovirus constructs with an empty vector. This comparison demonstrated enrichment of protein transport pathways associated with transgene expression as well as antigen-specific TLR stimulation, type I IFN production, enrichment of CD4⁺ T cell signalling, and BCR signalling, all of which function in the initiation of a humoral response ⁴³⁶. Among the TLRs activated by the delivery of exogenous antigens were TLR9, which plays a role in the activation of pDCs, and TLR2, which drives NF-κB activity, both of which strongly influence the nature of the adaptive immune response through inflammatory signals ⁴³⁷.

MVA-induced gene expression changes have been better characterised than those of adenovirus serotypes used as vaccine vectors. A longitudinal study employing cDNA arrays to assess gene expression changes in MVA-infected HeLa cells showed enrichment of a variety of cytokines and NF-κB signal transduction pathway components ⁴³⁸. A comparison between MVA and a related attenuated poxvirus confirmed these findings in human monocyte-derived DCs and additionally found type I IFN, TLR, and IL-6 pathways to be specifically associated with MVA infection ⁴³⁹. A similar comparison between MVA and other related

poxvirus strains, this time in macrophages, highlighted MVA as being particularly adept at inducing IFN- β and activating innate immune response pathways ⁴⁴⁰. These findings are in line with the characterisation of MVA-induced systemic responses presented here, with MVA-fHbp demonstrating superior induction of type I IFN and corresponding IFN response genes to Ad-fHbp. However, the magnitude of gene expression changes was lower following MVA-fHbp. This may be attributable to differences in antigen expression kinetics associated with these vectors, as has been observed *in vivo* through real-time photon imaging ²²⁶.

Investigative viral vectored vaccine regimens are typically administered as heterologous prime-boost combinations in clinical trials, with the rationale of inducing stronger immune response following exposure to the same antigen while avoiding the possibility of this being dampened by a neutralising and/or cellular memory response to the vector ²¹⁶. This principle was first proven in humans when an MVA vaccine administered three weeks after a plasmid DNA vaccine prime increased antigen-specific CD8⁺ T cell responses five–ten-fold compared with either vaccine given on its own ⁴⁴¹. Since adenovirus vectors have taken centre stage, priming with an adenovirus and boosting with an MVA have become the orthodox approach to prime-boost regimens. This strategy has been used successfully in multiple clinical trials to elicit strong cytotoxic responses to intracellular pathogens ^{244–248}. However, unpublished observations from a pre-clinical study at OVG and the results of recent studies of heterologous prime-boost combinations of viral vectored Ebola vaccines have demonstrated that robust cellular and humoral immunity can be achieved by priming with MVA and boosting with adenovirus, although the initial humoral immune response to the MVA prime is low thus limiting the potential utility of this strategy in an outbreak setting ^{442,443}. A comparison was made between these two prime-boost strategies in the present

study to shed more light on the differential immunogenicity between them. While it appears that the limited immune response elicited by MVA is reflected in the gene expression data, with the number of significantly DEGs approximately 4-fold lower than adenovirus at 24 hours, the transcriptional changes following an MVA boost are similar to the single dose, while the adenovirus prime induces almost double the number of significantly DEGs as it does as a single dose. This would suggest that an MVA prime appears to augment the molecular response to a subsequent adenovirus boost to a greater degree than an adenovirus prime influences an MVA boost. Further characterisation of transcriptional differences between the prime-boost strategies revealed substantial changes to the host epigenetic profile following the MVA-fHbp/Ad-fHbp regimen, as had been observed for the single dose of Ad-fHbp. These epigenetic changes appear to be reflected in the differential usage of TFBS between the two combinations, but further epigenetic profiling would be required to make conclusions based on these observations and attribute markers to specific cell populations. The MVA/Ad prime-boost also appeared to induce greater IFN- γ signalling, stimulation of preferential TLRs, and antigen processing and presentation compared with Ad/MVA. Finally, a comparison of the whole blood immune cell signatures indicates that differences in gene expression may indicate differences in the relative abundance of immune cell populations. Both groups with MVA-fHbp as the final dose were found to induce CD103⁺ DC signatures, indicating potential cross-presentation of exogenous antigen to CD8⁺ T cells ⁴²⁵, while CD4⁺ and CD8⁺ T cell signatures were detected in the single dose groups, suggesting T cell priming activity. The splenic NK cell fraction was most enriched in the single dose groups but the Ly49H⁺ phenotype, associated with an immunological memory response in this cell type ⁴⁴⁴, was enriched across all vaccine groups, particularly those in which

adenovirus was the final immunisation. A recent study of NK cell responses to homologous prime-boost with MVA in cynomolgus macaques has reported modification of the NK cell phenotype following the prime that influences the cytotoxicity of this population after the boost ⁴⁴⁵. A systems vaccinology analysis of the responses to an rVSV-based EBOV vaccine also described a higher frequency of NK cells with specific phenotypic alterations post-immunisation ⁷⁰.

Taken together these data provide many interesting hypotheses for further exploration of the relationship between gene expression changes and the nature of the immunological response to heterologous prime-boost vaccine regimens. These data also provide insight into the molecular mechanisms underpinning the immunogenicity of the adenoviral vectored candidate MenB vaccine antigen in mice ahead of their characterisation in humans.

Chapter 7

Discussion

The modern era of vaccine development has been accompanied by significant technological advances, particularly in the area of sequencing. The advent of whole-genome sequencing ⁴⁴⁶ provided researchers with the means to design candidate vaccines *in silico* using pathogen sequence information and algorithms as opposed to the previously conventional methods of pathogen isolation and attenuation. The first major proof-of-principle for this *in silico* or ‘reverse vaccinology’ approach to vaccine design was the pioneering development of the 4CMenB vaccine from the MC58 *N. meningitidis* reference genome in 2000 ^{194,447}. This advance represented a promising strategy for the development of prophylactic interventions against diseases that conventional strategies had failed to yield. Not only have such technological advances in sequencing allowed for innovation in vaccine design, but they have also been instrumental in shaping our current understanding of how established and candidate vaccines induce protection. RNA-seq technologies have laid the foundations for systems vaccinology by allowing early transcriptional changes in whole blood, PBMCs, and single cells to be profiled in high resolution and serve as predictor variables for subsequent responses to immunisation. The best characterisation of these vaccine-induced responses to date remains the systems analysis of the response to YF-17D immunisation in humans ^{66,67}. The accessibility of pathogen sequence data and the sequence data arising from immunisation of model organisms or human

participants, are integral components of the current era of vaccine development and hold much promise for the development of the next generation of vaccines against ‘difficult’ pathogens.

Biological and technical variation arising from the measurement of low abundance transcripts have been identified as limitations of the transcriptomics component of systems vaccinology analyses ⁴⁴⁸. The issue of dealing with problematic sequence data in the form of machine-generated or technical noise is one of central importance in improving our ability to make interpretations based on data generated from high-throughput sequencing. Sequencing technologies are inherently prone to the generation of ‘noise’ due to known and unknown factors before ³⁰⁸, during ³⁰⁹, and after the sequencing reaction, particularly as the increase in sequencing depth for RNA-seq experiments ³⁰⁵ increases the sensitivity of detection of minor perturbations to the transcriptome between samples ³⁰⁶. Some of the noise introduced in RNA-seq data analyses is contributed by computational choices at the various stages of read processing ³¹¹. Noise can arise from differences between tools based their ability to deal with spliced variants or reads that map to multiple locations on the genome ³⁰⁷. In theory, if noise introduced in the analysis pipeline can be accurately identified in a sample-specific manner it can and be accounted for or removed from the analysis. In chapter 3, a novel method for noise detection was introduced based on the distribution of the p2pPCC ²⁹⁷ versus the abundances of genes with the aim of determining user-defined thresholds of signal-to-noise. This was done by generating expression profiles constructed from the sum of the abundances of reads across the length of a gene from which the point-to-point Pearson correlation between sample-specific expression profiles can be calculated. The specific contributions of RNA-seq read processing tools to computational noise were assessed in a comparative manner by quantifying the

number of genes that fell below a user-defined signal-to-noise threshold for a given combination of read processing tools. Further to this, it was demonstrated that a user-defined threshold-based adjustment approach offers a more empirical alternative to conventional *ad hoc* filtration methods ^{449,450} which do not account for variability within the experiment or additional sample-specific factors ⁴⁵¹. As most statistical methods developed for RNA-seq analysis have focused mainly on mitigating the impact of known biases such as batch effects ⁴⁵², sequence composition ⁴⁵³, and sequencing depth ⁴⁵⁴ on DGE analyses ³¹⁰, the noise detection and adjustment pipeline is instead focused on accounting for unknown sources of noise to refine the determination of genes as DE. The development of such tools for other types of big data, such as scRNA-seq which is subject to considerable technical noise due to the minute quantities of starting material ⁴⁵⁵, will be instrumental in dealing with these data as further technological advances increase the depth and sensitivity of sequencing.

Transcriptomic analysis has been used to evaluate the conservation of innate immune responses across species. The responses to endotoxin and viral nucleic acids have been compared at the cellular and tissue levels between mice, rats, rabbits, pigs, macaques, and humans ³⁶³. This study provided further evidence for evolutionarily conserved patterns of gene regulation underlying inflammatory responses to immune stimuli, a point of contention in the early days of comparative transcriptomics ^{357,358,361}. There is, however, considerable divergence in other aspects of the innate immune response between model organisms, such as mice, and humans ³⁶⁹, particularly in the relative abundance of cell populations and expression of key mediators of antigen recognition ⁴⁵⁶. This is an important consideration when evaluating the toxicity of vaccines and their associated adjuvants in animal models ⁴⁵⁷. The failure of such models to predict adverse events

following immunisation (AEFI) in humans highlights the need for novel methods for identifying inflammatory markers. One approach is based on conserved responses to immunisation in mouse models and other organisms used for the pre-clinical evaluation of preventive interventions ⁴⁵⁸. Systems vaccinology may offer solutions to this dilemma and this approach has recently been applied to assess the early gene expression changes underlying the inflammatory response to a range of well-tolerated licensed vaccines and toxigenic stimuli ⁴⁵⁹. A different approach was taken in chapter 4 whereby a highly reactogenic licensed vaccine was administered on its own and in combination with well-tolerated routinely-administered vaccines to explore the phenomenon of exacerbated reactogenicity following concomitant immunisation ²⁰². A comparative transcriptomic approach was first employed to assess the conservation in whole blood transcriptional changes induced by this vaccine regimen between the mice and human infants after receiving the same vaccine combination, validating the use of the mouse immunisation model to further explore 4CMenB-induced reactogenicity. The comparative assessment of the vaccine components and other related vaccines and immune stimuli led to the attribution of a pro-inflammatory gene signature to the outer membrane vesicle (OMV) component of the vaccine, and complementary systems analyses of physiological temperature changes, cytokines in serum, and gene expression on brain tissue attributed the reactogenicity of the vaccine specifically to the membrane bound hexa-acylated endotoxin. These data demonstrate the utility of the systems vaccinology approach for the elucidation of the immunological pathways underlying and the causal components of vaccine AEFI that would be difficult to assess in detail in a human study. This rationale was applied to investigate the cause of febrile reactions associated with an influenza vaccine; a variety of model organisms underwent transcriptional and immune profiling with

the aim of identifying molecular signatures that would explain the phenomenon observed in a paediatric cohort ⁴⁶⁰. It has also been shown that non-human primate innate immune response gene expression patterns and cytokine production following immunisation correlate well with AEFI observed in a human study ⁴⁶¹. By collecting, correlating, and standardising data acquired from pre-clinical systems vaccinology analyses, it may be possible to establish robust biomarkers for more accurate prediction and investigation of AEFI in humans ⁴⁶².

The reactogenicity of the 4CMenB vaccine, while not severe or concerning enough to affect its recommendation or uptake, nonetheless has the potential to deter hesitant parents in the future as disease incidence declines and the risk of AEFI outweighs the occurrence of an increasingly rare infection in their minds. This story has played out in the case of the whole cell pertussis vaccine where concerns about safety forced the development of a better tolerated, though less effective, vaccine ⁵⁵. Two important lessons can be learned from this saga, the first being that vaccine reactogenicity and AEFI should be thoroughly investigated prior to and monitored continuously after licensure to mitigate public concern, and second, it is crucial that alternative vaccines do not sacrifice effectiveness for benign and transient adverse events. However, 4CMenB presents the additional considerations of multiple doses being required to induce protection, waning antibody titres ^{212,213}, and the economic challenges of introducing immunisation programmes, particularly for teenagers ²⁰⁷. Chapter 5 presents a candidate vaccine antigen that is currently being assessed for safety and immunogenicity in a phase I clinical trial. If the adenovirus vector-based novel vaccine performs as well in humans as the pre-clinical studies of the vaccine antigen would suggest, this would represent a starting point for the development of a viable alternative to the other two licensed vaccines containing fHbp, 4CMenB and MenB-fHbp. The FL SS fHbp

antigen is capable of inducing protective antibody titres more rapidly and with greater persistence than 4CMenB after a single dose of AdHu5 containing the transgene in mice. Given the fulminant nature of the disease and the high incidence within the first months of life, the early induction of bactericidal antibody is particularly important for protecting against invasive meningococcal disease (IMD). Adenovirus-based vaccines, non-human or rare serotypes in particular, have both an excellent safety and immunogenicity profile in humans, as evidenced by their use in numerous clinical trials ⁴⁶³, and their production is relatively inexpensive making them an attractive platform for cost-effective immunisation programmes ⁴⁶⁴. However, an adenovirus-based MenB vaccine would have to incorporate at least one more antigen in order to increase the coverage of such a candidate vaccine, and a demonstrable impact on colonisation and carriage would certainly be a favourable attribute of any alternative to the currently licensed vaccines. Nonetheless, the reductive analyses presented in chapter 5 demonstrate the impact of the N-terminal signal sequence that forms part of this antigen and further experiments placing this sequence ahead of heterologous bacterial antigens demonstrates that this immune-enhancing sequence has the potential for inclusion in other candidate vaccine antigens. This chapter adds to a growing body of literature characterising the transgene expression kinetics of adenovirus vectors and how these relate to immunogenicity ^{226,234,237,465,466} and demonstrates the impact of transgene sequence on influencing these kinetics, and therefore the antigen-induced immune response. These data also provide further examples of the application of signal peptide sequences to enhance processing, presentation, and immunogenicity of antigens ^{387,392,395}. This work exemplifies how increased availability of pathogen sequence data, novel vaccine platforms, and functional

characterisation of the immune response to antigens are critical elements of rational vaccine design in the current era of vaccinology.

Building on from this work is the characterisation of the gene expression responses induced by the pre-clinical vaccine candidate to better understand the molecular mechanisms of novel vaccine platforms, another key aspect of contemporary vaccine design, presented in chapter 6. By comparing empty and transgene encoding adenovirus vectors, the transgene product-specific transcriptional response could be delineated relative to the vector-specific response. In doing so, several immunological pathways were defined that may be of functional relevance to the immunogenicity observed following Ad-fHbp immunisation in mice. By applying this approach to other viral vectored antigens, the optimal immune response for a particular disease can be determined through comparative transcriptomic assessment of antigens encoded by the same viral vector. A distinct response was observed for the RSV prefusion F antigen, demonstrating that this approach is sufficiently sensitive to distinguish the host responses to transgene products, even at the pathway level. In addition to defining the transcriptional response to the candidate MenB vaccine antigen in mice, the molecular mechanisms underlying the response to this antigen encoded by a heterologous viral vector and prime-boost combinations of both vaccines were defined in this chapter. Adenovirus and MVA vectors are associated with distinct expression kinetics which are known to influence humoral immunity by differential induction of germinal centre responses, with adenovirus exhibiting far greater stimulation of antigen-specific B cell and follicular helper T cell (T_{FH}) responses than MVA ²⁵⁰. This difference may be reflected in the greater enrichment of innate immune pathways observed in adenovirus-immunised mice, such as TLR2 and TLR9 stimulation ⁴³⁷ and production of IFNs known to act on B cells ⁴³⁶, that

precede these adaptive immune responses. On the other hand, MVA appeared to stimulate greater cytotoxic T cell response pathways, in line with what is observed by *in vivo* analysis of vector kinetics and corresponding immune responses ²²⁶. The immune response to adenovirus prime, MVA boost vaccine regimens has been characterised in numerous clinical trials and found to potently induce CD8⁺ T cell and antibody responses ^{240,247,467,468}. The immunological pathways enriched by this and the alternative MVA prime, adenovirus boost regimen were also characterised and the gene expression changes induced by the latter were of greater magnitude than the conventional combination, with profound differential epigenetic regulation characterising the augmented response to the adenovirus when administered as a boost. Further epigenetic profiling, beyond the scope of the current study, will be required to validate these findings. The observation of robust immune responses induced by this vaccine combination has been of recent interest ^{442,443} and may be more suited to diseases that require less immediate protection.

In conclusion, the work presented in this study represents the application of several conventionally employed methods in 21st century vaccinology toward the improved understanding of licensed and novel vaccines, with the ultimate aim of informing the rational design of better tolerated and more immunogenic vaccines. The use of transcriptomics, both exploratory and comparative, was central to the current study and its application was refined by the development of a novel pipeline to reduce the influence of problematic data on conclusions drawn from these studies. This pipeline has the potential to be commonly used in transcriptomic analyses, improving the comparability of datasets to which different computational methods are applied. By using a licensed MenB vaccine as a starting point, recapitulating observations from a human study in a mouse immunisation model, and defining aspects of the inflammatory response that are

common to preparations that induce fever but absent from those that dissociate reactogenicity from immunogenicity, the undesirable characteristics of the vaccine were elucidated. These data will inform antigen selection for meningococcal or OMV-based vaccines that are well-tolerated and minimise side effects. The functional characterisation of a novel vaccine antigen, rationally designed through modification of the antigen's binding domain and N-terminal signal sequence, enabled the understanding of how this antigen's sequence influences its expression and contributes to its superior immunogenicity. The signal sequence has demonstrated immune-enhancing effects when incorporated ahead of other adenoviral vectored bacterial antigens, making its inclusion a strong consideration for other candidate vaccines of this nature. Finally, by returning again to whole blood transcriptomics, the early gene expression changes induced by a less reactogenic and more immunogenic meningococcal vaccine than 4CMenB in mice were determined. Validation experiments will be designed around these findings to investigate the pathways associated with the Ad-fHbp-induced and antigen-specific innate immune response – these data will lay the foundations for further delineation of the functional genomics associated with ChAdOx-fHbp in humans.

Future directions

The next step will be to comparatively assess the whole blood gene signatures associated with ChAdOx1-fHbp, 4CMenB, and MenB-fHbp in humans following completion of the ongoing phase I trial at OVG. This will provide a comprehensive analysis of the early gene expression changes that underlie, and

potentially predict, the degree to which these distinct vaccine platforms induce protective immune responses in humans.

By performing RNA-sequencing on whole blood samples taken from participants prior to and 24 hours after each dose of vaccine and conducting differential gene expression analyses, we aim to address the following questions: are there unique gene signatures associated with each vaccine that explain differences in their immunogenicity and/or reactogenicity? Can a 24 hour post-immunisation gene signature be used to predict protective SBA titres and T cell responses measured at later timepoints? Can these genes tell us more about the innate immune responses that shape the desired adaptive immune responses to meningococcal vaccines?

Comparative transcriptomic analysis of whole blood gene signatures induced by the three distinct vaccines – 4CMenB, MenB-fHbp, and ChAdOx1-fHbp – will define the immunological pathways favourable for a protective immune response against meningococcal disease and highlight those associated with the reactogenicity of the licensed vaccines. The findings will support development and selection of the best vaccines for children. By identifying genes that correlate with clinically relevant outcomes, we will define gene signatures that are predictive of vaccine immunogenicity with the aim of establishing alternative correlates of protection for meningococcal vaccines development that can be measured at earlier time points post-immunization than the gold standard SBA.

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