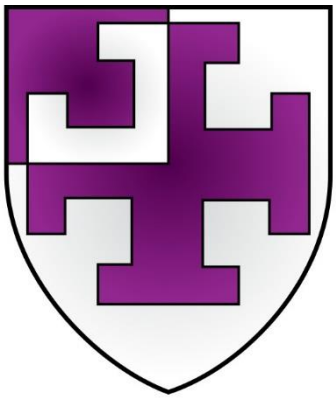
CHARACTERISING IMMUNE RESPONSES TO VIRAL VECTORED VACCINES AGAINST INFLUENZA AND HEPATITIS C



Dr Richard Antrobus, MBChB, BMedSc, MRCP, St Cross College and,

The Jenner Institute, Nuffield Department of Medicine, University of Oxford

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ABSTRACT

CHARACTERISING IMMUNE RESPONSES TO VIRAL VECTORED VACCINES AGAINST INFLUENZA AND HEPATITIS C

Thesis submitted for the degree of Doctor of Philosophy, Trinity Term 2014

Dr Richard Antrobus, St Cross College, University of Oxford

For both influenza viruses and hepatitis C viruses, T cell responses to conserved antigens are one strategy for the human host to control the spread of infection. Such T cell responses can be generated with the use of viral vectored vaccines.

Initially I show that the viral vectored vaccine MVA-NP+M1 can boost memory T cell responses to influenza A virus in adults aged over 50 years old. However within this group, MVA-NP+M1 had reduced immunogenicity in adults who were aged over 70 years old. The influenza virus-specific T cell responses comprised both CD4 and CD8 T cells, and were capable of secreting multiple Th1 cytokines. I then show that MVA-NP+M1 can be safely co-administered alongside seasonal influenza vaccine. The combination does not interfere with the peak T cell response that normally occurs 1 week following MVA-NP+M1. There was a statistically significant increase in antibodies to the H3N2 strain when the vaccines were co-administered, suggesting that the MVA-NP+M1 can act as an adjuvant.

The efficacy of MVA-NP+M1 in humans had been previously evaluated in an influenza virus challenge study. I used a whole blood transcriptome approach to improve the classification of outcomes following influenza virus challenge. For subjects with laboratory-confirmed influenza, individuals with moderate/severe symptoms were found to have a distinct transcriptional signature comprising over 2,000 genes. I used a machine learning algorithm to reduce this variation down to just six genes (CCL2, SEPT4, LAMP3, RTP4, MT1G and OAS3). I validated this finding using expression data from an independently conducted challenge experiment. Data from these six genes was successfully able to predict symptomatic and asymptomatic cases with 89% and 100% accuracy respectively.

To induce T cell responses to hepatitis C virus, I used the vaccines ChAd3-NSmut and MVA-NSmut in a prime-boost regimen. While the combination was highly immunogenic in healthy young adults, MVA-NSmut alone was unable to prime immune responses. The magnitude of T cell responses to the vaccine immunogen was correlated with the breadth of the T cell responses to different epitopes. Re-administration of the same two vaccines after a short time interval (8 weeks) did not improve upon previous peaks in T cell response. However with a longer time interval (> 34 weeks), some individuals were able to achieve higher frequencies of virus-specific T cells compared to the first round of vaccines. A whole blood transcriptome approach was used to study gene expression in volunteers vaccinated with ChAd3-NSmut and MVA-NSmut. Vaccination with MVA-NSmut results in a very strong, but relatively short-lived host gene expression signature. In contrast, the transcriptional response seen following ChAd3-NSmut was much less pronounced. A comparison of the functional analysis of gene lists from both vaccines showed that similar pathways were being activated and repressed.

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STATEMENT OF ATTRIBUTION

IFN-γ ELISpot assays

In the FLU001 extension trial approximately half of the IFN-γ ELISpot assays were performed by the author, with the majority of the remainder being performed by Dr T Berthoud. In the FLU003 trial the majority of IFN-γ ELISpot assays were performed by Dr T Berthoud and Pooja Mange. For the HCV003 trial IFN-γ ELISpot assays were performed by Anthony Brown, Rachel Townsend and Leo Swadling.

Anti-TIV ELISAs

Anti-TIV ELISAs were performed in the Jenner laboratories by Caitlin Mullarkey.

HI titres

HAI assays were performed at the laboratories of the Health Protection Agency/Public Health England by Claudia Rosenow, Surita Gangar and Janice Baldevarona.

Flow cytometry

Approximately one-quarter of samples were assayed by the author with the assistance of Dr A Spencer. The remainder of samples were assayed by Dr T Berthoud.

RNA extraction

RNA extractions and enrichments for FLU002 clinical trial samples were performed by Emma Davenport. All RNA extractions and enrichments for FLU003 and HCV003 clinical trial samples were performed by the author.

Microarray hybridization

Hybridisation of RNA to bead chip arrays and scanning was performed by Dr Dilair Baban, High-Throughput Genomics, WTCHG, Oxford.

PUBLICATIONS ARISING FROM THIS RESEARCH

Antrobus RD, Lillie PJ, Berthoud TK, Spencer AJ, McLaren JE, Ladell K, Lambe T, Milicic A, Price DA, Hill AV, Gilbert SC. A T cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years. PLoS One. 2012; 7(10): e48322.

Antrobus RD, Berthoud TK, Mullarkey CE, Hoschler K, Coughlan L, Zambon M, Hill AV, Gilbert SC. Coadministration of seasonal influenza vaccine and MVA-NP+M1 simultaneously achieves potent humoral and cell-mediated responses. Molecular Therapy. 2014; 22(1): 233-8.

Davenport EE, Antrobus RD, Lillie PJ, Gilbert S, Knight JC. Transcriptomic profiling facilitates classification of response to influenza challenge. Journal of Molecular Medicine. 2015; 93(1): 105-14.

Swadling L, Capone S, Antrobus RD, Brown A, Richardson R, Newell EW, Halliday J, Kelly C, Bowen D, Fergusson J, Kurioka A, Ammendola V, Del Sorbo M, Grazioli F, Esposito ML, Siani L, Traboni C, Hill A, Colloca S, Davis M, Nicosia A, Cortese R, Folgori A, Klenerman P, Barnes E. A human vaccine strategy based on chimpanzee adenoviral and MVA vectors that primes, boosts, and sustains functional HCV-specific T cell memory. Science Translational Medicine. 2014; 6(261): 261ra153.

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ABBREVIATIONS

Ad6	Adenovirus 6
ChAd3	Chimpanzee adenovirus 3
ChAd63	Chimpanzee adenovirus 63
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DEG	Differentially expressed genes
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
FITC	Fluorescein isothiocyanate
GCP	Good clinical practice
GMP	Good manufacturing practice
GMR	Geometric mean ratio
GMT	Geometric mean titre
GO terms	Gene ontology terms
GSK	GlaxoSmithKline plc.
GTAC	Gene therapy advisory committee
GWAS	Genome-wide association studies
HA	Haemagglutinin
HAI	Haemagglutination inhibition (assay)
HCV	Hepatitis C virus
HLA	Human leukocyte antigen

IL	Interleukin
ILI	Influenza-like illness
IFN-γ	Interferon gamma
IRF	Interferon regulatory factor
IRP	Immune Risk Phenotype / Profile
ISG	Interferon-stimulated genes
LDL	Low density lipoprotein
LCI	Laboratory-confirmed influenza
M1	Matrix protein 1
M2	Matrix protein 2
MHC	Major histocompatibility complex
MIV	Monovalent inactivated influenza vaccines
MHRA	Medicines and Healthcare Products Regulatory Agency
MVA	Modified Vaccinia virus Ankara
NA	Neuraminidase
NP	Nucleoprotein
NS	Non-structural
ORF	Open reading frame
PCA	Principal components analysis
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cells
PE	R-Phycoerythrin
PFU	Plaque forming units
PHA	Phytohaemagglutinin

PKR	Protein kinase R
QIV	Quadrivalent inactivated influenza vaccine
RIG-I	Retinoic acid-inducible gene 1
SEB	Staphylococcal enterotoxin B
SNP	Single nucleotide polymorphism
SFC	Spot forming cells
SFU	Spot forming units
TIV	Trivalent inactivated influenza vaccine
TLR	Toll-like receptor
TRIM	Tripartite motif-containing protein
VLDL	Very low density lipoprotein
VP	Virus particles
VSN	Variance Stabilization and Normalization
WHO	World Health Organisation
WTCHG	Wellcome Trust Centre for Human Genetics

CHAPTER 1: INTRODUCTION

1.1 INFLUENZA VIRUS

Influenza viruses (A, B and C) are members of the *Orthomyxoviridae* family of RNA viruses. All three viruses share the characteristics of having segmented, negative-strand RNA genomes, however over the course of evolution they have diverged to the extent that they can no longer successfully exchange gene segments between these different types (Bouvier and Palese, 2008). Influenza A virus is responsible for the majority of human influenza disease, however the virus is zoonotic with several reservoirs in other species. Aquatic birds harbour most types of influenza A viruses, but reservoirs also exist in pigs, horses, dogs and seals (Nicholson et al., 2003). There is considerable diversity amongst influenza A viruses, fuelled by the lack of proof reading from its RNA polymerase enzymes. Influenza B viruses mutate slower and are antigenically less diverse compared to influenza A viruses. However they still contribute to the burden of influenza disease, particularly in children and young adults (Lambe, 2012). Influenza C virus is distinct from A and B viruses both in terms of its physical and genetic structure, and while capable of causing typical disease in humans, it contributes least to the overall number of cases compared with A and B viruses.

The classification of influenza A viruses into subtypes is based on the antigenic properties of the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). There are currently 17 HA subtypes and 9 NA subtypes known (Lambe, 2012), however only a minority of these circulate in humans. For the last four decades H1N1 and H3N2 have been the two dominant strains in humans. However H3N2 only took over from H2N2 in 1968, which in turn only emerged in 1957 (Palese, 2004). In contrast to this diversity amongst influenza A viruses, only

one subtype of haemagglutinin and neuraminidase is recognised for influenza B virus (Nicholson et al., 2003).

1.1.1 INFLUENZA VIRUS EPIDEMIOLOGY

In temperate regions of the world including the United Kingdom, influenza virus activity occurs in seasonal cycles, with peak incidence (in the UK) occurring between November and February. The reasons behind this pattern of activity are not well understood, but potential contributing factors include seasonal variation in host susceptibility (e.g. vitamin D levels), environmental factors (e.g. vapour pressure) and social factors (e.g. time spent indoors during winter) (Lipsitch and Viboud, 2009). Even factors which only make small contributions to the transmission rate have the potential to result in a magnified effect. This phenomenon is called dynamic resonance (Dushoff et al., 2004). It occurs because immunity to influenza viruses is not permanent. As influenza viruses drift (see section 1.3.3), immunity is lost and there is a build-up of susceptible individuals, but when an epidemic does occur, it leads to the rapid depletion of susceptible individuals who become immune after recovering from the disease. If influenza was simply associated with wintry conditions (low vapour pressure, low temperature, short days etc.) then that would imply that influenza in the tropics was rare (Lipsitch and Viboud, 2009). This is not the case, although patterns of activity can vary between different climates. For example in subtropical Southeast Asia there are biannual epidemic cycles (with January/February being the main peak in incidence and a second peak occurring in July/August) and furthermore in some equatorial regions there is a lack of seasonality altogether (Lipsitch and Viboud, 2009).

In addition to causing seasonal epidemics, the influenza virus has the potential to cause global pandemics. The strains with pandemic potential are those which have only recently emerged from animal reservoirs of influenza viruses. Thus, within the human population, there is a very low prevalence of pre-existing neutralising antibodies and a high proportion of the population who would be susceptible to infection. There were three pandemics in the course of the twentieth century and there has already been one pandemic in the twenty-first century. Pandemic influenza is a major global threat, with wide ranging impacts. It has been estimated that during a pandemic UK gross domestic product could fall by some 0.75% and that the overall cost to the nation could be in the region of £170 billion (Coker, 2007).

One of the potential dangers of pandemic influenza is for the case fatality ratio to be significantly higher than that of seasonal influenza. In the 2009 H1N1 pandemic, the case fatality ratio was only 0.015-0.025% (Presanis et al., 2011) however for the 1918 Spanish 'flu, the case fatality ratio was approximately 2% and resulted in an estimated 40 million deaths over 2 years (Mills et al., 2004). The case fatality ratio of H5N1 highly pathogenic avian influenza has been over 50%, and therefore it would be a grave concern if this virus were to develop into a pandemic.

The population cohorts most at risk from pandemic influenza can be different to those of seasonal influenza, for example the risk of severe disease can be higher in pregnant women or young children (Nguyen-Van-Tam et al., 2010). Nevertheless the elderly are still at risk, particularly as they are likely to have a greater prevalence of pre-existing medical co-morbidities.

1.1.2 LABORATORY MODELS OF INFLUENZA

Before continuing to describe how the human host responds to influenza virus infection, it is important to consider the merits of the various animal models which have been the basis of so much research on influenza. Viral attachment and the subsequent infection of host cells is dependent on receptor specificity. Attachment is mediated by influenza virus haemagglutinin binding to sialic acid residues, however the nature and distribution of sialic acid molecules varies between species and varies between cell types within an individual host (Matrosovich et al., 2004). Thus, the manifestations of influenza disease vary according to the species it infects. For example, many types of influenza virus circulate in aquatic birds such as ducks and in these birds infections are frequently asymptomatic (Magor, 2011). The site of infection in ducks is the intestine rather than being (in the case of humans) a disease of the respiratory tract. Avian influenza viruses affect the intestine because duck epithelial cells exclusively express sialic acid with alpha 2,3 galactose linkages, and these linkages are the preferential targets for binding (Magor, 2011).

Human influenza viruses show preferential binding to sialic acid molecules with alpha 2,6 galactose linkages. These glycoproteins are mostly expressed on non-ciliated cells and are more prevalent in the upper respiratory tract. Hence influenza is a disease of the upper respiratory tract in humans. However, cells of the human lower respiratory tract (and to a lesser extent cells of the upper respiratory tract) also express sialic acid with alpha 2,3 galactose linkages (O'Donnell and Subbarao, 2011). Hence, avian influenza viruses can infect human hosts and may result in pneumonitis. The above viral tropism is important in understanding how well animal models pertain to the human situation, because the distribution of sialic acid residues in a species will govern the pattern of disease (O'Donnell and Subbarao, 2011).

Mice

Mice are the most commonly used experimental model for influenza. They have the advantages that they are relatively inexpensive and easy to work with. There is an abundance of reagents to help study their responses to infection, and there are well characterised inbred strains with which to work. However, different strains respond heterogeneously to infection, partly because there are strain-dependent differences in the galactose linkages used by sialic acid receptors, but also because host resistance to infection is in part genetically determined. Regardless of the strain, mice are not natural hosts to influenza viruses and do not develop a pattern of disease that mimics the human phenotype. Specifically, while they can develop lower respiratory tract symptoms they will not develop upper respiratory tract symptoms. For the purposes of challenge experiments the typical outcome measures are either weight loss or lethality (O'Donnell and Subbarao, 2011).

Ferrets

Influenza disease in ferrets is more comparable to influenza disease in humans, with animals developing sneezing, coughing and rhinorrhoea. This pattern of disease probably reflects the distribution of sialic acid galactose linkages which is similar to that of humans. In addition to symptom quantification it is possible to measure weight loss, lethality, activity and vital signs as outcome measures. Unfortunately, as ferret models are not used for many other diseases, the animals are relatively expensive and available only from a limited number of suppliers. They require specialist housing, are outbred, and there are limited ferret-specific reagents that can be used to study their responses (O'Donnell and Subbarao, 2011).

Non-human primates

Non-human primates (NHPs) are the closest match to humans in terms of their genetics and physiology. However, there are significant differences between the sialic acid receptor usage between NHPs and humans, which may contribute to the fact that NHPs are less susceptible to infection with human influenza virus strains. This susceptibility also varies between the limited number of NHP species available for use in the laboratory. NHPs are both expensive and difficult to work with, partially due to the restrictions in place for their use (O'Donnell and Subbarao, 2011).

Pigs

Domestic pigs are an important host of influenza A viruses, and it has been suggested that they have facilitated the emergence of human pandemic influenza viruses (Vijaykrishna et al., 2011). Specifically the pandemics of 1957, 1968 and 2009 were caused by a reassortment influenza virus that had been generated in pigs (Crisci et al., 2013). Pigs are one of the natural hosts of influenza viruses, with H1N1, H1N2 and H3N2 being the causative subtypes (Crisci et al., 2013). Pigs are susceptible to infection with both human influenza viruses and avian influenza viruses (Crisci et al., 2013). While all the three subtypes mentioned have been associated with influenza disease, subclinical infection is very common and many, if not most infected pigs do not demonstrate any physical signs of illness (Crisci et al., 2013).

The sialic acid receptor distribution is very similar between humans and pigs, i.e. alpha 2,6 galactose linkages predominate in the upper respiratory tract, whereas alpha 2,3 galactose linkages are restricted to the lower respiratory tract (Meurens et al., 2012).

- In comparison to mice, porcine models are far more similar to humans in terms of their anatomy and physiology (Meurens et al., 2012). Importantly for the study of

infectious diseases, pigs are also more similar to humans immunologically. This is true across a wide range of immunological parameters from cytokines (such as IL-8) to the distribution of viral pattern recognition receptors (including TLR-7 and TLR-9) (Meurens et al., 2012).

- In comparison to non-human primates, porcine models are considered more ethically acceptable and are significantly cheaper to work with (Meurens et al., 2012). They are also easier to breed, with shorter gestation periods and generation intervals, and have larger litter sizes (Meurens et al., 2012).
- In comparison to the ferrets, porcine models have the advantage that there are a much wider range of immunological reagents and methods available to use. Pigs are also available in both inbred as well as outbred strains (Meurens et al., 2012).

1.1.3 HOST SUSCEPTIBILITY AND IMMUNITY TO INFLUENZA VIRUSES

Antigenic drift and antigenic shift

As an RNA virus, influenza viruses have a high mutation rate due to the lack of proof reading from their RNA polymerase enzymes. Host resistance or susceptibility is largely determined by the presence or absence of neutralising antibodies to the influenza virus HA molecules. Over time, point mutations accumulate which alter glycosylation at exposed sites of the HA molecules, and these confer a selection advantage to the virus – a process called antigenic drift.

Pandemic influenza occurs on the infrequent occasions when the virus undergoes an antigenic shift, as opposed to drift which is constantly occurring. Antigenic shift describes a major re-assortment of the genetic material between two different influenza virus subtypes.

This may occur when two genetically diverse influenza viruses infect the same host cell. The segmented genetic material from the two viruses is replicated and is then free to assemble into new daughter particles. Pre-existing immunity to these new antigens will be negligible and therefore the majority of the population will be susceptible to infection. This therefore explains why attack rates are generally higher in pandemic influenza compared with a typical winter influenza epidemic.

Humoral immunity to influenza viruses

The natural immune response to influenza virus involves the generation of neutralising antibodies targeted against haemagglutinin epitopes on the viral spike. While these antibodies mediate host protection, they do not provide long-lasting immunity (Nabel and Fauci, 2010) due to the antigenic drift of the virus as discussed earlier. The haemagglutinin viral spike consists of two structural regions, the head and the stem; the latter being antigenically more conserved between drifted strains. While natural exposure and seasonal influenza vaccination result in HA-specific antibodies directed against the head region, more broadly neutralising antibodies can be induced by focusing the humoral immune system on the haemagglutinin stem region (Nabel and Fauci, 2010).

While HA-specific antibodies are able to confer protection against influenza viruses, other types of antibody responses are also recognised as being important to host defence (Dormitzer et al., 2011). Although not neutralising, neuraminidase-specific antibodies can impair viral replication and reduce the severity of disease upon infection (Marcelin et al., 2012). One example of the clinical significance of anti-NA antibodies comes from observations of the 1968 pandemic. This event heralded a major change to the dominant circulating strains,

with H3N2 replacing H2N2. During the pandemic, individuals who had higher titres of anti-NA antibodies were both less susceptible to disease, and if infected had a reduced likelihood of suffering with severe symptoms (Monto and Kendal, 1973).

Mucosal immunity

Secretory IgA is the predominant immunoglobulin isotype at mucosal surfaces, as unlike IgG it can be translocated across epithelial cells (Janeway et al., 2008). As the influenza virus is spread through air-borne droplets via the respiratory tract, specific IgA is believed to be important for host defence at mucosal sites. Many murine studies support the importance of mucosal IgA responses in protection against influenza virus infection and disease (Arulanandam et al., 2001). Parentally administered influenza vaccines produce weak IgA responses at mucosal sites, hence there is considerable interest in developing novel vaccines that are delivered intra-nasally (Vajdy et al., 2007).

The UK have recently introduced the live attenuated influenza vaccine (see section 1.1.4) into the NHS childhood vaccination programme. A meta-analysis of high quality randomised controlled trials has shown that the live attenuated influenza vaccine has moderate to high efficacy (70-90%) in children aged 7 years and younger (Osterholm et al., 2012). This efficacy is seen despite the fact that the live attenuated influenza vaccine does not reliably cause increases in serum HI titres. However, mucosal immune responses are induced. In one study using FluMist, a third of vaccinated subjects developed a ≥2-fold increase in influenza virus-specific IgA antibodies from nasal wash samples (Barría et al., 2013). In addition, the expression of interferon-induced genes (including CXCL10 (IP-10) and MX1) was increased in the nasal wash cell pellet, suggesting that the vaccine had induced a biologically relevant inflammatory state (Barría et al., 2013).

Innate immune responses to influenza viruses

If a human host does not have sufficient concentrations of specific antibody to neutralise the virus, infection will occur in cells of the upper respiratory tract. The innate immune system is able to respond immediately to influenza virus infection by recognising pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). These consist of both cytoplasmic molecules and cell surface receptors and are germline encoded. Somatic recombination of gene segments, which characterises the adaptive immune response, is therefore not required.

Epithelial cells of the human respiratory tract are able to send danger signals when infected by influenza viruses. The cytoplasmic RNA recognition protein, RIG-1 and Toll-like receptor (TLR)-7 are the key sensory pathways for these epithelial cells (Tripathi et al., 2013). RIG-1 detects 5' capped single-stranded RNA in the cytoplasm, and then interacts with MVAS, NFKB and IRF3 to result in the secretion of pro-inflammatory cytokines. Specifically, infection of human alveolar type II cells with influenza virus results in the secretion of large quantities of interferon-lambda, also known as IL-29 or type-III interferon (Tripathi et al., 2013).

The innate immune system also consists of specialised cells, which may either be resident in, or be recruited to the sites of infection. The most important resident innate cells for influenza viruses are thought to be alveolar macrophages, however dendritic cells also have a key role (Tripathi et al., 2013). Neutrophils, monocytes, natural killer cells, as well as further dendritic cells can then be recruited to the site of infection. Plasmacytoid dendritic cell (pDCs) are thought to be crucial to innate immune response to viruses. They express a range of TLRs including both TLR7 and TLR9 in the endosomal compartment which allows for the detection of viral RNA and viral DNA respectively (Bao and Liu, 2013). Upon detection of viral infection, pDCs are capable of secreting massive quantities of type I interferons (Reizis et al., 2011).

However, when the role of pDCs have been explored in murine models of influenza, they have been found to be dispensable for the control of disease. Compared to wild type mice, pDC deficient mice experienced a similar degree of weight loss and a similar extent of lung pathology (Wolf et al., 2009).

T-cell responses to influenza viruses

The adaptive immune response to influenza virus results in the generation of antigen-specific CD4 and CD8 T cells. The principal function of CD8 T cells, or cytotoxic T lymphocytes (CTLs), is to kill virally infected cells prior to the release of further viable virions. Hence CTLs can contain, control and eliminate an established infection, but do not provide a means of sterile protection. CD4 T cells provide help to B cells and are therefore essential to the production of class switched high affinity neutralising antibodies.

While protective neutralising antibodies are focussed on the antigenically most variable parts of the virus, the immunodominant peptides for CTLs are derived from internal influenza virus proteins. The amino acid sequences of these proteins are relatively conserved between influenza virus subtypes because significant variations in their sequence would affect enzymatic functions (or other core functions) and therefore viral fitness. The importance of this for influenza vaccinology is that CTL responses can theoretically provide heterosubtypic immunity.

Evidence for heterosubtypic immunity by CTLs begins by demonstrating that CTLs can be cross-reactive to different influenza virus subtypes. There are many studies, both in mouse models and in humans, which show that CTLs can be cross-reactive by using either ELISpot assays (Lee et al., 2008, Weinfurter et al., 2011) or more traditionally, cytotoxicity (Chromium-51 release) assays (Hillaire et al., 2011, McMichael et al., 1983b).

Evidence for the protective effect of cross-reactive influenza virus-specific T cells against infection has also been demonstrated, albeit mostly in animal models (Weinfurter et al., 2011). Influenza virus-specific T cells adoptively transferred to naïve recipients, result in increased rates of protection against influenza virus challenge both in mice and in chickens. Equally, when primed animals are depleted of influenza virus-specific T cells, influenza virus challenge results in worse outcomes (Hillaire et al., 2011).

Although limited, there is some evidence from human studies that T cells can protect against influenza disease. For many years the only such evidence was a study by McMichael et al. which showed an association between T cell cytotoxicity (in chromium release assays) with protection from influenza virus challenge (McMichael et al., 1983b). More recently, Wilkinson et al. have performed influenza virus challenges with either H1N1 or N3N2 and managed to correlate the magnitude of CD4 T cells specific for nucleoprotein and matrix protein with protection (Wilkinson et al., 2012).

Thus the rationale exists for a vaccination strategy whereby strong T cell responses directed to the conserved antigens of influenza A viruses are stimulated. A vaccine which could provide protection across heterosubtypes (i.e. heterosubtypic immunity) is sometimes referred to as a *universal* influenza vaccine.

1.1.4 INFLUENZA VACCINES

Current influenza vaccination strategies

Seasonal influenza vaccination programmes are used in many countries to lessen the burden of winter epidemics. Two principal types of influenza vaccine are available; live attenuated vaccines derived from cold-adapted influenza virus strains (LAIV) and inactivated vaccines

which may be presented as whole virions or split after treatment with detergents or solvents (Dormitzer et al., 2011).

The traditional seasonal vaccine (called trivalent inactivated influenza vaccine, or TIV) consists of three different influenza virus strains, which have been predicted as being the most likely to be circulating that season. The three strains comprise two influenza A virus strains and one influenza B virus strain. However in recent years, quadrivalent inactivated influenza vaccines (QIV) have been developed (McKeage, 2013). Marketing authorisation from the MHRA for the GSK product FLUARIX™ TETRA▼ was announced to the UK in April 2013. Quadrivalent influenza vaccines include an additional influenza B virus strain. Given the fact that two different B viruses may circulate concurrently, quadrivalent vaccines reduce the risk of the dominant B strain being omitted from the vaccine (McKeage, 2013). Whether three or four influenza virus strains are to be incorporated into a vaccine, the predictions for these circulating strains are made by the WHO twice a year (once for each hemisphere), and from these candidates high-growth seed strains are prepared (Gerdil, 2003). The vaccine viruses are then grown in embryonated eggs, before being inactivated (typically with formalin) and then purified. If TIV is going to be made as a split preparation (which is more typical) then either ether and/or a detergent are used for this process.

Vaccine evaluation

In the UK (and many other European countries) the safety and immunogenicity of the new TIV formulation must be evaluated in a clinical trial. The immunological outcomes required for marketing authorisation to be granted are specific increases in antibodies to the influenza virus haemagglutinin molecules measured by the haemagglutination-inhibition (HAI) assay (Gerdil, 2003).

The HAI assay utilizes the fact that influenza virus can agglutinate red blood cells from certain species of birds and mammals (for example turkeys and guinea pigs). Viral particles in effect cross link red blood cells by HA molecules binding to sialic acid residues. Thus, specific antibody against influenza virus HA molecules will inhibit this haemagglutination.

The origins of why HAI titres are used as the basis of influenza vaccine licensing go back many years. In 1943, challenge studies were performed by a group of investigators in Michigan, USA lead by Jonas Salt (Francis et al., 1945). These resulted in some of the first publications to show that higher HAI titres were associated with protection against influenza. These findings were true for both vaccinated and unvaccinated volunteers. This association was corroborated shortly afterwards in studies conducted in military recruits (Meiklejohn et al., 1952) however the HAI titres associated with protection were lower than previously reported. Approximately 20 years later a study was published which reported the outcome of influenza virus challenge studies in over 1,000 adults (Hobson et al., 1972). The HAI titre associated with 50% protection against influenza was between 1:18 and 1:36. These historical data, combined with more recent studies (Ohmit et al., 2011) have resulted in a post-vaccination HAI titre of 1:40 being accepted as a marker of seroprotection to allow regulatory authorities to evaluate and license seasonal influenza vaccines.

There are however, concerns with attempting to define protection as being heralded as having a HAI titre above a certain threshold. One such concern is that although the HAI assay generally has good reproducibility within a laboratory (i.e. it is relatively precise), the same samples often generate divergent results when tested in different laboratories (Stephenson et al., 2009). Hence the same subject may be deemed to be protected when tested in one laboratory, but unprotected when tested in another. Furthermore as described above, the

1:40 threshold was only found to be the level at which 50% of the adult population were protected. For many individuals the post-vaccination titres required to be protected may be a lot higher. Another concern is that the 1:40 titre threshold was not derived from studies in children and may therefore not be relevant to this population.

Improving on current influenza vaccination strategies

While the above vaccination strategy can be effective, it has some noteworthy disadvantages:

- The manufacturing process is slow – taking approximately 6 months from start to finish. This timescale is inadequate for a pandemic influenza vaccine, where the final product is needed in a matter of weeks. Instead, it would be preferable to have a vaccine stockpiled, which could be issued as soon as the pandemic is identified.
- Manufacturing is entirely reliant on a supply of embryonated eggs. This part of the manufacturing chain is particularly difficult to upscale or downscale according to sudden changes in demand. It is also potentially vulnerable to disruption by influenza itself, as the chickens needed to provide these eggs may be effected by the emergence of a novel influenza virus strain.
- Because the vaccine needs to be reformulated each year, this incurs greater development costs which overall detract from the cost-benefit of the programme.
- Approximately 1 in 20 times the WHO predictions do not sufficiently match the strains which subsequently dominate that influenza virus season. As the immune responses produced by vaccination are highly specific, vaccine efficacy in these years is substantially reduced.
- Sometimes the selected candidate seed strains do not grow well. This can cause a significant delay before the required doses of vaccine are ready.

In addition to the problems listed above, current seasonal influenza vaccines also have the significant disadvantage that they are less immunogenic in the elderly compared to the young adult population (Goodwin et al., 2006). The evidence for this as well as its consequences are discussed in more detail in Chapters 3 and 4, along with potential explanations and solutions.

For all the reasons stated above alternative vaccination strategies for influenza viruses are desirable. Vaccines that can induce broad immune responses, are particularly relevant to addressing the clinical need, as they could protect against drifted strains and potentially against pandemic strains of influenza viruses. I have already introduced the concept of a universal influenza vaccine as the goal in providing protection against a wide range of influenza virus subtypes. It is perhaps unrealistic to think that a truly universal influenza vaccine will be the first to emerge. More probably, a vaccine that provides limited protection to a restricted range of influenza virus subtypes could be developed. There are a number of strategies being pursued with this goal and I will now describe those which aim to provide protection by generating T cell responses to conserved influenza virus antigens. However, in addition to stimulating a different aspect of the immune system, developments in manufacturing technologies may help to address the issues with slow production of the traditional influenza vaccines.

The development of T cell vaccines against influenza viruses

There are many different strategies for generating T cell responses in vaccinated subjects. The focus of this thesis is viral vectored vaccines and therefore they are discussed in greater detail

in Section 1.4 and in subsequent chapters. However the basic principles of viral vectored vaccines will be introduced here.

Before being considered for use as vaccines, viral vectors were identified as a potential strategy for delivering gene therapy. The induction of immunity against the transgene severely limited that approach to gene therapy but suggested their use as vaccines. A suitable virus is genetically engineered to express the antigen of interest from the pathogen against which protection is required. The viral vector is then able to deliver that antigen into host cells where it can be appropriately processed and presented by MHC molecules. In addition the viral vector will activate the innate immune response through pattern recognition receptors and this signalling will subsequently shape the adaptive immune response.

Viral vectored vaccines which generate T cells specific for influenza virus-NP and M1 are capable of protecting mice against heterologous influenza virus challenge (Lambe et al., 2013). Mice have a number of disadvantages as a model for influenza (outlined in section 1.1.2) and therefore it would be of interest to see whether similar findings could be demonstrated in other animal models. While there is evidence that viral vectored vaccines can induce antibody-mediated protection against heterologous influenza virus challenge in pigs (Wesley et al., 2004), ferrets (Scallan et al., 2013) and non-human primates (Kreijtz et al., 2009), the same efficacy data does not exist for heterosubtypic T cell responses induced by viral vectored vaccines.

DNA vaccines have been extensively studied in humans and animals models. The principal of DNA vaccines is that the *in vivo* delivery of recombinant DNA, whether naked or in other forms, results in expression of the encoded protein (Gurunathan et al., 2000). For influenza,

it was demonstrated as early as 1993 that mice vaccinated with DNA encoding NP from influenza A/PR/8/34 (H1N1) virus were protected from heterologous challenge with influenza A/HK/68 (H3N2) virus (Ulmer et al., 1993). Protection against influenza virus challenge using DNA encoding NP has also been demonstrated in ferrets (Laddy et al., 2008) and non-human primates (Laddy et al., 2009). In the ferret experiments depletion of CD4 and CD8 T cells revealed the relative contributions of the two T cell subsets (Laddy et al., 2008). While DNA vaccines can prime naïve laboratory animals, in humans a vaccine is needed which can boost pre-existing T cell responses. In contrast to viral vectored vaccines, DNA vaccines do not generally boost pre-existing immune responses (Berthoud et al., 2011, Lambe, 2012).

Recombinant influenza virus proteins, generated in bacteria, plant or insect cell lines can be used to generate antibody responses to influenza viruses, and similarly recombinant peptides, linked together to form a string of epitopes, can be used to generate cellular immune responses to influenza viruses (Lambe, 2012). Unfortunately the success of these approaches is limited by the intrinsic lack of immunogenicity, although adjuvants can be used to compensate for this.

Virus-like particles have developed as a useful tool for novel vaccines as their size and the density with which epitopes are presented make them highly immunogenic. Currently licensed vaccines which use virus-like particles (namely the hepatitis B vaccine and the human papillomavirus vaccine), protect the vaccinee via the generation of neutralising antibodies. Similar approaches targeting influenza virus haemagglutinin have progressed to large phase II clinical trials and are robustly immunogenic (López-Macías et al., 2011). More recently, virus-like particles containing the HA and M1 proteins of the PR8 strain of influenza virus,

have been shown to protect mice from both homologous and heterologous influenza virus challenge (Hemann et al., 2013). The mechanism of this protection was shown to be dependent on CD8 T cells with the use of depletion experiments using specific monoclonal antibodies (Hemann et al., 2013).

The vast majority of evidence in both human and animal studies has shown that using vaccines to generate substantial antigen-specific T cell populations is safe. However immunopathology can be seen following influenza virus infection. This can be defined as occurring when the cytotoxic mechanisms (i.e. the actions of CTLs) necessary to destroy infected cells and clear the virus, also leads to damage to host tissues in addition to resolving the primary infection (Damjanovic et al., 2012).

Therefore prior to T cell generating vaccines earning a good reputation for safety there have been theoretical concerns that high frequencies of antigen-specific T cells may result in immunopathology when subjects are subsequently challenged. Two studies are sometimes cited to support this: In the first study, transgenic mice with artificially high frequencies of influenza virus NP-specific CTLs (Mamalaki et al., 1993) were challenged with influenza virus at two different doses. Animals challenged at low dose were protected, but those challenged with a high dose of virus developed increased pulmonary pathology and higher rates of mortality (Moskophidis and Kioussis, 1998). In a second study, the *in vivo* effects of influenza virus specific CTLs were explored through adoptive transfer experiments into T cell and B cell deficient BALB/c mice (Small et al., 2001). The recipient mice were transgenic for influenza virus-HA, which was expressed solely by pulmonary type II alveolar cells thanks to the transgene being under the control of the surfactant protein C promoter. The CTLs used in the adoptive transfer experiments were T cell clones derived from irradiated splenocytes that had

been infected with influenza A/Japan/305/57 (H2N2) virus. This second study has so many abstractions from the situation where polyclonal CTLs are induced by vaccination and recognise naturally infected respiratory epithelial cells, that it would be unreasonable to consider the results generalizable. As described above, the consensus of evidence from many studies in humans and multiple animal models (both inbred and outbred) demonstrates that T cells are protective and not pathogenic.

When evaluating novel influenza vaccines in clinical trials it is necessary to progress through one of two types of phase II study. Both types of study look for efficacy via the different rates of influenza disease between a vaccination group and a control group.

1. In the first type of study the subjects are based in the community and influenza disease is acquired by natural exposure. Unfortunately, because the rate of influenza can be less than 5%, and because the absolute risk reduction might be only in the region of 1-2%, large numbers of subjects are required for a study to be powerful enough to detect differences between the two groups with statistical significance. These difficulties, together with the technical problems associated with using serological measures to determine both post-vaccination responses and episodes of influenza virus exposure are discussed in Chapter 4.
2. The other method of efficacy study is to expose subjects deliberately to influenza virus by intranasal inoculation – called influenza virus challenge. Human challenge models of infectious diseases are not unique to influenza, with malaria (Roestenberg et al., 2009) and respiratory syncytial virus (DeVincenzo et al., 2010) having established methodologies. Subjects need to be managed in quarantine facilities for the duration of influenza virus challenge, as unlike with a malaria challenge, there would be a

significant risk of the pathogen entering the general population. Influenza virus challenge studies are therefore far more expensive to perform per subject. However the rates of influenza in the subjects are also much higher and therefore fewer subjects are required to make a study powerful enough to detect statistically significant differences between groups. In order to ensure this high attack rate, subjects selected for entry into a challenge study must be more susceptible to infection with influenza virus than the normal population. One method for ensuring this is to select subjects based on undetectable HI titres to the challenge strain of influenza virus. However, the concern with this approach is that it creates results which cannot be translated to the general population. Even with this targeted selection, the subjects of an influenza virus challenge (unlike those of a malaria challenge study) are highly unlikely to be immunologically naïve. During their lifetime, subjects will have been repeatedly exposed to influenza virus. On some occasions this will have resulted in symptomatic disease, however the majority of exposures will be asymptomatic. Nevertheless, asymptomatic exposures to influenza virus can activate the immune system and shape the individual's repertoire of influenza virus-specific T cells and antibodies. Even if the subjects do not possess strain specific neutralising antibodies, they will almost certainly have pre-existing T cell immunity. These issues are discussed further in Chapter 5, where I will also attempt to suggest improvements for how to classify subject outcomes following influenza virus challenge.

1.2 HEPATITIS C VIRUS

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family of RNA viruses and its genome consists of a single strand of positive-sense RNA. Most of the genome forms a single open reading frame which is translated into a large (approximately 3,000 amino acid) pre-protein, and then subsequently processed into three structural and seven non-structural proteins (Simmonds, 2004). HCV appears to have been circulating for hundreds of years at its geographical origins in West Africa and South East Asia (Klenerman and Gupta, 2012). However the route by which HCV entered the human population remains uncertain (Simmonds, 2013).

HCV is now extremely diverse, with seven distinct genotypes identified. These genotypes vary by some 30-35% at nucleotide sites, although more variation is seen in the E1 and E2 envelope glycoproteins than in the core protein or some non-structural proteins such as NS3 (Simmonds, 2004). Within the different genotypes there exist subtypes, which vary by some 20-25% of nucleotide sites (Simmonds, 2004).

1.2.1 HEPATITIS C VIRUS EPIDEMIOLOGY

The World Health Organisation estimate that 150 million people are chronically infected with HCV and that every year, 3–4 million people become infected and more than 350,000 people die from HCV related diseases (WHO, 2012).

HCV is a blood borne virus and the major risk factor for spread is the use of intravenous drugs. Additional routes of transmission include contaminated blood products (or organ transplants) that have not been adequately screened for HCV and by vertical transmission. Transmission through sexual contact can occur depending on the context (for example when there is HIV

co-infection), however there is thought to be no significant risk of sexual transmission among monogamous heterosexual couples (Tohme and Holmberg, 2010).

HCV has a worldwide distribution, with the highest prevalence being in Egypt (15%), Pakistan (4.8%) and China (3.2%) (WHO, 2012). Most cases in Egypt are genotype 4a and are a legacy of the mass campaigns of parenteral antischistosomal therapy which used non-disposable and poorly sterilized needles (Simmonds, 2004).

1.2.2 HCV INFECTION AND IMMUNITY

HCV infects and replicates inside human hepatocytes. In many cases a persistent infection is established and over a period of decades a proportion of these patients will develop cirrhosis and hepatocellular carcinoma (approximately 20% and 2% respectively). However, approximately 20% of subjects acutely infected with HCV are able to clear the virus spontaneously.

Some of the factors that influence the rate of spontaneous clearance are due to the virus, for example the HCV genotype and the diversity of HCV viral quasispecies. Non-immunological host factors associated with successful clearance include female sex, young age and non-black ethnic origin. Behavioural factors associated with viral clearance include the absence of an alcohol-use disorder and non-use of tobacco. The presence or absence of additional chronic viral infections is also important, with HIV co-infection being associated with a reduced rate of spontaneous clearance of HCV (Grebely et al., 2012).

The immunological mechanisms which influence spontaneous clearance of HCV infection are of great interest as they may suggest how the immune system could be targeted with a vaccine to induce a protective immune response:

Innate immune responses to HCV

The production of cytokines called interferons is essential to the control of many viral infections. For HCV, there has been much focus on a group of cytokines that are encoded in a cluster on the chromosomal region 19q13. In 2009, genome-wide association studies (GWAS) found that single nucleotide polymorphisms (SNPs) upstream of the *IL28B* gene could predict the likelihood of response to standard treatment with pegylated interferon-alpha and ribavirin (Ge et al., 2009). This gene encodes interleukin 28B, also known as interferon-lambda 3. Two closely related genes in the same cluster encode interleukin 28A (IL28A/interferon-lambda 2) and interleukin 29 (IL29/interferon-lambda 1). The most important SNPs (rs12979860 and rs8099917) are actually upstream of both *IL28A* and *IL28B* (as the two genes have an antiparallel orientation) but are physically closer to IL28B (Ge et al., 2009, Tanaka et al., 2009, Suppiah et al., 2009, Hayes et al., 2012).

The influence of these SNPs in the direct control of HCV infection is further validated by GWAS examining acute infection with HCV. The same alleles which confer improved treatment response to interferon-ribavirin therapy, also confer an increased likelihood of spontaneous clearance of the virus following acute infection (Thomas et al., 2009, Rauch et al., 2010).

A mechanism to link this genetic association with clearance of the virus has remained somewhat elusive (Booth and George, 2013). It had been hypothesized that individuals with the beneficial alleles do not trigger interferon regulatory pathways (via molecules such as SOCS) to the same extent. Indeed, individuals with the poor response genotype exhibit greater baseline expression of interferon-stimulated genes (Rau et al., 2012). Hence, when individuals with the favourable genotype receive treatment, interferon signal transduction is unimpeded and results in the greater expression of effector proteins stimulated by interferon (Hayes et al., 2012).

However this theory doesn't really explain why IL28B, but not IL28A, should be associated with enhanced clearance of the virus, given the fact that the two cytokines are virtually identical (Booth and George, 2013). It has recently been discovered that a further cytokine (called interferon-lambda 4) is encoded by a gene which lies between *IL28B* and *IL28A* (Prokunina-Olsson et al., 2013). This cytokine had previously escaped detection as it is only expressed transiently and at low abundance (Booth and George, 2013). Further work is needed to determine whether the effects of previously described SNPs are mediated through this cytokine.

Adaptive immune responses to HCV

Despite the difficulties of studying acute HCV infection, it is clear from the available published data that cell mediated immune responses are central to preventing persistent infection with HCV: There are strong HLA associations with the likelihood of developing resolved or persistent infection (McKiernan et al., 2004), suggesting that the processing and presentation of viral antigens to T cells is important to host resistance.

When comparing the adaptive immune responses between individuals with different outcomes following HCV infection, it is evident that those who have cleared HCV retain strong and broad T cell responses to HCV (with both CD4 and CD8 T cell responses), whereas those who remain persistently infected show weak and narrowly focussed responses (Spada et al., 2004, Lechner et al., 2000).

While HCV only circulates naturally in humans, it can cause infection when experimentally inoculated to some species of non-human primate. Experiments with chimpanzees have

corroborated the findings that strong and broad CTL responses are associated with spontaneous clearance of HCV (Cooper et al., 1999).

When protective immune responses have been induced in chimpanzees, depletion of CD4 and/or CD8 T cells results in a renewed susceptibility of the animals to subsequent HCV challenge (Grakoui et al., 2003)

In later studies, chimpanzees were vaccinated against HCV prior to heterologous HCV challenge (Folgori et al., 2006). These investigators were able to show a correlation between vaccine-induced cross-reactive T cells and prevention of chronic HCV infection (Folgori et al., 2006).

1.2.3 TRADITIONAL AND MODERN TREATMENTS FOR CHRONIC HEPATITIS C

Over the last few years, directly acting antiviral drugs have been developed for the treatment of HCV infection. Prior to this, the main form of treatment was with a combination of pegylated interferon-alpha and ribavirin. The standard of care treatment for genotype 1 HCV infection is 48 weeks of this dual therapy. The rate of sustained virological response following completion of treatment is typically 40-50%, which therefore leaves many patients for whom the virus is still detectable after treatment. Before the development of newer antiviral agents there were unsatisfactory second line treatment options. Patients offered re-treatment with dual therapy could expect a sustained virological response rate of only 10-20% (Ramachandran et al., 2012).

Boceprevir and telaprevir act by inhibiting the HCV non-structural 3 serine protease. Phase 2 and phase 3 studies using boceprevir/telaprevir have been successful in treatment-naïve patients infected with genotype 1 HCV. When these drugs are given alongside standard dual therapy (i.e. triple therapy), robust increases in the rates of sustained virological response are

observed (Ramachandran et al., 2012). Similarly, there are significant improvements in response rates when patients who have previously failed dual therapy are treated with triple therapy.

How do these developments impact on the case for a novel vaccine for hepatitis? Firstly, while these drugs may reduce the need for a vaccine that could be used therapeutically, they do not provide an alternative for a prophylactic vaccine. Secondly, these drugs are currently priced at a level that is unaffordable to many healthcare economies. Thus, there is still a need for an effective vaccine for HCV, which would complement these new developments in the treatment of chronic infection.

1.2.4 PREDICTED CHARACTERISTICS FOR A SUCCESSFUL PROPHYLACTIC VACCINE

Based on the available data from animal models of HCV challenge and human examples of resolved HCV infection, a HCV vaccine candidate should be able to induce strong and broad T cell responses to HCV (with contributions from both CD4 and CD8 subsets).

The required magnitude of such responses is not known, and nor is it likely that data to answer this question will become available in the next few years. Neither do we know how broad the immune response must be to confer protection, yet it is possible to infer the required breadth from observational studies of human cases of HCV and from interventional studies in non-human primates: In chimpanzees protected from HCV infection through vaccination, cell mediated immune responses against HCV have been specific for between 2 and 10 epitopes, whereas a vaccinated chimpanzee that was not protected only had HCV-specific T cells directed against a single epitope (Folgori et al., 2006). In the case of a human subject with

acute HCV who subsequently cleared the infection, HCV-specific T cells were found which recognised 8 different epitopes (Lechner et al., 2000).

Given the diversity of HCV, the vaccine induced T cell responses should be focussed on conserved epitopes within the HCV genome, and it would be desirable for the T cells generated to show cross-reactivity between different genotypes of HCV.

1.3 COMMON THEMES IN THE IMMUNOLOGY OF INFLUENZA VIRUS AND HEPATITIS C VIRUS

Failure of humoral immunity to provide adequate protection:

It is testament to the potency of neutralising antibodies that so many of today's successful vaccines work by activating the humoral arm of the adaptive immune system. High titres of neutralising antibodies can protect against viruses like polio and hepatitis B. Antibodies also protect against bacterial diseases, either by neutralising toxins such as tetanus or diphtheria toxoid, or by opsonising encapsulated bacteria such as *haemophilus influenza*. Another encapsulated bacteria, *streptococcus pneumonia* has evolved to "evade" the immune system by diversifying into a group of serotypes which have significant differences in the antigenic properties of the lipopolysaccharide capsules. Although the immune system can generate high affinity antibodies to any serotype upon infection, these antibodies are not cross-reactive between serotypes. Unlike viruses, *streptococcus pneumonia* is not able to undergo mutation at a sufficient rate to change the antigenic properties of its surface within the lifetime of a human host.

Influenza virus evades the immune system in a related way. Firstly it exists as different types and subtypes, which have antigenically distinct surface glycoproteins. Secondly, it is able to change the antigenic properties of those surface glycoproteins through the processes of antigenic drift and antigenic shift (see Section 1.1.3). Neutralising antibodies are certainly very capable of protecting the host. They contribute to arresting viral replication and can confer sterile protection against reinfection with the same strain. Over time however, those neutralising antibodies become redundant, as the virus they were originally developed to defend against no longer circulates in the population.

Antibody responses to influenza virus are focussed on the globular head region of the haemagglutinin spike which project from the surface of the virus. There is a great deal of sequence diversity between the haemagglutinin genes of different influenza virus subtypes, however the majority of this diversity is from the head region. Within the stem region there are conserved epitopes. Neutralising antibodies directed against these conserved regions are capable of providing heterosubtypic protection. Hence it is likely to be in the interest of the virus for the humoral immune response to target the head region of haemagglutinin molecules.

Many studies support the hypothesis that cellular immunity is essential to resolved infection from HCV. Indeed spontaneous resolution can occur without seroconversion in both humans and animal models (Di Lorenzo et al., 2011, Cooper et al., 1999, Post et al., 2004). But despite not being essential for the control of HCV infection, some evidence does support a role for neutralising antibodies in modification of the disease course (Di Lorenzo et al., 2011). Passive transfer of anti-HCV antibodies for example can delay virus replication following challenge in non-human primates (Krawczynski et al., 1996).

How does HCV prevent itself from being eliminated from the human host by antibodies? Firstly, glycans play an important role in shielding HCV from potentially neutralising antibodies (Helle et al., 2011). Viral envelope proteins are heavily glycosylated with glycans produced by host cell machinery. These glycans (e.g. 11 N-linked glycans on the E2 proteins) are identified as "self" by the host immune response.

HCV is thought to use lipoproteins to help avoid antibodies: Firstly, by associating with LDL and VLDL important epitopes are shielded from binding by antibodies. Secondly, by using HDL to enhance entry into target cells, the time that free virions are exposed to host antibodies is reduced. Time spent outside cells is also reduced by the cell-to-cell transmission of HCV (Di Lorenzo et al., 2011).

Both neutralising and non-neutralising antibodies can be generated by the immune system in response to HCV, and the functional nature of the antibody is strongly influenced by the epitope. An epitope that leads to non-neutralising antibody may therefore shield an adjacent epitope which may have elicited neutralising antibodies. The non-neutralising antibody is termed an interfering antibody as it prevents subsequent binding by neutralising antibody (Di Lorenzo et al., 2011)

Immunodominance in T cell responses:

Despite viral pathogens encoding many potentially immunogenic peptides, the host CTL response frequently chooses to focus on a narrow range of epitopes. This phenomenon is called immunodominance (Schmidt et al., 2011). Understanding this phenomenon is relevant to our broader understanding of how CD8 T cells contribute to immune surveillance. However, in wanting to develop vaccines that can induce protective CTL responses, which

may need to be sufficiently broad in specificity that the selection of viral escape variants is avoided, a clear understanding of immunodominance is particularly relevant (Yewdell, 2006). There are several factors that determine which epitopes will be immunodominant. The abundance of foreign protein is an important factor, particularly as this will have an impact on the subsequent abundance of peptide-MHC complexes on the cell surface. The peptide binding qualities of different MHC molecules are very different, and therefore the epitopes that bind most readily will tend to be immunodominant. Yewdell states that: "Over 90% of immunodominance can be explained by the finding that only ~1% of peptides bind with sufficient affinity" to any given MHC molecule for the peptide:MHC complex to be stable enough to activate T cells (Yewdell, 2006).

The relative frequencies of MHC alleles in the population will also dictate which individual epitopes are immunodominant. Antigen-specific T cell responses are primed when naïve T cells encounter cognate peptide presented (in the context of MHC) by an activated antigen presenting cell. Thus the number of naïve T cells in the circulation will also dictate which peptides are immunodominant.

Influenza virus immunodominance has been well studied in humans and animal models. As would be expected from the factors that influence immunodominance, different epitopes are immunodominant in different strains of inbred mice. For example in B57BL/6 mice the nucleoprotein ($NP_{366-374}$) and RNA polymerase ($PA_{224-233}$) derived peptides are immunodominant, while for BALB/c mice the nucleoprotein ($NP_{147-155}$) derived peptide is immunodominant (Staneková and Varečková, 2010). For humans, the best characterised immunodominant epitope is $M1_{58-66}$ (derived from matrix protein 1) and restricted by HLA-A*0201. Once established, it nevertheless remains possible for the hierarchy of

immunodominant responses to be altered (Chen et al., 2004). In Chapter 3 I explore whether vaccination with viral vectors maintains immunodominant responses or allows previously subdominant responses to emerge.

Despite influenza viruses being the archetypal model of immunodominance, this phenomenon is also seen with T cell responses to HCV. However the different properties of the two viruses influence how immunodominance affects the subsequent shape of the host immune response. Firstly, HCV is more diverse than influenza virus in its non-structural proteins. There is approximately 90% sequence identity for NP (or M1) between different influenza A virus subtypes (Berthoud et al., 2011). This contrasts with 60-80% sequence identity for NS3a (or NS5a) between different genotypes of HCV (Anwar et al., 2013). One epitope that is conserved across all genotypes is the HLA-A*01-restricted epitope $NS3_{1436-1444}$ (Neumann-Haefelin et al., 2008). However, because the virus causes chronic infection, the evolution of viral escape mutations has a longer period of time over which to emerge. This results in the formation of quasispecies of virus which is quite distinct from the virology of influenza viruses. For the $NS3_{1436-1444}$ epitope, the selection pressure exerted by CTLs results in an escape variant with a Y1444F substitution (Neumann-Haefelin et al., 2008). This is particularly relevant when conceiving of a T cell vaccine to control HCV. Other immunodominant epitopes for HCV include the HLA-A2 restricted epitopes DLMGYIPLV (core protein), KLVALGINAV (NS3 protein) and ALYDVVTKL (NS5b protein) (Alanio et al., 2010, Schmidt et al., 2011). Precursor frequency has been shown to be the major determinant of these immunodominant responses in HCV (Schmidt et al., 2011, Alanio et al., 2010), and based on mouse data, precursor frequencies are thought to be similarly important for influenza viruses (Obar et al., 2008). Unfortunately studying precursor frequencies in human populations is difficult due to the high rates of natural exposure.

Immune evasion strategies:

I have already described the processes of antigenic drift and antigenic shift which are the most important methods that influenza viruses use to evade the host immune response. The process of antigenic drift is driven by the RNA polymerase, which has no proofreading capability. The rate of mutation is approximately one error per replicated genome (Drake, 1993), or approximately 0.001 substitutions/site/year. This error rate is very similar to the rate of substitutions seen with HCV and many other RNA viruses (Jenkins et al., 2002). Although for HCV, there are substantial differences in the evolutionary rates of different portions of the genome (Gray et al., 2011).

Both viruses use an RNA-dependent RNA polymerase for replication and transcription. For influenza viruses the polymerase is a trimeric structure consisting of an acidic subunit (PA) and two basic subunits called PB1 and PB2 (Boivin et al., 2010). The complex has additional functions which help to evade the host immune response, including a process termed “cap snatching”. During transcription, the PB2 subunit binds host pre-mRNA molecule at their “cap”. The short capped RNA primers are retained by the trimer, while the remainder is cleaved off by the endonuclease action of PA. The polymerase then works to create chimeric mRNA molecules which are exported to the cytoplasm for translation into viral proteins (Boivin et al., 2010). This process reduces the production of host cell defence proteins such as interferon molecules.

In addition to the above strategies, these molecules and other conserved influenza virus proteins have a range of actions to inhibit host innate immune responses as shown in Figure 1-1 (van de Sandt et al., 2012). Both PB2 and PB1-F2 (an alternate ORF near the 5' end of the

PB1 gene) are able to limit the production of IFN-β by associating with the molecule MAVS (van de Sandt et al., 2012).

The PKR molecule is an important component in defence against viruses as it prevents the recycling of GDP and stops translation, thus inhibiting viral replication (Sadler and Williams, 2008). Influenza virus NP has an inhibitory effect on PKR by causing P58(IPK) to dissociate from Hsp40 (Sharma et al., 2011). Viral infection also causes upregulation of SOCS (suppressor of cytokine signalling) proteins which act by inhibiting IFN-α and IFN-β receptor signalling pathways (van de Sandt et al., 2012).

The influenza virus NS1 protein is a key virulence factor (Jackson et al., 2008), and antagonises the host innate immune response at multiple points: NS1 inhibits RIG-I receptor recognition of viral ssRNA, prevents TRIM25 oligomerisation, and impairs the activation and nuclear translocation of essential transcription factors such as IRF-3 that are required to generate a type I interferon response (van de Sandt et al., 2012). NS1 also interferes with the export of mRNA and alters the expression of host cell genes.

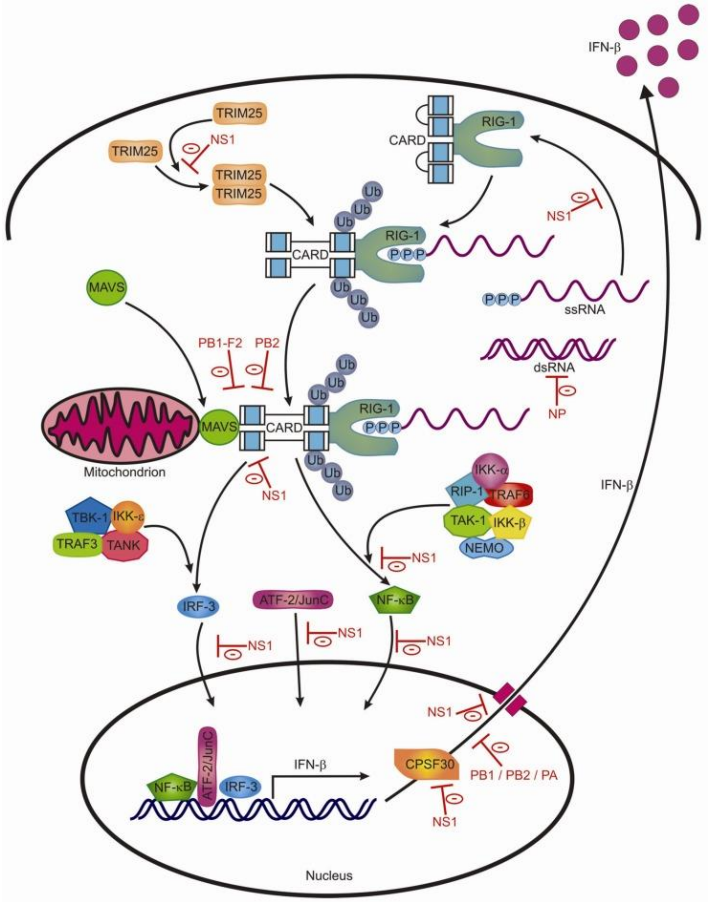
HCV has also evolved multiple mechanisms to evade the innate immune response. Similarly to influenza virus, HCV has also evolved to evade host cell recognition by RIG-I, as well as other microbial recognition pathways. The NS3/4A protease is able to cleave both MAVS and TRIF which therefore inhibits the RIG-I and TLR3 signalling pathways respectively (Horner, 2013). Other TLR mediated signalling may also be disrupted as a consequence of HCV targeting TRIF.

I have described how influenza virus is able to inhibit the actions of PKR. HCV also evades this host defence strategy, albeit via an entirely different mechanism. PKR kinase activation results in translational suppression throughout the cell, with the intention of limiting viral replication at the cost of producing less host defence proteins including the effector proteins stimulated by interferon (ISGs). Unfortunately for the host, the translation of HCV RNA can be initiated from an internal ribosome entry site (IRES). This consists of a nucleotide sequence located within the 5' untranslated region HCV RNA and is able to permit cap-independent translation to occur (Horner, 2013).

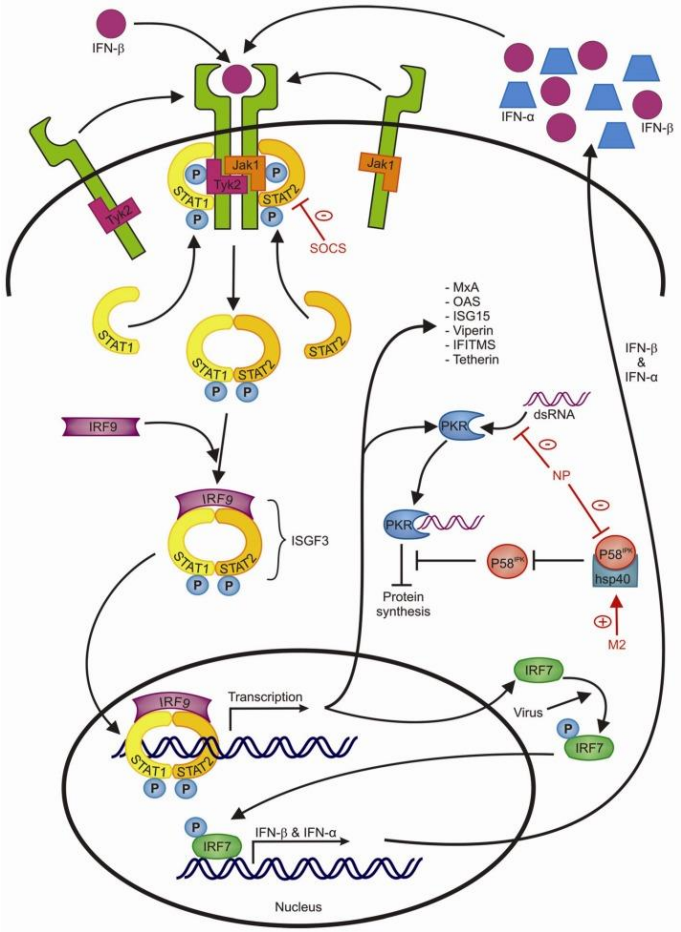
Other evasion pathways are likely to exist but yet to be discovered. For example, while it is known that the expression of HCV proteins blocks IFN-α signalling, and that this is likely to be occurring at the level of the JAK/STAT pathway, it is not clear how this is mediated (Heim et al., 1999). Recent speculation is that miRNAs may be responsible for some of the immune evasion exhibited by HCV (Horner, 2013).

Figure 1-1: Immune evasion strategies employed by the influenza virus

A



B



The multiple actions of influenza virus proteins in evading the immune system are shown schematically (reproduced with permission from van de Sandt). Specific pathways targeted by the virus include the detection of viral nucleic acids by the RIG-I pathway (left panel, A) and intracellular signalling cascade induced by type-I cytokines (right panel, B). The inhibitory actions of viral proteins are shown in red (van de Sandt et al., 2012).

1.4 VIRAL VECTORED VACCINES

1.4.1 PRINCIPALS AND POTENTIAL ADVANTAGES

Viral vectors were initially proposed as a platform for delivering gene therapy. A recombinant virus is engineered to express a transgene of interest, and this is administered to the subject. The virus then infects host cells and begins to translate the relevant protein. The host immune responses elicited in this process are detrimental to the success of gene therapy but can be exploited as a vaccine technology.

Viral vectored vaccines have the advantage of being able to express large quantities of full length protein. In addition to promoting T cell responses through the intracellular production of antigen, a leader sequence may be added to the transgene should a humoral immune response also be desirable. These sequences would allow protein to be trafficked to the cell surface where it would be available for binding by soluble antibody.

1.4.2 ADENOVIRAL VECTORED VACCINES

Adenoviruses are DNA viruses which cause mild acute respiratory tract infections. They have several properties that make then suitable for use as viral vectors: Firstly they are relatively easy to manipulate and grow *in vitro*; they have large insertion sites (in the region of 7 kb) which can express high levels of the required transgene; they can be rendered replication incompetent through the deletion of the E1 gene; they are genetically stable; and they do not integrate into the host genome (Lambe, 2012, Dicks et al., 2012).

The primary disadvantage associated with adenoviruses is the prevalence of anti-vector immunity in healthy populations. Neutralising antibody titres of above 1:200 have been

associated with reduced immune responses to adenoviral vectored vaccines in clinical trials (McElrath et al., 2008). The frequency of anti-vector immunity is variable, and is dependent on the demographics of the population (particularly age and geography), and the serotype of the vector (Dicks et al., 2012, Dudareva et al., 2009).

In addition to humoral immune responses, individuals previously exposed to adenovirus will have pre-existing T cells that are specific for adenoviral peptides. Studies in mice have shown that while pre-existing antibodies to the vector are the major factor in determining anti-vector immunity, T cells are also implicated. Specifically, B cell deficient mice that are repeatedly vaccinated generate increasingly large CD8 T cell responses to the adenoviral vector but have lower frequencies of transgene-specific CD8 T cells (Steffensen et al., 2012).

However as antibody mediated anti-vector immunity has a stronger influence than T cell mediated anti-vector immunity it has become desirable to use serotypes of adenovirus that do not circulate widely in human populations. This has led to the use of chimpanzee adenoviruses as vectors, which are phylogenetically indistinguishable from human adenoviruses. Clinical trials using the chimpanzee adenoviral vectors ChAd3 and ChAd63 expressing hepatitis C virus (Barnes et al., 2012) and malaria antigens (O'Hara et al., 2012) have now demonstrated that these vectors are both safe and capable of priming immune responses with excellent potency.

1.4.3 MVA VECTORED VACCINES

During the smallpox eradication programme of the 1960s, a live vaccinia virus vaccine was used to induce protective immunity against the related variola (smallpox) virus. Vaccinia infection is typically mild or asymptomatic in healthy individuals, although a small number of recipients (approximately 1 per million) can develop severe and potentially fatal

complications, typically due to the presence of a previously undiagnosed immunodeficiency. Modified Vaccinia virus Ankara (MVA) was first established as an alternative to this highly successful vaccine. Passage of the virus (chorioallantois vaccinia virus Ankara) through serial replication cycles in a non-mammalian cell line (chicken embryo fibroblasts) resulted in MVA. Although this virus is able to replicate its DNA and synthesize both early and late viral proteins, it is unable to produce infectious progeny (Sutter and Moss, 1992). It was subsequently used as part of the smallpox vaccination programme and established a good reputation for safety amongst some 120,000 recipients (Mayr et al., 1978).

MVA has several properties which make it suitable for use as a vector in viral vectored vaccines: i) It achieves high levels of transgene expression in human cells, ii) it is genetically stable, iii) it can be manufactured on the large scales required for mass vaccination programmes and iv) it can be given repeatedly with a good boosting effect despite the development of humoral and cellular immune responses to the MVA vector (Cottingham and Carroll, 2013).

Recombinant MVA vaccines expressing tuberculosis (McShane et al., 2004) or malaria antigens (McConkey et al., 2003) have demonstrated potent immunogenicity and have progressed to phase II clinical trials (Tameris et al., 2013, Hill et al., 2010).

1.5 SYSTEMS BIOLOGY APPROACHES TO VACCINOLOGY

1.5.1 OVERVIEW OF TECHNIQUES

A systems biology approach is one which attempts to measure and analyse the complex network of molecular interactions caused by a stimulus or a dynamic environment, rather than focusing on the individual processes occurring to single genes, proteins or cell types.

There are multiple different technologies that fit within the sphere of systems biology and these are complementary to one another. However, they typically rely on three common scientific developments: Firstly, they require sophisticated technologies that are able to simultaneous measure tens of thousands of variables; secondly, they depend upon having the computational power to interpret these data in ways that can be meaningful to the user; and finally, the interpretative step entirely depends on having a sufficiently accurate source of reference information. For most of these "-omic" technologies the "source of reference" has been the sequencing of the human genome.

Genomics

Genetic predictors of vaccine response have long been recognised: HLA alleles which associate with the seroconversion rate to hepatitis B vaccine are one such example, although more recently non-HLA genetic associations have been identified (Hennig et al., 2008). Variations between genomes can be measured using DNA microarrays that detect single nucleotide polymorphisms. Using such approaches genetic variation has been linked to immune responses to smallpox vaccine (Kennedy et al., 2012a, Kennedy et al., 2012b, Ovsyannikova et al., 2012), and anthrax vaccine (Pajewski et al., 2012).

Genomic technologies have also been used in an approach called “reverse vaccinology”. Traditionally, one or more targets from a pathogen (e.g. highly conserved cell surface proteins) would be selected to make a vaccine, and then that vaccine would be tested for efficacy (Seib et al., 2012). In a reverse vaccinology approach, the entire genome of a pathogen is analysed and the results are screened for targets which are predicted to have the characteristics of a relevant immunogen (Seib et al., 2012). A good example of this approach has been the sequencing of the serogroup B meningococcus genome (Pizza et al., 2000) which eventually led to the development of a potent vaccine (Giuliani et al., 2006) and its translation into large clinical trials (Santolaya et al., 2012).

Transcriptomics

Transcriptomics or more broadly functional genomics, examines the dynamic response of genes to a particular environment or stimulus, rather than the fixed component of the genetic sequence itself. Because the dynamics of the host response are the focus of investigation, experimental designs will often utilise repeated samples from subjects over time. Gene expression can be determined using microarray technologies consisting of tens of thousands of oligonucleotide probes fixed to a single chip. Alternatively, RNA sequencing technologies have become increasingly available and have the advantages of producing less background noise and are able to quantitate RNA expression over a greater dynamic range (Wang et al., 2009).

Proteomics

Proteomics have evolved from transcriptomics to evaluate biological systems on a protein level. This approach seeks to improve upon some underlying problems with examining

biological systems at the mRNA level. Firstly, mRNA expression does not always correlate with the amount of protein being produced; secondly, more than one protein may be produced from a single mRNA due to alternative splicing; and thirdly, proteins may undergo post-translational modifications which significantly influence their effect in a biological system.

1.5.2 PREVIOUS INSIGHTS INTO INFECTIOUS DISEASES AND HOST-PATHOGEN INTERACTIONS USING TRANSCRIPTOMICS

On the basis that different infectious agents will induce different molecular immune pathways, a study was performed to determine whether the infective aetiology of acutely septic patients could be distinguished on the basis of the transcriptional signature of peripheral blood leukocytes (Ramilo et al., 2007). For the 131 patients and 7 controls, a single (acute) time point sample of whole blood was collected for subsequent RNA extraction. The study found firstly that transcriptional signatures discriminated cases of influenza from cases of bacterial infections. There were 854 differentially expressed genes (DEGs), of which 394 were relatively overexpressed in cases of influenza and were characteristic of a strong type I interferon response (Ramilo et al., 2007). Secondly, it was found that transcriptional signatures could distinguish between cases of bacterial infection that were either due to *E coli* or *S aureus*. These microarray experiments were performed initially using Affymetrix GeneChips, but then reproduced on a second set of samples using Illumina whole genome Sentrix Hu6 bead chips (Ramilo et al., 2007).

As well as differentiating the aetiology of different infections, transcriptional profiling has also been used in clinical studies to examine the host-pathogen interactions from specific viruses. For respiratory syncytial virus (RSV) this approach was particularly relevant. A lack of understanding of the disease pathogenesis has impeded successful vaccine development for

RSV and the clinical management of patients acutely unwell with RSV is difficult because there are no available tools to accurately assess prognosis on an individual level. Mejias examined a cohort of 135 children with RSV and collected peripheral blood into Tempus™ tubes, from which RNA could subsequently be extracted (Mejias et al., 2013). Microarray analysis identified a transcriptional profile characterised by the overexpression of neutrophil and myeloid genes, together with the suppression of B cell and T cell specific genes (Mejias et al., 2013). The alterations in gene expression profile were surprisingly long-lasting, remaining detectable one month after acute infection. With relevance to the clinical management of these patients, the severity of disease was found to be associated with suppression of T cell responses (Mejias et al., 2013).

1.5.3 PREVIOUS INSIGHTS INTO VACCINOLOGY USING TRANSCRIPTOMICS

Bali Pulendran has coined the term systems vaccinology to describe the application of systems biology approaches to vaccinology (Pulendran, 2009, Pulendran et al., 2010). His group have used Affymetrix microarrays to explore dynamic changes in gene expression following vaccination with the yellow fever vaccine YF-17D (Querec et al., 2009) and following vaccination with seasonal influenza vaccine (Nakaya et al., 2011).

YF-17D is a live attenuated vaccine, which is highly effective at providing protective immunity, and stimulates both cellular and humoral arms of the immune system. Querec at al. vaccinated 15 subjects with YF-17D and examined the gene expression profile from PBMC at 0, 1, 3, 7 and 21 days post-vaccination (Querec et al., 2009). In addition to describing a network of anti-viral genes that were induced by vaccination, the group were able to identify early gene signatures that correlated with and could predict the cellular and humoral immunogenicity outcomes of individual vaccinees.

A similar approach has since been reported using seasonal influenza vaccines (Nakaya et al., 2011), with microarrays being performed on RNA extracted from PBMC at days 0, 3 and 7 post-vaccination. Both trivalent inactivated influenza vaccines and live attenuated influenza vaccines were used and found to have distinct signatures of gene expression, providing an interesting insight into the different mechanisms by which they act to stimulate an immune response. For example, the investigators found that expression of CAMK4 (encoding the kinase CaMKIV) was inversely correlated with HAI titers. This protein had never before been linked with a role in regulating B cell responses, however novel experiments with *Camk4*$^{-/-}$ mice were able to demonstrate that the knockout mice had significantly greater antibody responses compare to those of the wild-type mice (Nakaya et al., 2011). In addition, the investigators were able to use small numbers of genes (n=4) in multiple different combinations to predict those vaccinees that would develop protective immunity (defined by HI titre) following vaccination with TIV (Nakaya et al., 2011)

A separate group has also examined the response to TIV at a transcriptional level (Bucasas et al., 2011). In this study over a hundred subjects were vaccinated with TIV and peripheral blood samples were collected (into PAXgene tubes) at 0, 1, 3 and 14 days post-vaccination. These investigators also sought (and indeed found) a pattern of gene expression that correlated with increased vaccine response (a function of HI titre). However in contrast to the findings by Nakaya et al., Bucasas et al. found that it was an early interferon response (at day 1) which was the best correlate of immunogenicity. An explanation for these different findings may lie in the fact that different leukocyte subsets were being analysed and at different time points.

As a final example, transcriptional profiling has also been used to explore the mechanisms through which a human adenovirus vectored vaccine stimulates the immune system (Zak et al., 2012). RNA was extracted from PBMCs collected at 0, 4–6, 24, 72, and 168 hours post-

vaccination with the Merck Ad5/HIV vaccine. Microarrays were performed on each of these samples from 10 subjects. The investigators found more than 2,000 differentially expressed genes, which exhibited a peak response at 24 hours. A subset of these genes remained differentially expressed at 72 hours, but all traces of differential expression had been lost by 168 hours (Zak et al., 2012). Interestingly, the investigators found that this signature was attenuated in subjects with pre-existing immunity to the vector. They also attempted to correlate T cell immunogenicity with genome-wide changes in expression. Surprisingly, differential expression was only identified at the 72 hour time point, with approximately 200 genes being differentially expressed in high responders compared to low responders (Zak et al., 2012).

1.6 IMMUNOLOGY OF AGEING

Immunosenescence describes the ageing of the immune system, which in humans is characterised by impaired responses to new antigens, unsustained memory responses, an increased tendency towards autoimmune responses and persistent low-grade inflammation (Goronzy and Weyand, 2013).

Many scientific reports that attempt to characterise immunosenescence begin by highlighting the association between ageing and susceptibility to infectious disease. Unfortunately while it is true that the incidence, morbidity and mortality of many infectious diseases increases with age, it is difficult to attribute these observations solely to an ageing immune system. As our bodies age so do the many non-immunological defence mechanisms that protect us: For example as our skin ages and the prevalence of ulcers increases (Forssgren et al., 2008) we are more at risk of developing cutaneous infections, and as our gastrointestinal tract ages the

prevalence of diverticulosis increases (Peery et al., 2012) and we are more prone to infectious diverticulitis. Elderly individuals are more likely to die from a number of infectious agents because of medical co-morbidities which make them physiologically less resilient to sepsis. In addition, the presence of immunologically mediated co-morbidities may require immunosuppressant medication to be taken which also increase the risk of infectious disease. One of the most commonly cited clinical examples of immunosenescence is the increased rate of tuberculosis (due to reactivation rather than primary infection) in the elderly. The original studies that documented these finding have significant limitations. However in recent years the rates of tuberculosis reactivation have been re-examined (Horsburgh et al., 2010). This work utilised molecular typing (restriction-fragment-length polymorphism analysis) for *Mycobacterium tuberculosis* isolates to more accurately classify whether infections occurred as a consequence of primary infection or reactivation. Clustered tuberculosis isolates occurred due to primary infections and unclustered tuberculosis isolates occurred due to reactivation of latent infection (Horsburgh et al., 2010). They found that significantly increased rates of tuberculosis due to reactivation were seen among adults over the age of 50 years old.

The risk of reactivation of latent *mycobacterium tuberculosis* is also seen with immunosuppression (e.g. due to medication or HIV disease) suggesting that a loss of cellular immune surveillance may explain the observation. The other consistent and reproducible clinical feature of immunosenescence is the diminished responses to certain vaccines. Influenza vaccines are the archetypal example of this (Goodwin et al., 2006), however other vaccines such as the Hepatitis B vaccine also have lower success rates in the elderly for otherwise unexplained reasons (Chen et al., 2009).

Ex vivo studies from different human immune cell types have found functional deficiencies with innate (Panda et al., 2009), humoral (Frasca and Blomberg, 2009) and cellular (Haynes and Maue, 2009) components of the aged immune system. However the difficulty remains to consistently correlate any individual finding with a clinically relevant outcome.

Mouse models have been extensively studied in an attempt to develop a better understanding of immunosenescence. Unfortunately the obvious differences between species in terms of life span and exposure to environmental pathogens make it difficult to translate results. Nevertheless, mouse models have been found where the lethality of infections is increased with an aged immune system (Brien et al., 2009). Importantly these mouse models use adoptive transfer of immune components from either adult or aged mice into young mice in order to establish the specific impact of an aged immune system. However, while these models work well for studying the specific effects of aged lymphocytes, it is more complex to study certain aspects of the innate immune system (e.g. dendritic cells).

Although in recent years age-related changes have been described in the innate immune system, the most significant and reproducible changes to date have been associated with T cells. The origins of these findings were longitudinal studies conducted in Sweden, which associated an Immune Risk Phenotype (IRP) with mortality in those aged 85 years old at baseline (Olsson et al., 2000). The IRP included an inverted CD4/CD8 ratio, poor proliferative responses to T cell mitogens and seropositivity for CMV. As humans age there is a natural progression towards a T cell pool that has fewer naive T cells and a greater proportion of terminally differentiated memory cells. The changes are a consequence of thymic involution (which means naive T cells cannot be replaced), exposure to infection (which consumes naive

T cells and results in memory T cells) and a physical limit on the total lymphocyte pool. Terminally differentiated memory T cells eventually re-express the cell surface molecule CD45RA and have been shown to be dysfunctional in terms of their cytokine responses, cytotoxic activity and ability to proliferate (Fülöp et al., 2013).

CMV causes persistent infection and the virus is thought to intermittently reactivate without causing symptoms. This persistent infection drives the accumulation of large populations of late-stage differentiated CD8+ T cells. In fact up to half of CD8 T cells and 30% of CD4 T cells can be CMV-specific in elderly subjects (Pourgheysari et al., 2007). The crucial question is whether these changes have clinical consequences, and as mentioned above this is by no means clear cut. Investigators have tried to associate CMV-driven T cell changes with poor responses to influenza vaccination however results have not been consistent (Fülöp et al., 2013).

1.7 PROJECT AIMS AND HYPOTHESIS STATEMENTS

The aims of this project have been to develop improved vaccination regimens for influenza virus and HCV. For influenza vaccines I have focused on older adults, as this group is in most need of vaccines with greater immunogenicity. Influenza virus challenge studies are an important tool for testing novel influenza vaccines. I was therefore interested to explore how the outcomes of subjects following influenza virus challenge could be better defined. I performed an analysis of host transcriptional responses to influenza virus challenge with the aims of defining clearer phenotypes and exploring the impact of vaccination. For hepatitis C vaccines, I focussed on the use of viral vectored vaccines in seronegative individuals. The aim

was to prove that a heterologous prime-boost regimen would have sufficient immunogenicity to warrant progression to efficacy studies. A further aim was to improve the immunogenicity of standard prime-boost regimens by repeated rounds of vaccination. Finally I wanted to improve understanding of host responses to viral vectored vaccines, and address why reactogenicity and immunogenicity outcomes are so different between individuals and across different vectors. I therefore analysed genome wide transcriptional responses to vaccination in a cohort of subjects and determined genetic signatures that correlated with reactogenicity and immunogenicity.

In summary, I intend to address the following hypothesis statements in this thesis:

- The viral vectored vaccine MVA-NP+M1 is equally immunogenic in young adults and older adults.
- Co-administration of MVA-NP+M1 and seasonal influenza vaccine can adjuvant the humoral immune responses to the seasonal influenza vaccine without vaccine interference.
- Gene expression signatures in whole blood can distinguish influenza virus infected and non-infected subjects; and such gene expression signatures can be used to improve the biological classification of subjects in an influenza virus challenge study.
- Gene expression signatures following influenza virus challenge are significantly altered by prior vaccination with MVA-NP+M1.
- Heterologous prime-boost vaccination with ChAd3-NSmut and MVA-NSmut is safe and immunogenic in hepatitis C virus seronegative subjects.
- HCV-specific T cell responses can be boosted to higher levels by repeated administration of ChAd3-NSmut and MVA-NSmut.

- Following administration either ChAd3-NSmut or MVA-NSmut, reactogenicity and immunogenicity can be correlated with (or predicted by) gene expression signatures.

1.8 OUTLINE OF THESIS

- Chapter 2 describes the material and methods used in this study.
- Chapter 3 describes the safety and immunogenicity of MVA-NP+M1 in a population of adults over the age of 50 years. I compare data generated here with previous data from young adults and examine the effects of advancing age on immunogenicity.
- Chapter 4 describes the effects of vaccine co-administration for MVA-NP+M1 and seasonal influenza vaccine.
- Chapter 5 describes the host response to influenza virus challenge at the transcriptional level. I explore whether clinical phenotypes are associated with distinct genetic signatures and examine whether vaccination with MVA-NP+M1 impacts on this response.
- Chapter 6 describes novel prophylactic vaccination regimens for HCV. The candidate vaccines ChAd3-NSmut and MVA-NSmut are used in prime-boost combinations and repeatedly administered with differing time intervals.
- Chapter 7 describes the host response to vaccination with ChAd3-NSmut and MVA-NSmut at the transcriptional level. I explore whether reactogenicity and immunogenicity can be associated with or predicted from distinct genetic signatures.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 SOLUTIONS

ELISpot Coating Buffer: 15 mM sodium carbonate and 35 mM sodium bicarbonate capsules were dissolved in dH_2O and autoclaved.

Phosphate buffered saline (PBS) 0.01 M: NaCl 0.138 M, KCl 0.0027 M, pH 7.4: made by dissolving tablets in dH_2O.

R0 media: RPMI 1640 containing 100 IU/ml penicillin, 100 µg/ml streptomycin (all Sigma), and 2 mM/L-glutamine (Life Technologies).

R10 media: RPMI 1640 containing 10% heat-inactivated foetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin (all Sigma), and 2 mM/L-glutamine (Life Technologies).

PBS/Tween (PBS/T) 0.01 M: NaCl, 0.138 M, KCl 0.0027 M, pH 7.4 Tween-20 0.05%. Sachets dissolved in dH_2O.

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

2.1.2 ANTIBODIES USED FOR FLOW CYTOMETRY

Antibody	Supplier	Fluorochrome	Clone
αCD3	eBioscience	Alexa-Fluor-700	UCHT1
αCD8	eBioscience	APC-Alexa-Fluor 780	RPAT8
αCD4	Life Technologies	QD655	S3.5
αIFN-γ	eBioscience	FITC	4S.B3
αIL-2	eBioscience	PE	MQ1-17H12
αTNF	BD Biosciences	PE-Cy7	MAb11
αCD107a	eBioscience	PE-Cy5	eBioH4A3
αCD14	Life Technologies	Pacific-Blue	TuK4
αCD19	Life Technologies	Pacific-Blue	SJ25-C1
Dead cell marker	Life Technologies	LIVE/DEAD® Violet	

2.1.3 KITS USED

Human IFN-γ ELISPOT kits (Mabtech)

PAXgene Blood RNA kits (Qiagen)

GLOBINclear™ Whole Blood Globin Reduction Kits (Life Technologies)

2.2 ESTABLISHING CLINICAL TRIAL COHORTS

2.2.1 CONSENT, ETHICS AND REGULATORY APPROVAL

All volunteers provided written informed consent.

All clinical trials received approval from the appropriate ethics committee (either Oxfordshire Research Ethics Committee or GTAC).

All clinical trials and subsequent amendments were approved by the MHRA.

2.2.2 FLU001 EXTENSION TRIAL

The original FLU001 trial was a first in man study of MVA-NP+M1 in healthy adults aged 18-45 years (Berthoud et al., 2011). Its aims were to establish whether the vaccine could be safely administered and to determine a dose and route of administration that would balance reactogenicity with immunogenicity. Safety data from the FLU001 trial was used to select a dose of 1.5 x 10^8 pfu, which was taken forward into future clinical trials. The FLU001 extension trial was introduced by means of a substantial amendment to the FLU001 trial. Recruitment of a further 30 volunteers was approved, who were to be subdivided into three cohorts aged 50-59 years, 60-69 years and 70 years and over. Volunteers were screened and those with significant medical co-morbidities were excluded. A complete list of the inclusion and exclusion criteria is available via the website www.clinicaltrials.gov (identifier: NCT00942071). After receiving a single vaccination with MVA-NP+M1, follow-up visits were scheduled at 2 days, 1 week, 3 weeks, 8 weeks, 12 weeks, 24 weeks and 1 year. For this trial I screened, recruited and vaccinated approximately half of the volunteers.

Table 2-1: Demographic characteristics of volunteers in the FLU001 study

Group	Actual age range (years)	Mean age (years)	Females
Group 1 (aged 50–59 years)	50 – 59	55.2	50%
Group 2 (aged 60-69 years)	60 – 66	63.3	70%
Group 3 (aged 70+ years)	72 – 85	79.0	50%

2.2.3 FLU002 TRIAL

The FLU002 trial was a phase IIa efficacy study of MVA-NP+M1. Healthy volunteers aged 18-45 were recruited into one of two groups. The vaccination group received MVA-NP+M1 at 1.5 x10^8 pfu whereas the control group received no vaccination. Volunteers were pre-screened to ensure they had undetectable HI titres to H3N2 influenza (A/Wisconsin/67/2005) virus. They were subsequently admitted to a quarantine facility and challenged with an intranasal

administration of the same influenza virus strain at a dose $10^{5.25}$ $TCID_{50}$ in 1 ml. Self-reported symptoms were collected twice daily using a standardised scoring system. A modified Jackson score of ≥4 indicates mild influenza disease, and a score of ≥29 indicates moderate to severe influenza disease. Nasal lavage fluid for quantification of viral shedding was obtained daily and laboratory confirmed influenza was defined as at least mild symptoms plus shedding of influenza virus on at least one day post-challenge. Peripheral blood was collected into PAXgene tubes and stored at -80 °C for subsequent RNA extraction. Samples were available from the day prior to challenge and then 12 hours, 24 hours and 48 hours post challenge. Additionally, peripheral blood samples were collected for PBMC isolation and evaluation of T cell responses to influenza virus antigens, however these will not be discussed in this thesis. Management of this trial and its participants was conducted by Dr Patrick Lillie.

2.2.4 FLU003 TRIAL

The FLU003 trial was a phase I study involving co-administration of the vaccines MVA-NP+M1 and TIV (season 2011/12). The aims of the study were to establish whether this combination of vaccines could be safely co-administered, and what effect co-administration would have on immunogenicity. Healthy volunteers over the age of 50 years were eligible to take part provided they had not already received vaccination with the 2011/12 season TIV. A complete list of the inclusion and exclusion criteria is available via the website www.clinicaltrials.gov (identifier: NCT01465035). Eight volunteers were vaccinated with TIV and a saline placebo. Nine volunteers were vaccinated with TIV and MVA-NP+M1. After vaccination, follow-up visits were scheduled at 1 week, 3 weeks and 26 weeks. For this trial I screened, recruited and vaccinated all the volunteers in the study.

2.2.5 HCV003 TRIAL

HCV003 was a first in man study of the vaccine MVA-NSmut and its use as part of a heterologous prime-boost vaccination regimen with ChAd3-NSmut. The principals of such viral vectored vaccines are introduced in Section 1-4. The trial comprised of multiple arms, of which Arm A concerned healthy volunteers and Arms B and C concerned subjects who were chronically infected with HCV. In this thesis I will only discuss the use of these vaccines amongst healthy volunteers rather than their potential use as therapeutic vaccines. The different groups in Arm A are outlined in Table 6-1 (Chapter 6) and for all these healthy volunteers I conducted both the screening visits and vaccination visits.

2.2.6 VACCINES AND VACCINATIONS

All vaccinations performed were intramuscular. For the FLU001 and HCV003 trials the deltoid was the site of injection. For the FLU003 trial, the combined volume of vaccines was 1.6-1.7 ml and therefore vaccinations were performed in the anterolateral thigh.

VACCINE	**DOSE**	**DESCRIPTION / COMPOSITION**
ChAd3-NSmut	2.5 x 10^{10} vp	ChAd3 and MVA vectors expressing the non-structural regions (NS3-NS5b) of HCV genotype 1b. As a safety measure a mutation was introduced to the NS5b region to inactivate the viral polymerase.
MVA-NSmut	2 x 10^{8} pfu	
MVA-NP+M1	1.5 x 10^{8} pfu	MVA expressing a fusion protein of nucleoprotein (NP) and matrix protein 1 (M1) derived from influenza virus A/Panama/2007/99.
Influenza vaccine (split viron) Sanofi Pasteur MSD	15 micrograms of each HA protein	The 2011/2012 seasonal influenza vaccine comprised: H1N1 (A/California/7/2009), H3N2 (A/Perth/16/2009) and, Influenza B (B/Brisbane/60/2008).

2.3 CHARACTERISING T CELL RESPONSES TO VACCINATION

2.3.1 ISOLATION OF PBMC

The preparation of 50 ml Leucosep tubes (Greiner) was performed by the addition of 15 ml Lymphoprep™ (Axis-Shield), and then centrifugation at 2,780 x *g* for 1 minute to force the Lymphoprep™ below the porous filter disc. Heparinised blood samples (standard volume 50 ml) were then poured into two prepared Leucosep tubes, and centrifuged at 1,000 x *g* for 13 minutes without the brake. Plasma samples were pipetted from the surface and stored at -80 °C. PBMC were then collected from the interface using a Pasteur pipette and transferred to a fresh 50 ml Falcon tube (BD Biosciences). R0 media was added to the tubes up to a total volume of 40 ml. Samples were centrifuged at 2,780 x *g* for 5 minutes, resuspended and then washed again in 40 ml of R0 media. PBMC were then resuspended in 10 ml of R10 media for counting using a CASY Cell Counter.

2.3.2 ANTIGEN SPECIFIC T CELL ENUMERATION BY INTERFERON GAMMA ELISPOT

2.3.2.1 Procedure

The primary antibody (1-D1K) was prepared by making a 1:100 dilution with coating buffer (Section 2.1.1) to achieve a final concentration of 10 µg/ml. MAISP ELISpot plates (Millipore) were then coated with 50 µl/well of prepared primary antibody and incubated at 3-6 °C for 8 hours - 10 days. On the day of sample collection ELISpot plates were blocked: First the coating solution was flicked off and the plates were then washed 3 times with 100-200 µl sterile PBS. Blocking was then performed by addition of 100 µl R10 media for 30 minutes at room temperature. After blocking, the R10 media was flicked off and peptides (see below) or controls (both 50 µl) were added to each well. PBMC (2×10^5 in 50 µl) were then added to

each well to make a final volume of 100 µl. Each condition was assayed in triplicate. ELISpot plates were then incubated for 18-20 hours at 37 °C. The following day, the cells and peptides were flicked off the ELISpot plates, and they were washed 6 times in PBS/T solution. The secondary antibody (7-B6-1-Biotin) was diluted to 1:1,000 in PBS and then 50 µl was added to each well. After incubation for 2-4 hours at room temperature, the antibody solution was flicked off and the plates were washed 6 times in PBS/T solution. The streptavidin alkaline phosphatase was diluted 1:1,000 in PBS and then 50 µl was added to each well. After incubation for 1-2 hours at room temperature, the streptavidin alkaline phosphatase solution was flicked off and the plates were washed 6 times in PBS/T solution. The developing reagent was prepared according to the manufacturers' instructions and 50 µl was added to each well. The plates were developed for 5-30 minutes, until spots had developed at which point the plates were washed with tap water to halt the reaction.

2.3.2.2 Peptides

For the influenza vaccine trials, peptides of 15-20 amino acids in length, overlapping by 10 amino acids and spanning the whole of the NP+M1 insert, were used to stimulate PBMC at a final concentration of 10 µg/ml in 8 pools of 10 peptides.

For the hepatitis C vaccine trials, peptides of 15 amino acids in length, overlapping by 11 amino acids and spanning the ORF from NS3-NS5B (1,985) of HCV genotype 1b strain BK were used to stimulate PBMC. The peptides were divided into six pools, containing between 73 and 112 peptides each, and with a final concentration of 3 µg/ml.

2.3.2.3 Positive and negative controls

For the influenza vaccine trials R10 medium alone was used as a negative control, and a mixture of PHA (10 µg/ml) and SEB (1 µg/ml) was used as a positive control. For the hepatitis

C vaccine trials concavalin A was used instead of PHA/SEB as a positive control and DMSO was used instead of R10 as a negative control.

2.3.2.4 Reading plates, defining responses, and setting pass/fail criteria

Dried ELISpot plates were counted with an AID ELISpot reader (AID Diagnostika). Results were expressed as spot-forming units (SFU) per million PBMC. For an individual peptide pool the response was calculated as the mean of the triplicate peptide wells minus the mean of the negative control wells. The total response to the vaccine immunogen is the sum of the responses to the corresponding peptide pools.

For influenza vaccine trials ELISpot plates were deemed to have failed if either; (1) responses were greater than 100 SFU/million PBMC in the negative control wells, or if (2) responses of less than 1,000 SFU/million PBMC were recorded in the positive control wells.

For the hepatitis C vaccine trial cut offs were established as follows: 74 healthy HCV seronegative volunteers were screened for responses to HCV genotype 1B. The mean response plus three standard deviations was calculated as 48 SFU/million PBMC. Responses below this threshold were considered negative. Responses were also only considered positive if they were greater than three times the background.

2.3.2.5 Statement of attribution for ELISpots

In the FLU001 extension trial approximately half of the ELISpot assays were performed by the author, with the majority of the remainder being performed by Dr T Berthoud. In the FLU003 trial the majority of ELISpot assays were performed by Dr T Berthoud and Pooja Mange. For the HCV003 trial ELISpot assays were performed by Anthony Brown, Rachel Townsend and Leo Swadling.

2.3.3 IMMUNOPHENOTYPING BY FLOW CYTOMETRY

Intracellular cytokine staining (ICS) was performed with samples from the FLU001 extension trial. Assays were performed on the day of vaccination and then at week 1 and week 3 post vaccination using fresh PBMC. A panel of antibodies was selected to detect Th1-type cytokines (IFN-γ, IL-2 and TNF) and the mobilisation of CD107a. Reagent details are provided in Section 2.1.2.

Fresh PBMC (1–2 $x10^6$) were stimulated for 18 hours at 37 °C with either a single pool comprising of all the NP+M1 peptides at a final concentration of 4 µg/ml, SEB (1 µg/ml) or medium alone. The costimulatory monoclonal antibodies αCD28 and αCD49d (1 mg/ml each; BD Pharmingen) were added prior to incubation. After 2 hours, brefeldin A and monensin (both eBioscience) were added. The cells were then washed with FACS buffer, stained with the reagents listed in Section 2.1.2 according to standard procedures and acquired using an LSR II flow cytometer (BD Biosciences). Unstained cells and compensation beads (BD Biosciences) stained singly with the individual monoclonal antibodies were used as controls to calculate compensation. All monoclonal antibodies were titrated for optimal staining. Between 13,000 and 700,000 live, $CD14^-/CD19^-$ lymphocyte events were collected and analysed per condition. The majority of samples were assayed by Dr T Berthoud. Approximately one-quarter of samples were assayed by the author with the assistance of Dr A Spencer. Data were analysed using FlowJo version 9.4 (Tree Star, Inc.).

2.4 CHARACTERISING ANTIBODY RESPONSES TO SEASONAL INFLUENZA VACCINES

Both humoral immunogenicity assays were performed on serum samples from subjects in the FLU003 clinical trial. The pre-vaccination samples were taken on day 0 and the post-vaccination samples were collected at day 21.

2.4.1 ANTI-TIV ELISA

Nunc-Immuno Maxisorp Plates (Thermo Scientific, UK) were coated with 0.75 μg/ml TIV and incubated overnight at 4 °C. The following day plates were washed 6 times with PBS/T. Plates were blocked with 100 μL/well of Casein block solution (Pierce) for 2 hours at room temperature and then washed. Serum samples underwent two fold dilutions (from 1:1,000), were added to the plates for 2 hours at room temperature and then washed. Alkaline phosphatase conjugated goat anti-human IgG (1:5,000, Sigma) was added to the plates and incubated for 1 hour at room temperature. Bound antibodies were detected by adding p-nitrophenylphosphate substrate (Sigma) diluted in diethanolamine buffer (Fisher Scientific). A reference serum was used to generate a standard curve and volunteer serum with low HI titres was used as a negative control. Plates were allowed to develop until the 5^{th} dilution of standard reference serum reached an OD_{405} of 1 using an ELx800 microplate reader (Biotek). This point was defined as 1 relative antibody unit and units were read off of the standard curve similar to published methodology (Miura et al., 2008). Samples were diluted to fall on the linear part of the standard curve. This assay was performed in the Jenner laboratories by Caitlin Mullarkey. Fold changes were calculated to compensate for differences in baseline anti-TIV ELISA response.

2.4.2 HAI ASSAY

Two groups of egg-grown viruses were initially prepared. H1N1-like virus (A/California/7/2009), H3N2-like virus (A/Perth/16/2009) and B-like virus (B/Brisbane/60/2008) represented the strains included in the TIV for the Northern Hemisphere in 2011/12. In addition, A/Brisbane/10/2007(H3N2), A/Victoria/36/2011(H3N2), B/Wisconsin/1/2010, A/Brisbane/59/2007(H1N1) and NIBRG23 (rgA/turkey/Turkey/1/2005 (H5N1)) were prepared as drifted or heterosubtypic strains. Serum samples were initially treated with receptor destroying enzyme (RDEII, Denka Seiken Co Ltd, Japan) according to manufacturer's recommendations. Two-fold serial dilutions of serum were incubated for one hour with each of the above viruses. Turkey erythrocytes (25 µl of 0.5% red blood cell suspension) were then added and incubated for 1 hour before the result was read. Samples were tested in duplicate and negative values (titres <10) were given a nominal value of 5. In accordance with European Agency for the Evaluation of Medicinal Products (EMEA) guidelines results were expressed as percentage seroprotection, seroconversion and geometric mean titre (GMT). HAI assays were performed at the laboratories of the Health Protection Agency/Public Health England (Colindale, London) by Claudia Rosenow, Surita Gangar and Janice Baldevarona.

2.5 RNA METHODS

2.5.1 RNA EXTRACTION

Whole blood samples in PAXgene tubes were thawed from either -20 °C or -80 °C and equilibrated to room temperature. Samples were centrifuged and each pellet was dissolved in 4 ml RNase-free water. Samples were again centrifuged and each pellet was next dissolved

in 350 µl resuspension buffer. Samples were combined with a binding buffer and 40 µl of proteinase K, and incubated at 55 °C. Lysates were then passed through a PAXgene Shredder spin column to remove cellular debris. Supernatants from the flow-through fraction were then combined with 350 µl of ethanol (96–100%) and pipetted into PAXgene RNA spin columns. After centrifugation, the RNA samples were retained on the column membrane and thus the follow through fractions were discarded. DNase I incubation mix was prepared from stock and pipetted onto the PAXgene RNA spin column membrane. After a 15 minute room temperature incubation samples were washed three times. Elution buffer was pipetted directly onto each spin column membrane and samples were centrifuged to elute the RNA. The sample was then used to re-elute any remaining RNA from the spin columns. Samples were then incubated for 5 minutes at 65 °C and then immediately chilled on ice. RNA extractions for FLU002 clinical trial samples were performed by Emma Davenport, whereas RNA extractions for FLU003 and HCV003 samples were performed by the author.

2.5.2 GLOBIN mRNA DEPLETION

A relatively high proportion of globin mRNA will be present in the RNA samples eluted from whole blood, and this can subsequently decrease the sensitivity of RNA microarrays. Globin mRNA was therefore removed from the samples using a GLOBINclear™ kit.

The manufacturer's instructions state that the starting concentration of RNA should be greater than or equal to 70 ng/µl. Approximately one quarter of these samples therefore needed to be concentrated using a centrifugal evaporator (SpeedVac, Thermo Scientific).

Each sample was combined with hybridization buffer and a biotinylated capture oligonucleotide mixture before incubation at 50 °C to allow hybridisation with the globin mRNA. Streptavidin magnetic beads were then added to each RNA sample, mixed by

vortexing and then incubated for 30 minutes at 50 °C. Samples were placed on a magnetic stand and the streptavidin magnetic beads were pulled to the side of the sample tube. After clearing the samples were then combined with prepared RNA binding beads and again placed on a magnetic stand. At this stage the RNA samples were bound to the beads, so the supernatants were carefully aspirated and discarded. Samples were washed and after magnetic capture the supernatants were discarded again. Then the samples were air dried for 5 minutes. To elute the RNA from the beads 20 µl of warm RNAse free water was added to each sample, and then incubated at 58 °C for 5 minutes. The RNA Binding Beads were then captured on a magnetic stand before the supernatants (containing the purified RNA) were transferred to fresh tubes.

Globin mRNA depletion for FLU002 clinical trial samples were performed by Emma Davenport, whereas globin mRNA depletion for FLU003 and HCV003 samples were performed by the author.

2.5.3 RNA QUANTIFICATION AND QUALITY ASSESSMENT

The quantity of RNA obtained after both extraction and globin mRNA depletion was determined by spectrophotometry using a NanoDrop-1000 instrument (Thermo Scientific). Samples were then diluted (where possible) to the same concentration. Prior to microarray analysis the quality of RNA was determined by electrophenogram using a 2100 Bioanalyser instrument (Agilent). Concentrations and RIN values for the samples are shown in Table 2-1.

Table 2-2; Concentration and RIN values for RNA samples			
Trial	Number of samples	Concentration	Mean RIN
FLU002	96*	50 ng/µl	6.73
FLU003	32	45 ng/µl	8.11
HCV003	64	45 ng/µl	8.28
* for FLU002 samples only 32 samples were checked for quality			

2.5.4 RNA MICROARRAYS

Illumina Human-HT 12v4 expression bead chips were used for genome-wide expression analysis. Hybridisation of samples to Illumina bead chip arrays and scanning of the chips was performed by Dr Dilair Baban, High-Throughput Genomics, WTCHG, Oxford. Two microarray experiments were performed, each consisting of 96 samples. Each sample was run once, with 12 samples being loaded per chip and 8 chips being used each time.

RNA samples were loaded onto the bead chips and placed in a hybridization oven at 58 °C. After overnight incubation the bead chips were washed, initially at high temperature (55 °C for 10 minutes) and subsequently at room temperature. The bead chips were incubated with Streptavidin-Cy3 prior to the final wash, and then centrifuged at room temperature for 4 minutes to dry. Once the bead chips were dry they were read using the IScan microarray scanner.

2.5.5 MICROARRAY DATA ANALYSIS

Data from the FLU002 samples were transformed and normalised with assistance from Core Genomics at the WTCHG using the Variance Stabilization and Normalization (VSN) method (Huber et al., 2002). The same method was applied by the author to microarray data from FLU003 and HCV003 trial samples.

For the FLU002 data, two samples were identified as outliers and excluded as described below in Section 5.3.1. Subsequent analysis was performed on the re-normalised 94 remaining samples.

Analysis of gene expression was performed using R software (http://cran.r-project.org/).

The following R packages were downloaded, installed and used to investigate the data:

Limma was downloaded via http://www.bioconductor.org/biocLite.R and maSigPro was downloaded from: http://www.bioconductor.org/packages/2.2/bioc/html/maSigPro.html.

The pamr package (as utilised in Chapter 5) was downloaded from the website http://www.stat.stanford.edu/tibs/PAM/Rdist/.

Heatmaps were used to visually appreciate large quantities of expression data. Heatmaps were generated using the heatmap.2 function from the gplots package (also from http://cran.r-project.org).

Having established lists of significantly expressed genes, the functional relevance of these genes was explored using the bioinformatics resource DAVID (the Database for Annotation, Visualization and Integrated Discovery) which was available at http://david.abcc.ncifcrf.gov (Huang et al., 2009a, Huang et al., 2009b). Additional analysis and graphics were produced by Ingenuity Pathways Analysis and MetaCore™.

2.6 STATISTICS

Statistical analysis of ELISpot assay and flow cytometry data was performed using GraphPad Prism software version 6.02. Data was assumed to have a non-Gaussian distribution and therefore non-parametric tests were used. The Mann-Whitney U-test was used to test for significant differences between groups of volunteers, and the Wilcoxon signed rank test was used to test for significant differences between time points within the same group of volunteers.

For analysis of HI titres, Fisher's exact test was used to compare group differences in seroconversion and seroprotection. HI titre data were log transformed and tested for normality with the Shapiro Wilk test. For normally distributed data an analysis of covariance (ANCOVA) was performed on the log transformed data and adjusted for baseline value. Where log transformed data were not normally distributed, the non-parametric tests described above were applied.

Two-tailed p values were used, and a level of significance of 0.05 was applied unless otherwise specified. For analysis of gene expression data where multiple comparisons are being made, p values are adjusted by the false discovery rate method, and referred to as q values. The threshold of significance is also set at a q value of 0.05 unless otherwise specified.

CHAPTER 3: BOOSTING T CELL RESPONSES TO CONSERVED INFLUENZA VIRUS ANTIGENS WITH MVA-NP+M1 IN OLDER ADULTS

3.1 INTRODUCTION

3.1.1 DEVELOPMENT OF MVA-NP+M1

MVA-NP+M1 is a viral vectored vaccine, comprising MVA expressing nucleoprotein (NP) fused to matrix protein 1 (M1) via a flexible linker (GGGPGGG). This immunogenic insertion is situated at the TK locus under the control of the P7.5 promoter. Both the NP and M1 proteins are derived from influenza virus A/Panama/2007/99 (H3N2), but are over 90% identical with other H3N2 strains, H1N1 strains and H5N1 strains (Berthoud et al., 2011). Studies of healthy adults in the UK have shown that over 90% of individuals have responses to influenza virus proteins that are detectable by ELISpot assay, and over 70% have responses to NP and/or M1 (Lee et al., 2008).

MVA-NP+M1 was first manufactured in August 2007 to GMP standards by IDT Biologika. Clinical trials began in 2008, with volunteers receiving vaccinations at a dose of 5×10^7 pfu either intradermally or intramuscularly (Berthoud et al., 2011). A third group of volunteers then received a higher dose of 2.5×10^8 pfu intramuscularly, although this was associated with an unacceptably high rate of severe adverse reactions. The vaccine was demonstrated to be immunogenic, and a reduced dose of 1.5×10^8 pfu was proposed for future clinical trials (Berthoud et al., 2011).

This trial was given the abbreviated title FLU001. An amendment to explore the safety and immunogenicity of MVA-NP+M1 in older adults was subsequently approved, and this part of the trial is referred to in this thesis as the FLU001 extension trial.

3.1.2 MVA-NP+M1 AS AN ALTERNATIVE STRATEGY FOR VACCINATION AGAINST SEASONAL INFLUENZA

Seasonal influenza is thought to account for between 250,000 and 500,000 deaths per annum worldwide (WHO, 2009). Of these deaths, approximately 90% occur in the over 65 year old age group (Thompson et al., 2003). In addition to these mortality statistics, influenza also causes significant morbidity. In the UK during the 2010-11 season it was estimated that 9,000 hospital admissions were due to influenza, with 2,200 being admitted to intensive care or a high dependency unit (www.nhsemployers.org/HealthyWorkplaces/SeasonalFluCampaign). For these reasons, the elderly are an important target population in national vaccination regimens for seasonal influenza. What is debatable however, is whether vaccination is effective in this age group. Several sources of evidence suggest that seasonal influenza vaccination in the elderly is less effective than in young adults, or not effective at all:

a) Lower rates of seroprotection and seroconversion

Although antibody responses to vaccination measured by HI titre are by no means a perfect correlate of protection, new vaccines are given their manufacturing licenses based on rates of seroprotection and seroconversion. In a quantitative review of 31 vaccine antibody response studies, Goodwin et al. found that responses were 2-4 fold higher in young adults compared to the elderly (defined as study populations with a mean age of 65 and over) (Goodwin et al., 2006).

b) RCT data where age stratification has been performed

Unfortunately only one randomised placebo-controlled trial examining the efficacy of seasonal influenza vaccines has been performed in an elderly population in the last four decades (Govaert et al., 1994). Subjects over the age of 60 were included, with 41.5% of the study population being aged 60-64 years old. While the study did show

an overall efficacy of approximately 50%, an age-stratification suggested declining efficacy with age. In the over 70 year old age group, the confidence intervals for relative risk all ranged above 1 (Govaert et al., 1994).

c) Lack of impact on excess winter mortality

Because assessment of the number of influenza-related deaths in elderly people is a difficult task, an indirect (but robust) approach is used which attributes all excess deaths above an expected winter baseline to influenza. Thus, “excess mortality” (due to influenza) occurs when the mortality rate rises significantly above the expected seasonally variable baseline (Simonsen et al., 2007). Over the past four decades in the US this excess mortality has accounted for between 0.4-10% of winter deaths. If seasonal influenza vaccination were having an effect on mortality, then in situations where vaccination uptake significantly increased, we would expect the excess mortality rate to fall. Opportunities to observe this effect have come from two different countries (the US and Italy) where vaccination coverage rose from 5-15% to approximately 65%. However despite this increased coverage, no fall in the number of excess winter deaths was observed.

d) Unsound evidence base for mortality benefits (the dodgy dossier)

In contrast to the study designs methods mentioned above, which have relatively low risks of containing hidden biases, the evidence base for influenza vaccination reducing mortality comes from cohort studies. Recent retrospective cohort studies have estimated that influenza vaccination reduces all-cause mortality by an astonishing 50%. In order to adjust for selection bias based on health-status, multivariate models and analysis methods are used. This is meant to compensate for the fact that doctors will appropriately decline to vaccinate subjects with extreme medical co-morbidities

(e.g. terminal cancer or end-stage heart failure) where all interventions are being rationalised. However, other factors such as frailty are difficult to include in such models when they do not have a diagnostic code. Yet they do impact on the prescribing decisions made by physicians, and are major risk factors for mortality. Because of these selection biases, the quality of evidence supporting mortality benefits for influenza vaccination in the elderly is poor. The claims of 50% reductions in all-cause mortality jar with the fact that, at most, 10% of winter deaths can be attributed to influenza.

So why should seasonal influenza vaccines be less effective in the elderly? The answers to this are not known, however the consensus opinion is that the phenomenon is linked to the ageing of the immune system (immunosenescence). Indeed influenza vaccines are not the only vaccines which are less immunogenic in the elderly. A meta-analysis of immunological responses to hepatitis B vaccination found that older individuals had a significantly higher relative risk of being a non-responder (Fisman et al., 2002).

Viral vectored vaccines are a relatively novel mechanism for stimulating an antigen-specific immune response, and while studies have been conducted to examine whether the immature immune system can respond adequately to these vaccines, few studies have examined the impact on immunogenicity of an aged immune system. The results I describe here will not only demonstrate the immunogenicity of MVA-NP+M1 in the elderly, but will also be relevant to the wider use of viral vectored vaccines in the elderly.

3.1.3 VACCINATION WITH MVA-NP+M1 FOR PROTECTION AGAINST PANDEMIC INFLUENZA

Although I have outlined in detail the reasons why better seasonal influenza vaccines are needed, MVA-NP+M1 is not solely intended as an alternative to TIV. MVA-NP+M1 is capable of boosting circulating T cell responses to NP and M1 to high levels (Berthoud et al., 2011). It is known that the sequence homology for NP and M1 between different influenza A virus subtypes is over 90%, and therefore T cells induced by this vaccine have the potential to be cross-reactive between subtypes and therefore to provide heterosubtypic immunity. MVA-NP+M1 is intended as a "universal" influenza vaccine, which could also be applied to the setting of pandemic influenza (outlined in Section 1.1.4). Although pandemic influenza can effect younger populations to a greater extent than seasonal influenza, the older age group will most likely require vaccination during an influenza pandemic. It is therefore important to establish whether this vaccine candidate would be immunogenic in this population.

3.2 MVA-NP+M1 VACCINATION IS SAFE IN OLDER ADULTS

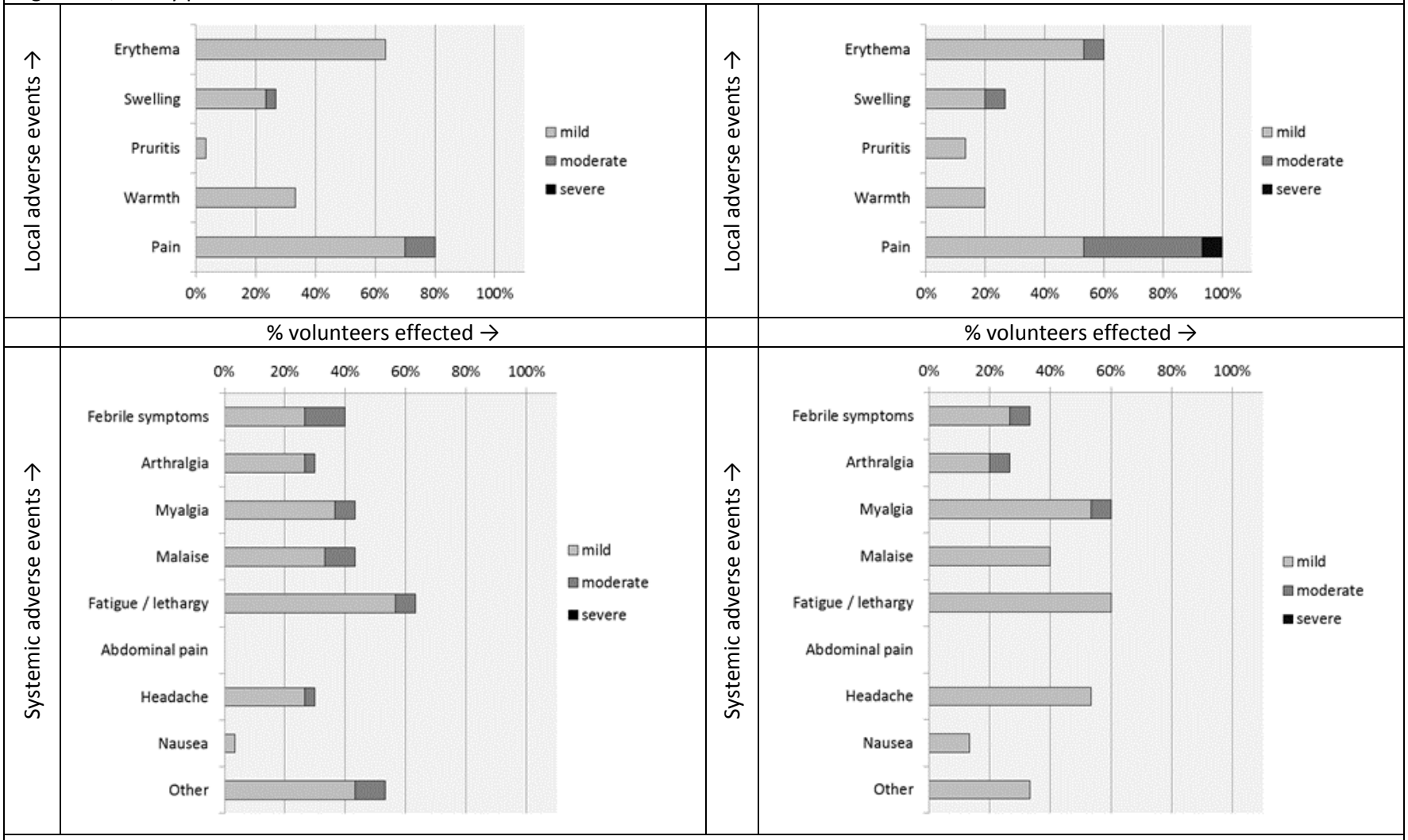
MVA has an excellent safety record, having been administered to more than 200,000 individuals during the smallpox eradication programme. Recombinant MVA vaccines also have an excellent safety profile. For example MVA85A has been administered to more than 2,500 infants (aged at least 18 weeks) (Tameris et al., 2013), and recombinant MVAs expressing HIV-1 antigens have been safely administered to HIV positive subjects (Cosma et al., 2003, Dorrell et al., 2007). Recombinant MVA vaccines expressing tumour antigens have successfully been administered to older adults in cancer trials (Amato et al., 2010). However, clinical trials of viral vectored vaccines against infectious diseases typically recruit adult subjects under the age of 55.

In Figure 3-1 I show that MVA-NP+M1 was safe and well tolerated amongst the 30 subjects in the FLU001 extension trial. There were no serious adverse events related to vaccination, and no persistent laboratory abnormalities were detected on routine haematological and biochemical profiling. Indeed, when evaluating solicited adverse reactions, there were no symptoms that were defined as severe (e.g. requiring a period of bed rest).

The most common adverse reaction was pain at the site of vaccination. This was mild in nature for the majority of volunteers. The next most common localised reaction was some mild erythema (redness), whereas pruritus (itching) was relatively uncommon. Onset of localised symptoms was either on the day of vaccination or the following day in 89% of occurrences. Median duration of localised reactions was 2 days. The most common systemic adverse events following MVA-NP+M1 were myalgia (muscle ache), fatigue and malaise. Onset of systemic symptoms was either on the day of vaccination or the following day in 86% of occurrences and the median duration of systemic reactions was 1 day.

I was able to compare this safety data with previous data from the FLU002 trial. These volunteers (15 in total) were aged 18-45 but had received the same dose of MVA-NP+M1 (1.5×10^8 pfu). The finding that the reactogenicity of MVA-NP+M1 was similar in older and younger age groups was particularly important for a vaccine that would need to include older adults as a target population for vaccination.

Figure 3-1; Safety profile for MVA-NP+M1



The profile of adverse events is shown for MVA-NP+M1. Adverse events with possible, probable or definite causation are shown. Local and systemic adverse events are shown in the top and bottom panels respectively. Data from the 30 volunteers aged 50 years and over and comparative data from younger volunteers aged 18-45 years old (FLU002 trial) are shown in the left and right hand panels respectively.

3.3 MAGNITUDE AND DURATION OF ANTIGEN SPECIFIC IMMUNE RESPONSES FOLLOWING MVA-NP+M1 ADMINISTRATION IN OLDER ADULTS

3.3.1 T CELLS WITH NP AND M1 SPECIFICITY ARE PRESENT AT BASELINE AND STABLE OVER SHORT TIME INTERVALS

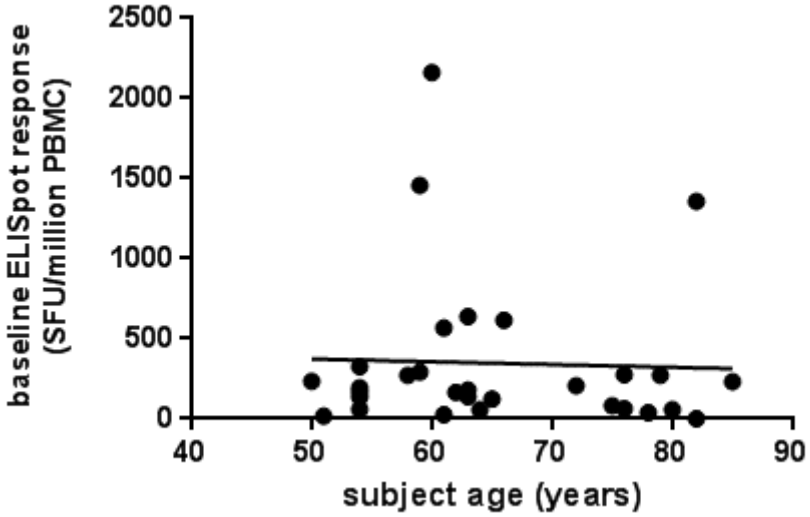
Previous studies have demonstrated that T cells with specificity to either influenza virus NP or M1 are present in the majority (approximately 70%) of healthy adults (Lee et al., 2008). Consistent with this I found that summed NP and M1 responses in the FLU001 extension subjects had a median response of 188 SFU/million PBMC on day 0 (immediately prior to vaccination).

Cumulative exposure to influenza virus increases with age. Children will develop T cell responses to influenza virus following their first exposure but it is unknown whether older adults have different baseline responses to internal influenza virus proteins compared to younger adults. Baseline levels of NP and M1 specific T cells are relevant because they may correlate with peak ELISpot assay response post-vaccination. Furthermore for other viral infections (notably cytomegalovirus) older adults can develop large expansions of oligoclonal T cells. I therefore performed a comparison of NP and M1 responses at baseline to age. Figure 3-2 shows that there does not appear to be a significant increase in these influenza virus-specific cells in older subjects.

In addition to day 0, ELISpot assays were also performed at screening visits. It was therefore possible to examine the stability of T cell responses to NP and M1 over time by comparing results at the two time points (Figure 3-3). This is important because clinical trials are being

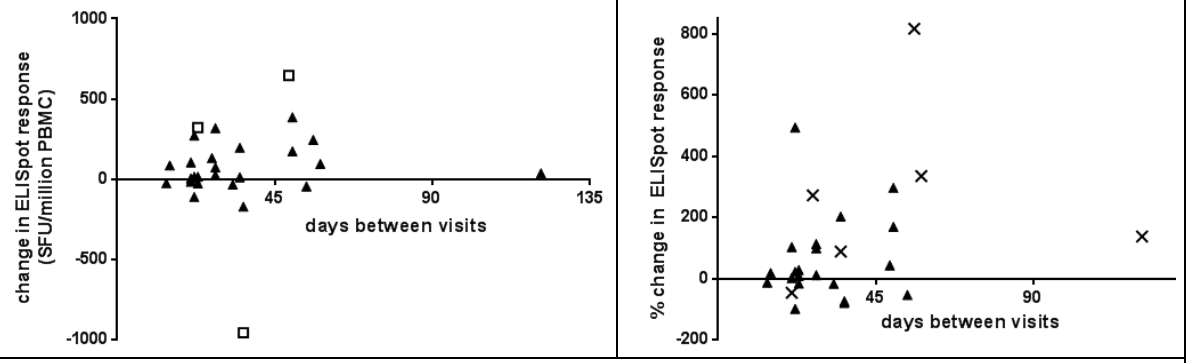
conducted over lengthy periods, and natural variations have the potential to mask responses induced by vaccination.

Figure 3-2; Pre-vaccination IFN-γ ELISpot responses by age of subject



Pre-vaccination T cell responses to NP and M1 (as determined by IFN-γ ELISpot assay) are shown on the y-axis with the age of the volunteer on the x-axis. Data from all 30 subjects in the FLU001 extension trial are shown. No significant correlation was identified (r = -0.102, p = 0.593).
Approximately half of the ELISpots in this figure were performed by the author.

Figure 3-3; Stability of T cell responses to NP and M1

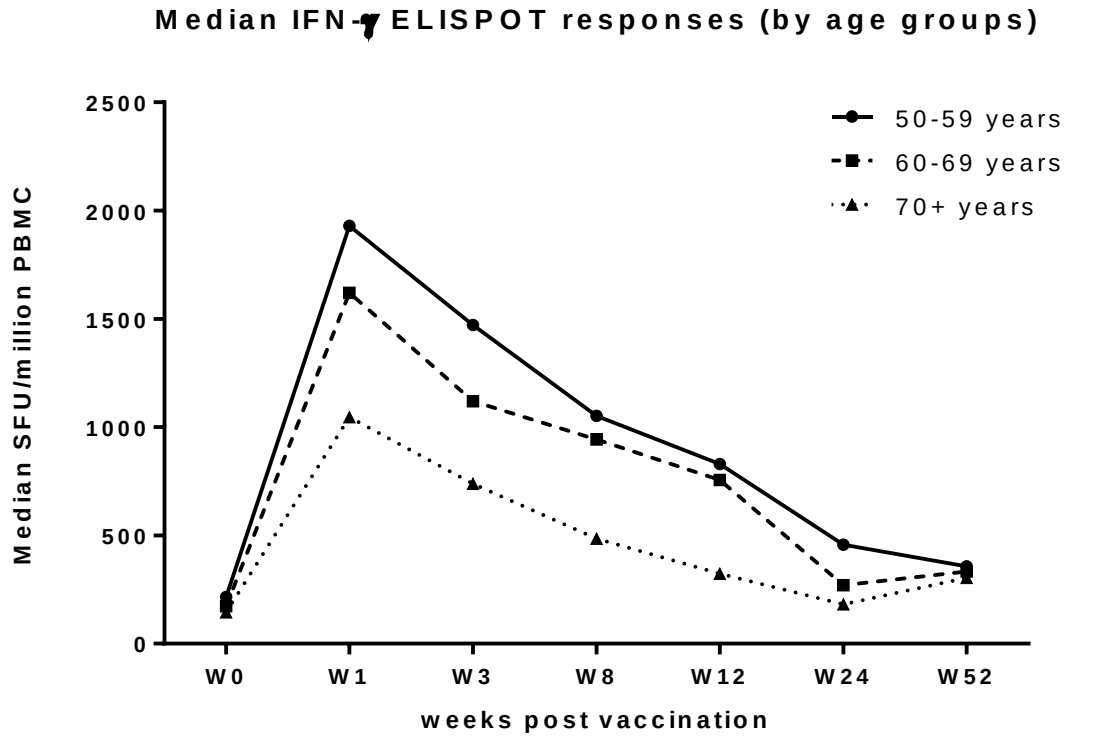


Differences in T cell responses to NP and M1 (as determined by IFN-γ ELISpot assay) are shown between the screening visit and the day of vaccination. For both graphs time (days) between visits is shown on the x-axis. On the left, absolute change in IFN-γ ELISpot assay responses is shown on the y-axis. The three individuals who had an initial response >1,000 SFU/million PBMC are shown as squares, with the remaining individuals represented by triangles. On the right, the percentage change in IFN-γ ELISpot assay response is shown on the y-axis. The six individuals who had an initial response <50 SFU/million PBMC are shown as crosses with the remaining individuals represented by triangles.

3.3.2 THE MAGNITUDE OF RESPONSE TO VACCINATION WITH MVA-NP+M1 VARIES WITH AGE

All subjects demonstrated an increase in NP and M1 responses following vaccination with MVA-NP+M1. Peak IFN-γ ELISpot assay response occurred on day 7 in 80% of subjects.

Figure 3-4; Median IFN-γ ELISpot responses stratified by age



This figure shows median T cell responses to the influenza virus proteins NP and M1 (γ-axis) as determined by IFN-γ ELISpot assay. These responses are shown over time (x-axis) following vaccination with MVA-NP+M1 at week 0 (W0). Volunteers are from the FLU001 extension trial are grouped by age with 10 volunteers per group.
Analysis of the differences between age groups at the different time points using the Kruskal-Wallis test gave p values of 0.0729, 0.6465, 0.0097, 0.0147 and 0.1334 respectively at 1 week, 3 weeks, 8 weeks, 12 weeks and 24 weeks post vaccination.
Approximately half of the ELISpot experiments used to construct this figure were performed by the author.

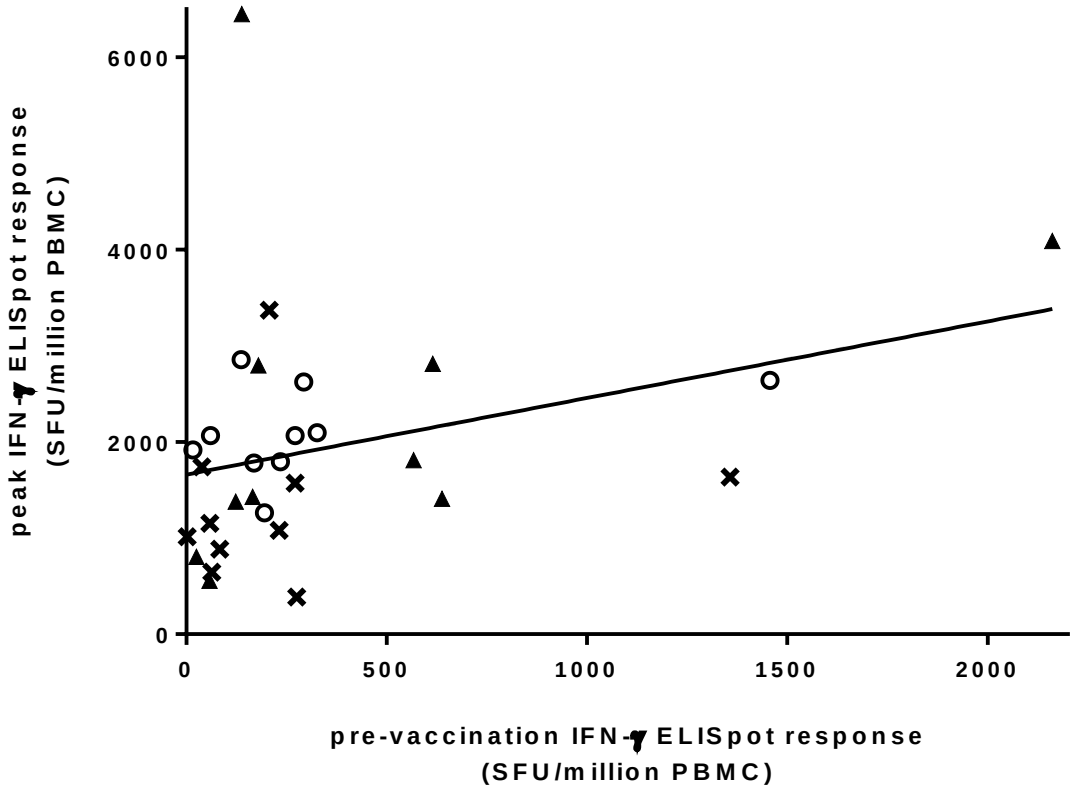
When the magnitude of IFN-γ ELISpot assay responses was compared, there was a trend towards lower responses in the oldest age group and higher responses in the youngest age group (Figure 3-4). Analysis of the differences between age groups at the different time points

using the Kruskal-Wallis test gave p values of 0.0729, 0.6465, 0.0097, 0.0147 and 0.1334 at 1 week, 3 weeks, 8 weeks, 12 weeks and 24 weeks post vaccination.

3.3.3 POST-VACCINATION RESPONSES CORRELATE WITH BASELINE RESPONSE

The magnitude of post-vaccination IFN-γ ELISpot assay responses may be influenced by the baseline pre-vaccination level of NP- and M1-specific T cells. Figure 3-5 shows a scatter plot of pre-vaccination and peak post-vaccination responses.

Figure 3-5; Correlation between pre- and post-vaccination IFN-γ ELISpot responses



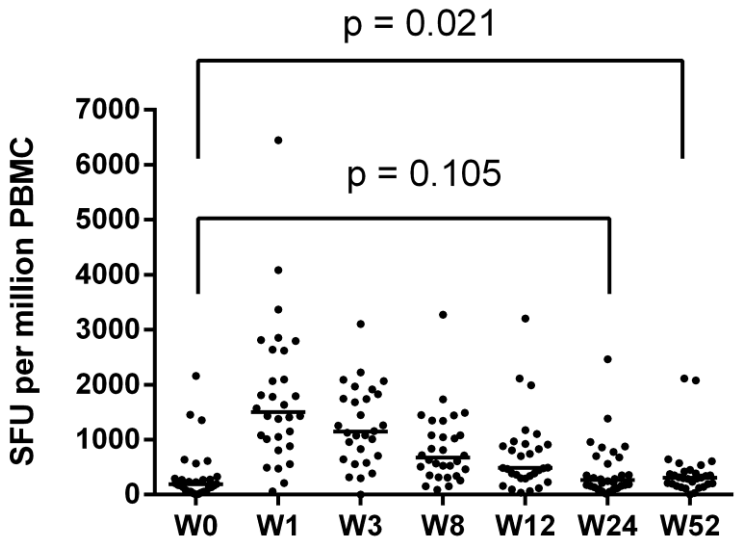
This figure shows T cell responses specific for the influenza virus proteins NP and M1 as determined by IFN-γ ELISpot assay. Each point represents one of the 30 subjects in the FLU001 extension trial. Subjects aged 50-59 years old are represented by circles, subjects aged 60-69 years old are represented by triangles and subjects aged 70 years and over are represented by crosses. The T cell responses before vaccination with MVA-NP+M1 (x-axis) correlate with the T cell responses after vaccination with MVA-NP+M1 (y-axis). Statistical significance was determined by Spearman's rank correlation coefficient (r = 0.4065, p = 0.0258).

The positive correlation between pre- and post-vaccination responses was just statistically significant (Spearman r = 0.4065, p = 0.0258).

3.3.4 DURATION OF ANTIGEN SPECIFIC IMMUNE RESPONSES FOLLOWING MVA-NP+M1 IN OLDER ADULTS

For a vaccine to be effective in practice it must be able to induce protection over a sustained period of time. I was therefore interested to examine the durability of the immune responses induced by MVA-NP+M1. When data from the 30 volunteers are analysed together median NP and M1 responses are increased compared to baseline at all time points. All these increases are significant (Wilcoxon signed rank test) with the exception of week 24 post vaccination (Figure 3-6).

Figure 3-6; Duration of IFN-γ ELISpot responses following MVA-NP+M1



This figure shows data from the 30 subjects in the FLU001 extension trial. T cell responses to the influenza virus proteins NP and M1 as determined by IFN-γ ELISpot are shown on the y-axis. Responses are shown over time (x-axis) following vaccination with MVA-NP+M1 at week 0 (W0). The p values for post-vaccination responses compared to week 0 were <0.001 at week 1, week 3, week 8 and week 12, p = 0.105 at week 24 and p = 0.021 at week 52. Approximately half of the IFN-γ ELISpots for this figure were performed by the author.

However, when the subjects are analysed according to three stratified age groups then differences in vaccine durability become apparent. For the 50-59 years old age group, responses to NP and M1 remained significant at all time points out to 52 weeks. For the 60-69 years old age group, responses were significantly elevated at 12 weeks post vaccination ($p = 0.037$), but not at 24 weeks post-vaccination. Finally, for the 70 years old and over age group, there was a much more rapid drop off in ELISpot assay responses, which were only significantly raised at 1 and 3 weeks post vaccination ($p = 0.006$; $p = 0.023$), but not at any of the other time points.

3.3.5 DIVERSITY OF T CELL RESPONSES AMONGST PEPTIDE POOLS

So far I have examined the magnitude of NP and M1-specifc T cell responses without reference to whether these responses are narrowly focused or broadly focused on multiple epitopes. Magnitude of response is particularly of interest because one of the few clinical studies to find a T cell correlate of protection from influenza virus (Wilkinson et al., 2012) was for the frequency of CD4 T cells specific for nucleoprotein. While a role for T cell breadth has not been identified for influenza virus, this does have a clear role in other human viral infections – notably HCV (Spada et al., 2004, Lechner et al., 2000).

T cell responses to influenza A viruses are characterised by immunodominance – a phenomenon whereby the majority of CTL are specific for a small number of epitopes despite multiple non-dominant epitopes being immunogenic (Akram and Inman, 2012). This phenomenon is seen both in humans who have had multiple natural exposures to influenza viruses and in mice that have been experimentally challenged with influenza viruses. It has also been reported that as humans age, gaps develop in the T cell repertoire to influenza virus

(Yager et al., 2008), and that the focus of the immune system on immunodominant epitopes becomes even narrower. Because of the relevance this has for a T cell inducing influenza vaccine in the elderly I examined the distribution of pre-vaccination ELISpot assay responses to NP and M1 amongst the eight peptide pools. As described in Section 2.3.2.2, the peptides in these pools were 15-20 amino acids in length, overlapped by 10 amino acids and spanned the whole of the NP+M1 insert. For each individual I calculated the percentage of the total response that was attributed to each of the eight different peptide pools. Interestingly, while the majority (80%) of individuals aged 50-59 had T cell responses that were dominated (>50% total) by responses in either one or two pools, only 40% of subjects aged 70 and over had responses that were dominated by one or two pools.

It is important to consider that HLA type is a major determinant of the specific nature of the T cell response to influenza virus. HLA types for the individuals in the FLU001 extension study are shown in Table 3-1 below. By chance there were no A1 genotype volunteers in Group 2 and no A11 genotype volunteers in Group 3.

Table 3-1; HLA types for volunteers in the FLU001 extension study

HLA	A1	A2	A3	A11	B7	B8
Group 1	40%	30%	20%	40%	40%	30%
Group 2	0%	50%	40%	20%	30%	0%
Group 3	50%	50%	30%	0%	40%	20%

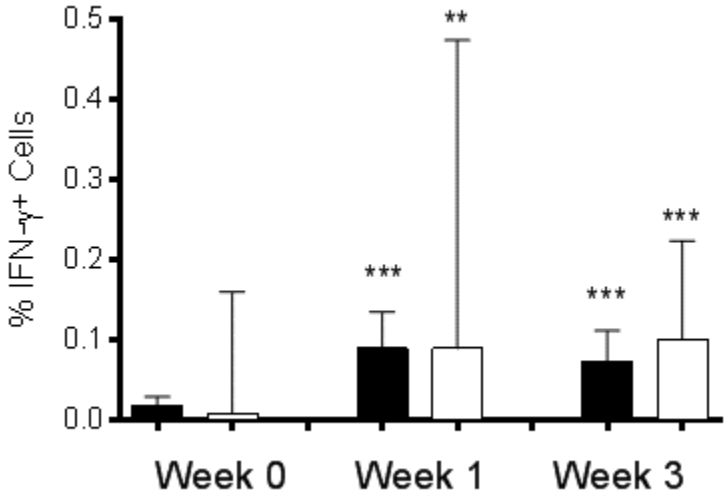
Vaccination with MVA-NP+M1 had a wide range of effects on pre-existing immunodominant T cell responses. In some individuals responses in a dominant peptide pool remained dominant after vaccination, whereas for other individuals a previously subdominant response took over from a dominant one. No consistent pattern was evident. The responses within volunteers of a particular HLA type were also variable. For HLA-A2 individuals the $M1_{58-66}$

epitope (in pool 6, or “p6”) is known to be immunodominant. Surprisingly only 7.7% (1/13) of A2 individuals had a pre-vaccination response in p6 which was >30% of the total response, and only 31% (4/13) of A2 individuals had a pre-vaccination response in p6 which was >20% of the total response. While responses to p6 peptides became substantially more represented in three A2 individuals post-vaccination with MVA-NP+M1, responses to other pools (p2, p3, p5) became more represented in other A2 individuals.

3.3.6 BOTH CD4 AND CD8 T CELL SUBSETS ARE BOOSTED BY VACCINATION WITH MVA-NP+M1

Earlier studies with MVA-NP+M1 had revealed that both CD4 and CD8 T cell subsets secreting IFN-γ were boosted by vaccination (Berthoud et al., 2011). However, the increases in the CD8 subset were more substantial than the CD4 subset (reaching a median frequency of 0.4% versus 0.098%) and achieved statistical significance. In the FLU001 extension I was able to analyse a larger number of vaccine recipients. In keeping with the results from the younger volunteers, pre-vaccination IFN-γ secreting T cell responses to NP and M1 were low in both CD4 and CD8 T cell subsets (median less than 0.02% of total CD4 or CD8). The median frequency of IFN-γ secreting T cell responses was similar in CD4 and CD8 T cell subsets, both at 1 week and 3 weeks post vaccination (Figure 3-7). However the range of responses both before and after vaccination is consistently greater in the CD8 T cell subset.

Figure 3-7; Interferon gamma secretion by CD4 and CD8 T cell subsets



This figure represents data from the 30 subjects in the FLU001 extension trial, where subjects were vaccinated with MVA-NP+M1 at Week 0. The frequency of interferon gamma secreting T cells is shown on the y-axis, as determined by intracellular cytokine staining. Assays were performed with fresh PBMC and used stimulation with NP and M1 peptides. Columns represent median percentage responses compared to total CD4 cells (black) or CD8 cells (white). Error bars indicate interquartile range. All increases at week 1 or week 3 are significant compared to baseline (Wilcoxon signed rank test):

** $p < 0.002$ *** $p < 0.001$

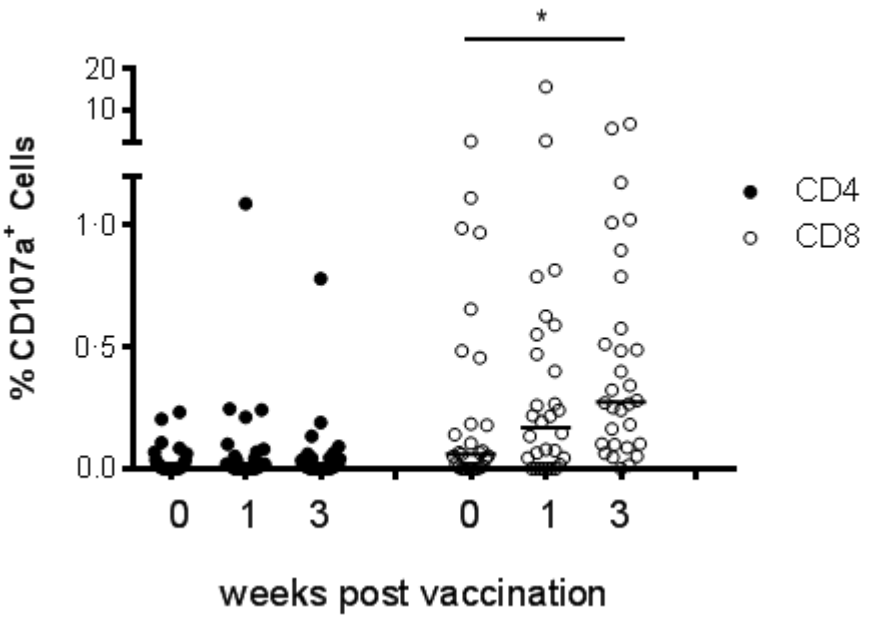
Approximately one-quarter of samples for this figure were assayed by the author with the remainder of samples being assayed by Dr T Berthoud.

3.3.7 CD8 T CELLS MOBILISE CD107a FOLLOWING VACCINATION

Cytotoxic T cells exert their effects principally through the release of specialised lysosomes. These structures are called cytotoxic granules and contain perforin and granzymes. These proteins are able to induce apoptosis in virally infected (or cancerous) host cells. CD107a (also known as lysosomal-associated membrane protein 1 (LAMP1)), is an integral membrane protein in cytotoxic granules and can be used as a marker for degranulation in CD8 T cells. Previous studies have demonstrated strong correlations between expression of CD107a and the ability for peptide-specific CD8 T cells to kill cognate target cells in chromium release assays (Rubio et al., 2003).

In the FLU001 extension subjects, CD107a expression by NP and M1 specific CD4 T cells was low prior to vaccination and did not increase following vaccination. Expression of CD107a by CD8 T cells was higher at baseline, and then increased following vaccination (Figure 3-8). This finding suggests that MVA-NP+M1 is able to boost antigen specific T cells with cytotoxic potential.

Figure 3-8; Mobilisation of CD107a in CD8 T cells compared to CD4 T cells

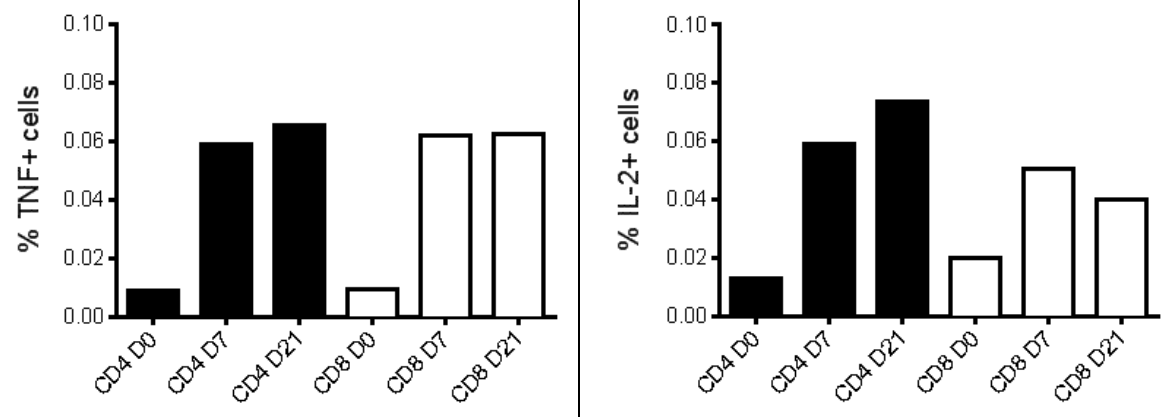


This figure represents data from the 30 subjects in the FLU001 extension trial, where subjects were vaccinated with MVA-NP+M1 at week 0. Surface mobilisation of CD107a was determined by flow cytometry following stimulation with NP and M1 peptides. Pre- and post-vaccination responses are shown separately for CD4 T cells (black circles) and CD8 T cells (open circles). CD107a expression by CD4 T cells was low and remained unaffected by vaccination. CD107a expression by CD8 T cells was higher at baseline but also increased following vaccination, peaking at 3 weeks. * p = 0.012, Kruskal-Wallis test
Approximately one-quarter of samples for this figure were assayed by the author with the remainder of samples being assayed by Dr T Berthoud.

3.3.8 BOTH CD4 AND CD8 T CELLS SECRETE MULTIPLE Th1 CYTOKINES FOLLOWING VACCINATION WITH MVA-NP+M1

In addition to secreting IFN-γ, NP- and M1-specific T cells also secreted the cytokines IL-2 and TNFα (Figure 3-9). Significant increases in the frequency of these cytokine secreting T cells were detected both at 1 week and at 3 weeks post vaccination.

Figure 3-9; CD4 and CD8 T cells secreting TNFα and IL-2

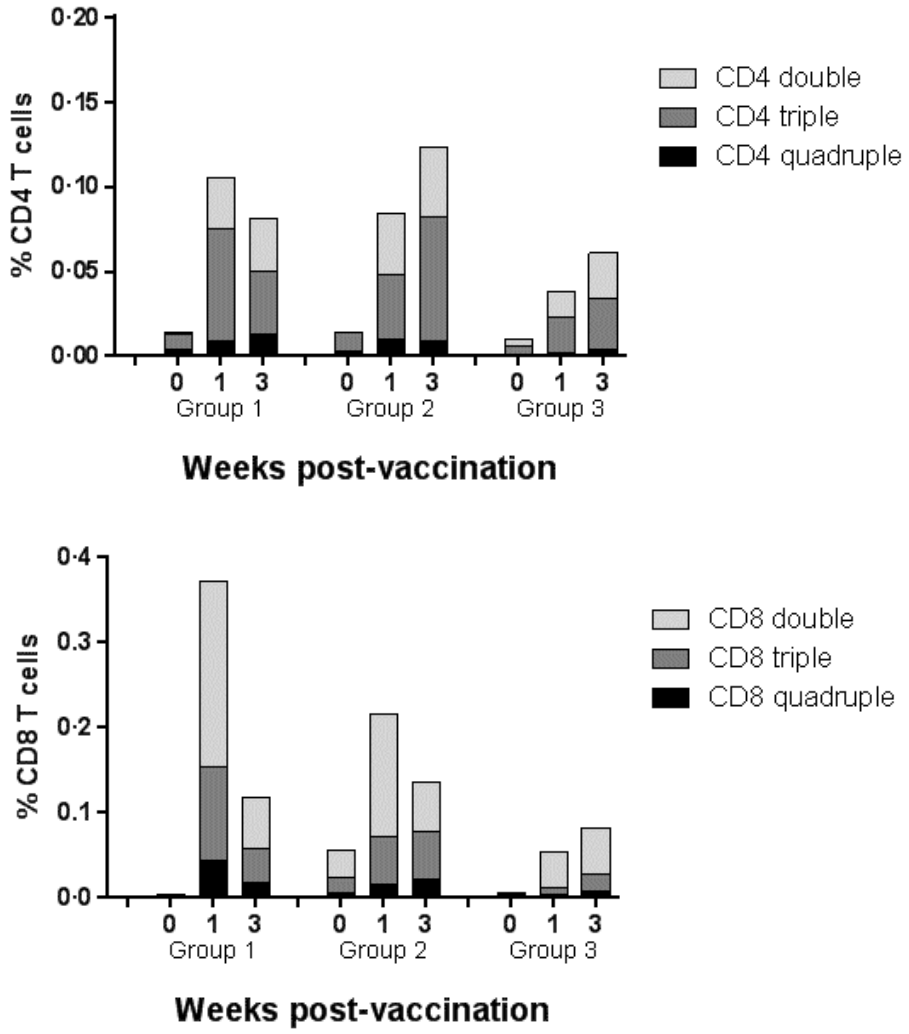


Expression of TNFα (left panel) and IL-2 (right panel) in NP- and M1-specific T cells as determined by intracellular cytokine staining. Median responses from all volunteers are shown at three time points (D0 / week 0, D7 / week 1 and D21 / week 3). Black bars represent CD4 T cells subsets and white bars represent CD8 T cell subsets. Significant increases compared to baseline were demonstrated at both 1 week and 3 weeks post-vaccination, for both cytokines and in both T cell subsets (Wilcoxon signed rank test). Approximately one-quarter of samples for this figure were assayed by the author with the remainder of samples being assayed by Dr T Berthoud.

T cells that secrete multiple cytokines have been termed polyfunctional, and have been associated with protective immunity in some models of infectious disease (Darrah et al., 2007). Figure 3-10 shows the frequency of T cells that are secreting multiple cytokines. Increases in the median frequency of polyfunctional T cells are observed in all three age groups at both post-vaccination time points compared to baseline. This is true of both CD4 T cell and CD8 T cell subsets. However the median frequency of double and triple cytokine

secreting T cells is lower in the oldest age group compared to subjects aged 50-69 years old. Again this is true of both CD4 and CD8 T cell subsets.

Figure 3-10; CD4 and CD8 T cell subsets with multiple functions



This figure represents data from the 30 subjects in the FLU001 extension trial, where subjects were vaccinated with MVA-NP+M1 at week 0. These data were obtained from flow cytometry experiments using intracellular cytokine staining following stimulation of fresh PBMC with NP and M1 peptides. The median frequency of T cells demonstrating more than one function are shown for CD4 T cells (top) and CD8 T cells (bottom) Functions include the secretion of the Th1 cytokines IFN-γ, TNF and IL-2 as well as the mobilisation of CD107a. Approximately one-quarter of samples for this figure were assayed by the author with the remainder of samples being assayed by Dr T Berthoud.

3.4 DISCUSSION

I have first shown that MVA-NP+M1 can be given safely at a dose of 1.5 x 10^8 pfu in healthy adults over the age of 50. The oldest volunteer in the study was 85 years old, and even when volunteers were stratified according to age, there was no increase in the mean number of adverse events amongst the oldest volunteers. These data therefore add to the previous experience with MVA-5T4 (Trovax) from the cancer immunotherapy field that recombinant MVA vectored vaccines are well tolerated in the elderly (Amato et al., 2010). Despite these encouraging findings it is important to note that this population of clinical trial volunteers has been highly selected: 37% of screened subjects were excluded, and 26% of screened subjects were excluded specifically due to either medical co-morbidities or abnormal haematological or biochemical indices. It is possible that in a more representative elderly population which included a proportion of frail subjects, the tolerability of the vaccine may be different.

I then examined the magnitude of IFN-γ ELISpot assay responses to the vaccine insert, with particular attention to the effects of age. MVA-NP+M1 was highly potent in older adults, and resulted in an 8.5 fold increase in NP- and M1-specific T cells. When the 30 subjects in the FLU001 extension trial were compared to the 15 subjects who had received the same dose of MVA-NP+M1 in the FLU002 trial no significant differences were observed (Antrobus et al., 2012). However this comparison could only be made at the day 21 post-vaccination time point, as this was the only common sample available.

I then examined the effects of advanced age on immunogenicity by comparing IFN-γ ELISpot assay responses within the three stratified age groups (50-59, 60-69 and 70+ years old). At 7 days post-vaccination the median IFN-γ ELISpot assay responses were 1,929, 1,620 and 1,047

SFU/10^6 PBMC ($p = 0.073$, Kruskal-Wallis test). However in an analysis comparing the 50-59 years old age group and the 70+ age group these differences are significant ($p = 0.035$, Mann Whitney test). Significant differences in ELISpot assay responses were also detected between the three age groups at 8 weeks and 12 weeks post-vaccination ($p = 0.0097$ and $p = 0.0147$, Kruskal-Wallis test). The power to detect any statistically significant differences between the age groups at 3 weeks post-vaccination was diminished by the loss of two data points in the 70+ age group.

These reductions in absolute magnitude are mirrored by reductions in the durability of response. For each of the three groups the baseline response at day 0 was compared to each post-vaccination time point. For the 50-59 year old age group, all post-vaccination time points (spanning 12 months follow up) were significantly raised above baseline. In contrast the 60-69 year old age group ceased to have a significantly raised response by week 24, and for the 70+ age group responses were no longer significant by week 8.

It would seem unlikely that lower post-boost responses were due to lower pre-vaccination levels of NP- and M1 specific T cells in the oldest age group, as baseline responses were similar between the age groups, and there was no overall correlation between baseline response and subject age. It would also seem unlikely that the lower magnitude of responses related to gaps in the T cell repertoire as ELISpot assays revealed a broad spectrum of responses across the peptide pools. However two possible explanations as to why these responses could be reduced with age are described below:

Anti-vector responses:

The immunogenicity of viral vectored vaccines may be influenced by anti-vector immunity. For recombinant MVA vaccines, anti-vector immunity may have been developed from either prior exposure to smallpox, or more likely, from smallpox vaccination. Could the oldest volunteers in the FLU001 extension study have had greater exposure to vaccination?

Routine vaccinations for smallpox took place in the UK in the 1960's, finishing in 1971. The youngest volunteer in the FLU001 extension cohort was born in 1960, so it would seem unlikely that this cohort would have been denied vaccination. Nevertheless with small group sizes, it would be possible for one cohort to have a difference in vaccination status. It is also plausible for anti-vector immune responses induced by vaccination to persist after decades without subsequent exposure (Hammarlund et al., 2003). The most reliable method for determining this would be to identify vaccinia-specific antibodies in screening samples. A robust, sensitive and specific ELISA method for detecting these antibodies has been reported (Hammarlund et al., 2003). In contrast, if volunteer history were to be used as the sole method for determination of vaccination status then it might prove difficult to obtain accurate results. The younger volunteers would be less likely to report a positive history due to recall bias.

Immunosenescence

Another possible explanation for the reduction in immunogenicity amongst older subjects is immunosenescence, which is described in more detail in Section 1.6. Is there any evidence from pre-clinical studies to support the role of immunosenescence in this vaccination setting? One might expect studies using animal models to provide consistent evidence about which vaccines exhibit reduced immunogenicity or efficacy with age, as inbred animals reduce the variation in responses that are due to host genetics. Unfortunately there is a lack of published data on the immunogenicity of viral vectored vaccines between young and aged mice, however several studies have been published describing the effects of using DNA vaccines in young and aged mice. However even these results are inconsistent, with some studies demonstrating reduced immunogenicity and efficacy (Klinman et al., 1998) and other studies showing equivalent immunogenicity (Bender et al., 1998) and/or efficacy (Ito et al., 2010). Thus there is little pre-clinical evidence to help hypothesise about whether a viral vectored vaccine should be equally immunogenic between young and old human subjects.

So why should a recombinant MVA-vectored vaccine be less immunogenic in the elderly? One explanation might come from the target cell of MVA. If DCs were less abundant, either initially at, or following recruitment to, the vaccination site, then potentially less antigen will be presented to memory T cells. Alternatively if DCs were functionally impaired, then they might respond less vigorously to and/or secrete lower quantities of pro-inflammatory cytokines. This might result in reduced expression of co-stimulatory molecules. Although this has not previously been established for MVA, it has been shown that DCs derived from elderly subjects secrete less TNF, express less maturation markers and lead to reduced T cell proliferation following stimulation with influenza virus (Liu et al., 2012). Alternatively there

may be an age associated impairment of T cells (Goronzy and Weyand, 2013) which make them proliferate less vigorously and contract more promptly.

After examining the magnitude and duration of T cell responses, I then examined the phenotype of boosted T cells. Both CD4 and CD8 T cells are induced by vaccination with MVA-NP+M1. The relative contribution that each T cell subset may provide for protection from influenza is not known, however evidence exists for both. For example Wilkinson et al. have correlated the magnitude of CD4 T cells specific for nucleoprotein and matrix protein with protection from influenza virus challenge (Wilkinson et al., 2012), while McMichael et al. have associated T cell cytotoxicity (in chromium release assays) with protection from influenza virus challenge (McMichael et al., 1983b). The latter study implies a predominant role for CD8 T cells expressing CD107a (Rubio et al., 2003), which are also shown to be significantly induced following vaccination with MVA-NP+M1. Finally, I have shown that T cells induced by MVA-NP+M1 are capable of secreting multiple cytokines. This is particularly relevant as these "multifunctional" or "polyfunctional" T cells have been associated with control of infection in some murine models (Darrah et al., 2007). Furthermore, in HIV infected individuals, those individuals termed "elite controllers" have been demonstrated to have a greater frequency of polyfunctional HIV-specific T cells compared to HAART suppressed, non-controllers or HIV seronegative individuals (Owen et al., 2010). This suggests that polyfunctional T cells can be clinically relevant as well as protective in animal models.

One of the reasons that MVA-NP+M1 is so immunogenic in this setting is that MVA is being used to boost existing T cell responses, which have been primed over a lifetime of natural exposure to influenza viruses. As I will later describe in Chapter 6, recombinant MVA is far less effective at priming T cells in immunologically naive subjects. Although the magnitude of

boosting was excellent, high levels of NP- and M1-specific T cells were not maintained for long. In the first 6 months following vaccination with MVA-NP+M1 T cells contracted with a half-life of approximately 9 weeks. Previous studies have reported that the half-life of influenza virus-specific T cells induced by natural influenza infection is in the order of 2-3 years (McMichael et al., 1983a), however the methods employed in the two studies were quite different making direct comparisons difficult. While it might be attractive to explore how the durability of these vaccine responses could be extended, ultimately we require a better understanding of T cell correlates of protection for influenza in humans, so that we can direct research towards the most efficacious endpoints.

In Chapter 6 I describe how MVA can be combined with adenoviral vectors in a heterologous prime boost regimen to produce high levels of circulating T cells specific for a recombinant antigenic insert. This may also prove to be an effective strategy for influenza vaccines and the clinical trial to address this question is now underway (ClinicalTrials.gov Identifier: NCT01818362).

CHAPTER 4: COMBINING TRIVALENT INACTIVATED VACCINES AND VIRAL VECTORED VACCINES AGAINST INFLUENZA VIRUS IN HUMANS

4.1 INTRODUCTION

The trivalent inactivated influenza vaccine is insufficiently immunogenic in certain circumstances. Firstly, as discussed in section 3.1 it results in significantly lower rates of seroconversion and seroprotection in the elderly (Goodwin et al., 2006). These serological outcomes are typically measured by haemagglutinin-inhibition assay and are defined in the box 1. Secondly, TIV is less immunogenic in young children (Schmidt-Ott et al., 2007), and two doses are recommended to ensure adequate protection. This is particularly true for children less than 2 years old, where the second dose of TIV results in a substantial improvement to seroprotection rates (Englund et al., 2005). In addition to this lack of immunogenicity, inactivated vaccines have similar efficacy rates to placebo in children under 2 years old (Jefferson et al., 2005). Additionally, influenza virus haemagglutinin molecules from non-TIV subtypes are naturally poor immunogens. This is typified by H5 subtypes, where 30 µg doses may be given twice and still produce poor rates of seroconversion (Nicholson et al., 2001).

Box 1:

Seroprotection is defined as a HI titre of >1:40.

Seroconversion is defined as a four-fold increase in HI titre. For example from 1:20 to 1:80

One method for improving immunogenicity in these vaccines is to use an adjuvant. Adjuvants act in a non-specific way to enhance adaptive immune responses to a specific antigen. The archetypal adjuvant has been alum, however there has been a rapid increase in availability of adjuvants (Table 4-1), which are increasingly being shown to be safe and effective.

Table 4-1; Examples of vaccines adjuvants in use					
Adjuvant	Components				
	Alum	MPL	Oil-in-water emulsion (e.g. squalene)	Saponin (e.g. QS21)	Liposome
Alum	+	-	-	-	-
MF59 / Addavax*	-	-	+	-	-
Matrix M / AbISCO*	-	-	-	+	-
AS01^	-	+	-	+	+
AS02^	-	+	+	+	-
AS03^	-	-	+	-	-
AS04^	+	+	-	-	-
* Addavax and AbISCO are research grade adjuvants for use in pre-clinical studies ^ AS = adjuvant system (manufactured by GSK)					

The innate and adaptive arms of the immune system co-operate in producing an immune response. Danger signals are sensed through pattern recognition receptors on innate immune cells. These signals are then conveyed through the release of cytokines and the expression of co-stimulatory molecules. One mechanism by which adjuvants can operate is by mimicking these danger signals. An example of this is the drug Imiquimod, which has been licensed to treat genital warts (due to human papilloma virus infection) and whose active ingredient is a toll-like receptor (TLR)-7 agonist. The natural agonist for TLR-7 is single stranded RNA, and via this interaction viral infections can be detected and signalling occurs to trigger a host antiviral responses.

These notions are particularly relevant when considering the use of viral vectored vaccines. Not only do these vectors provide a delivery system for the antigen of interest, they also provide a local environment that is immunostimulatory. In other words they have intrinsic properties which allow them to function as an adjuvant. It is this rationale that provides the hypothesis, whereby co-administration of an inactivated influenza vaccine (such as TIV)

together with a viral vectored vaccine (such as MVA-NP+M1) could result in enhanced antibody responses.

Evidence for adjuvanting through co-administration of viral vectors and protein vaccines was published by Hutchings et al. in 2005. Using BALB/c mice and hepatitis B surface antigen (HBsAg) vaccine, co-administration regimens were examined with plasmid DNA, recombinant adenovirus and multiple poxviruses. Both recombinant and non-recombinant MVA mixed with HBsAg, produced higher titres of anti-hepatitis B antibodies compared to HBsAg alone. This effect was also observed with the other poxviruses (NYVAC, FP9, ALVAC) and to a lesser extent with recombinant adenovirus (Hutchings et al., 2005).

Further data to support this hypothesis comes from Mullarkey et al. who have examined the ability for MVA-NP+M1 to act as an adjuvant in three separate animal models (Mullarkey et al., 2013):

In mice, the co-administration of MVA-NP+M1 alongside TIV, enhances the antibody responses to TIV as measured by both ELISA and HI titre. Antibody responses measured by ELISA remained significantly higher up to 6 months post-vaccination. In challenge experiments, mice receiving vaccine co-administration lost less weight and showed a trend towards increased survival compared to mice vaccinated with TIV alone.

In chickens, the co-administration of MVA-NP+M1 alongside H7 HA protein (from A/Netherlands/219/03) at 3 weeks post-hatch, produced significantly higher antibody responses to H7 protein as measured by ELISA.

Finally in pigs, the co-administration of a prime-boost regimen of viral vectored vaccines (ChAdOx1-NP+M1 and MVA-NP+M1) together with H5 HA protein (from A/duck/Hunan/795/03) was shown to be superior to H5 HA protein alone, in terms of the breadth of antibody response generated. Pseudotype neutralisation assays were used to

assess breadth, and significant increases in IC_{50} titres were observed in 80% of the 5 lentiviral pseudotypes tested.

In this Chapter I attempt to translate these pre-clinical findings into a clinical trial of vaccine co-administration.

4.2 VACCINE CO-ADMINISTRATION IS SAFE

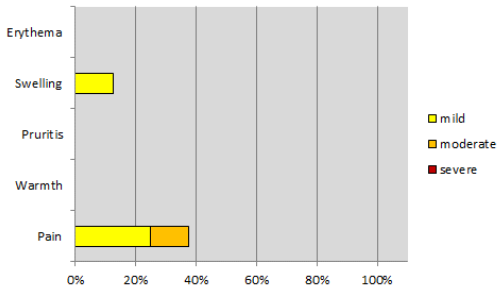
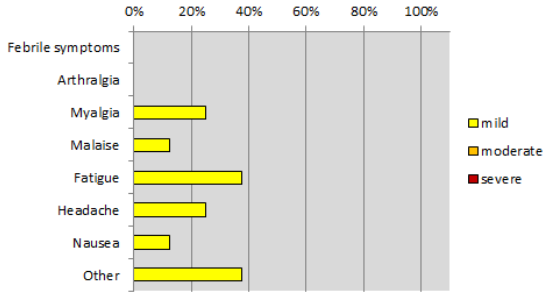
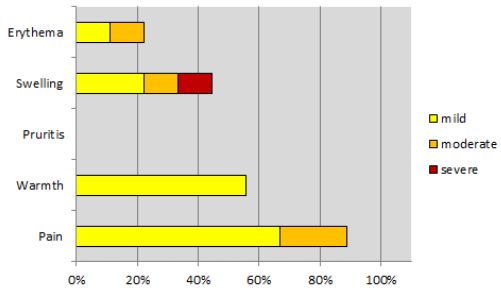
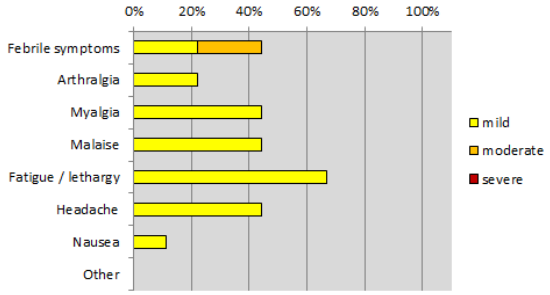
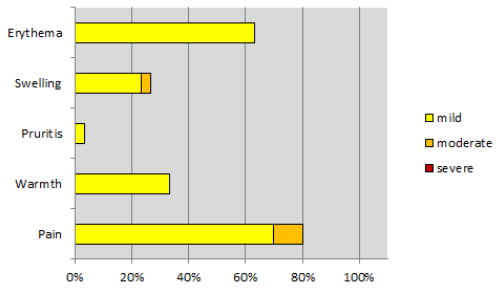
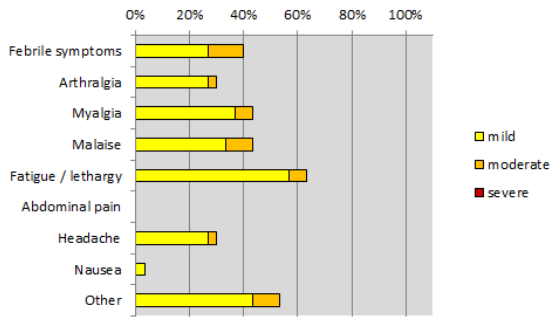
In order to establish the relative contributions made by each vaccine to the overall side effect profile, safety data from three groups was compared. In my control group, 8 volunteers received an injection of TIV followed by an injection of saline. This second injection was important to control for the placebo effect that may accompany receipt of an experimental vaccine and secondly to control for the fact that the co-administration group received a combined injection volume of 1.65 ml.

In the control group, vaccination with TIV and placebo was very well tolerated. The majority of volunteers (63%) experienced no localised adverse events. There were more systemic adverse events than local adverse events (mean = 1.5 vs 0.5 events per volunteer) however these were all of mild severity. The most common systemic adverse event was fatigue and there were no instances of fever or feverishness.

Co-administration of MVA-NP+M1 and seasonal influenza vaccine was safe in the 9 adults that were vaccinated. There were no serious adverse events and there were no adverse reactions detected from monitoring of routine haematological and biochemical parameters. The most common localised adverse event was pain, which occurred in 89% of volunteers. Onset of localised symptoms was either on the day of vaccination or the following day in 90% of occurrences and the median duration of localised reactions was 3 days. Localised adverse

events were mild in 74% of occurrences and only 1 severe adverse event was observed in the vaccine co-administration group (an episode of swelling).

Figure 4-1; Safety profile for three different vaccine regimens

	Local adverse events	Systemic adverse events
TIV only		
Co-administration		
MVA-NP+M1 only		

Adverse events are shown for three different vaccination regimens. From the FLU003 trial eight volunteers who received TIV only (top panels) and nine volunteers who received MVA-NP+M1 and TIV (middle panels) are shown. For comparison, thirty volunteers who received MVA-NP+M1 (bottom panels) are shown. Local adverse events are shown on the left and systemic adverse events are shown on the right. Only adverse events that are deemed related to vaccination are shown.

The most common systemic adverse event in the co-administration group was fatigue, but again the majority of adverse events were mild in severity.

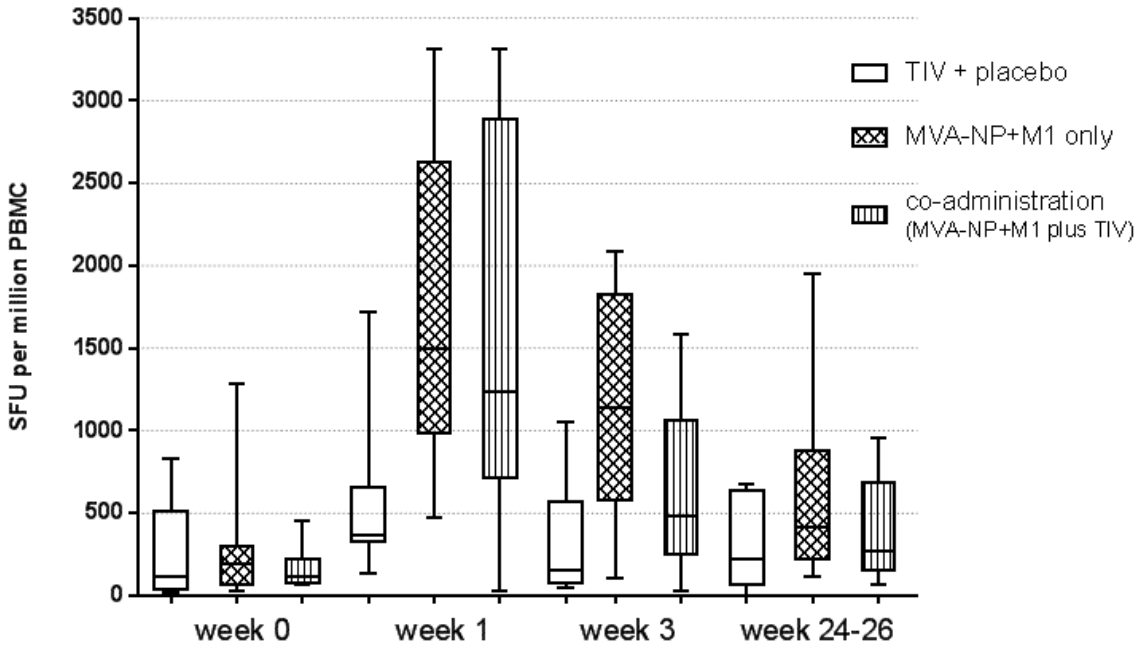
The adverse event profiles were then compared between the co-administration group and volunteers who received vaccination with MVA-NP+M1 alone as part of the FLU001 extension trial (Chapter 3). Overall the frequency and distribution of adverse events in the vaccine co-administration group was very similar to that observed when MVA-NP+M1 was administered as a single vaccination (Figure 4-1).

4.3 T CELL IMMUNITY IS NOT SIGNIFICANTLY IMPAIRED BY VACCINE CO-ADMINISTRATION

Data from the ELISpot assays show that co-administration of the two vaccines results in a greater magnitude of T cell responses to NP and M1 than TIV alone. This increase is statistically significant at day 7 but not at week 3 or week 26 post vaccination.

I then compared these responses to the results described in Chapter 3. The peak response (at one week post vaccination in all cases) is equivalent when MVA-NP+M1 is given alone, or when it is co-administered with TIV. The responses are also equivalent at the week 24-26 time point. There is however a significant drop off in the response at day 21 in the co-administration group (Figure 4-2).

Figure 4-2; IFN-γ ELISpot responses following vaccine co-administration



Data are shown from vaccinated volunteers from two clinical trials. From the FLU001 extension trial, data from 30 subjects who received vaccination with MVA-NP+M1 at week 0 are shown (cross hatched shading). From the FLU003 trial eight volunteers who received TIV only (clear boxes) and nine volunteers who received MVA-NP+M1 and TIV (vertical striped boxes) are shown. T cell responses to NP and M1 as determined by IFN-γ ELISpot assay are shown on the y-axis. Boxes represent the interquartile range, with horizontal bars at the median and whiskers representing 10-90th centile. Responses are shown for three different groups: Volunteers receiving TIV only are shown with clear boxes, volunteers receiving MVA-NP+M1 (from the FLU001 extension study) are shown with cross hatched boxes and volunteers receiving vaccine co-administration are shown with vertically striped boxes.

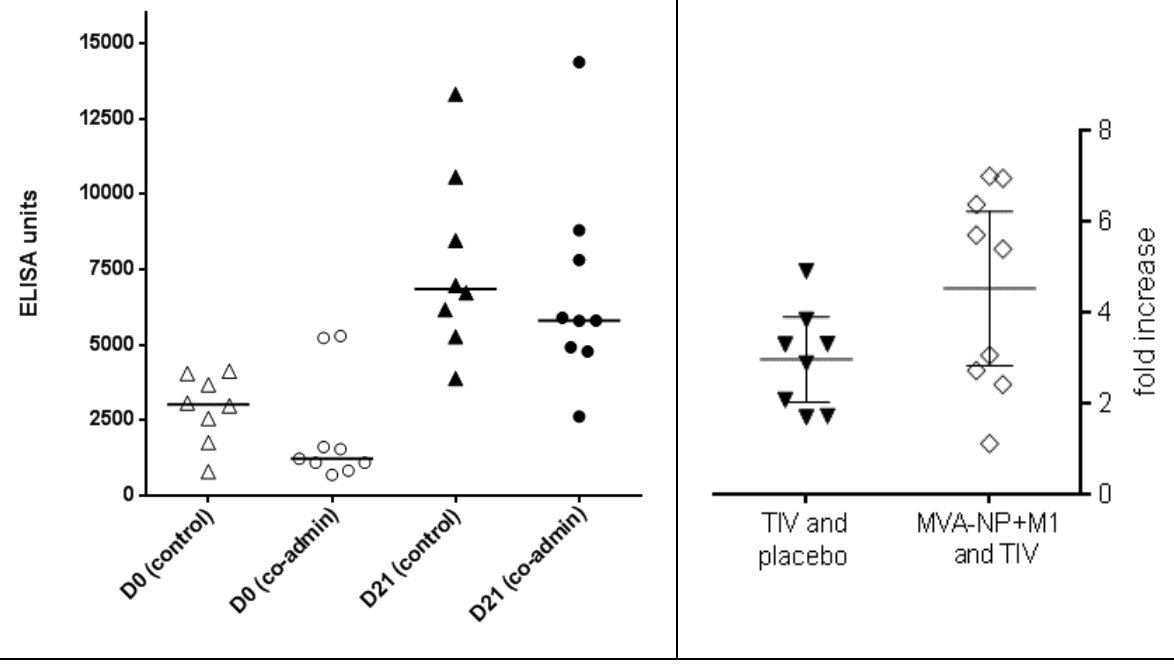
The following comparisons gave p values as follows (Wilcoxon signed rank test)

Vaccine group:	week 0 vs week 1	week 0 vs week 3
TIV + placebo	p = 0.0156	p = 0.3125
MVA-NP+M1	p < 0.0001	p < 0.0001
MVA-NP+M1 + TIV	p = 0.0078	p = 0.0273

4.4 ANTIBODY RESPONSES FOLLOWING VACCINE CO-ADMINISTRATION

Humoral immune responses to TIV were determined using two different methodologies. Firstly, an anti-TIV ELISA method was used (as described in Section 2.4.1). Serum samples from the day of vaccination (day 0) and 21 days post-vaccination were tested and responses from all individuals increased from day 0 to day 21. The results are shown in Figure 4-3 below in arbitrary units (left panel) and as fold change (right panel).

Figure 4-3; Antibody responses to TIV as measured by anti-TIV ELISA



Data are shown from the 17 volunteers in the FLU003 trial. The eight subjects in the control group were inoculated with TIV and a saline placebo at day 0 (D0). The nine subjects in the co-administration group were vaccinated with MVA-NP+M1 and TIV at day 0 (D0). Post-vaccination serum samples were taken at day 21 (D21). Left panel shows serum antibody concentration as measured by ELISA with arbitrary units on the y axis. The same batch of TIV that was used to vaccinate volunteers was used to coat the ELISA plates. In the left hand panel data from the two groups are shown at two time points (i.e. day 0 and day 21). There was no significant difference in the anti-TIV ELISA responses between the two groups at day 21 ($p = 0.4174$). The right panel shows the same data as a ratio of D0:D21 (i.e. fold increase), but again there is no significant difference between the two groups ($p = 0.1967$).
Anti-TIV ELISAs were performed by Caitlin Mullarkey.

It was particularly important to examine fold change as there were baseline differences in responses between the two groups. Fold increases were greater in the co-administration group compared to the control group, however the difference was not statistically significant ($p = 0.197$, Mann Whitney test).

Next, antibody responses to the three influenza virus strains in the vaccine were determined by HI titre (Section 2.4.2). For both groups, the HI titres were used to calculate rates of seroprotection (at each time point), seroconversion (between the two time points), and the geometric mean titre. No significant differences were observed between the two groups when either seroprotection or seroconversion were used as the outcome measures (Tables 4-4 and 4-5). When post-vaccination geometric mean titres were compared, greater responses at day 21 were observed in the co-administration group for the two vaccine strains of influenza A virus, although these did not reach statistical significance. However, from Table 4-2 it can be seen that despite randomising subjects to treatment group, there were baseline differences in geometric mean titre. To compensate for these baseline differences geometric mean ratios were calculated, representing the fold change in geometric mean titre. Analysis of the geometric mean ratios revealed a significantly greater response to the H3N2 component of TIV in the group that received vaccine co-administration (Table 4-2).

In addition to the three vaccine strains of influenza virus, HI titres were also measured to 4 drifted strains of influenza virus and one heterosubtypic strain of influenza A (H5) virus. Responses to the drifted H1N1 and H5 strains were very low, and although responses were detected for the other three drifted strains, no statistically significant differences were detected between the co-administration group and the group that received TIV alone (Table 4-3).

Table 4-2; HI titres for TIV strains of influenza virus							
	Co-administration group (TIV + MVA-NP+M1)			Control group (TIV + placebo)			P value
	GMT (day 0)	GMT (day 21)	GMR	GMT (day 0)	GMT (day 21)	GMR	
H3N2 (A/Perth/16/2009)	7.4	339.2	45.6	16.2	200.2	12.3	0.047
H1N1 (A/California/7/2009)	9.3	97.6	10.5	13.6	54.6	4.0	0.21
Influenza B (B/Brisbane/60/2008)	5.0	47.3	9.5	5.5	57.4	10.5	0.87
Abbreviations: GMT, geometric mean titre; GMR, geometric mean ratio; TIV, trivalent inactivated influenza vaccine. P values relate to the differences in GMR. HAI assays were performed at the laboratories of the Health Protection Agency/Public Health England by Claudia Rosenow, Surita Gangar and Janice Baldevarona.							

Table 4-3; HI titre data for non-vaccine strains of influenza virus							
	Co-administration group (TIV + MVA-NP+M1)			Control group (TIV + placebo)			P value
	GMT (day 0)	GMT (day 21)	GMR	GMT (day 0)	GMT (day 21)	GMR	
H3N2 (A/Brisbane/10/2007)	11.3	160.0	14.2	25.0	131.7	5.26	0.225
H3N2 (A/Victoria/361/2011)	5.4	11.3	2.09	5.9	11.1	1.86	0.760
H1N1 (A/Brisbane/59/2007)	5.0	5.0	1.00	10.0	10.5	1.05	0.346
H5N1 (rgA/turkey/Turkey/1/2005)	5.0	5.4	1.08	5.0	5.5	1.09	1.000
Influenza B (B/Wisconsin/1/2010)	5.0	16.1	3.22	2.9	9.8	1.65	0.415
Abbreviations: GMT, geometric mean titre; GMR, geometric mean ratio; TIV, trivalent inactivated influenza vaccine. P values relate to the differences in GMR. HAI assays were performed at the laboratories of the Health Protection Agency/Public Health England by Claudia Rosenow, Surita Gangar and Janice Baldevarona.							

Table 4-4; HI titres expressed as seroprotection

		Co-administration group (TIV + MVA-NP+M1) at day 0	Control group (TIV + placebo) at day 0	Co-administration group (TIV + MVA-NP+M1) at day 21	Control group (TIV + placebo) at day 21	P value
vaccine strains	H3N2 (A/Perth/16/2009)	0%	25%	100%	88%	0.47
	H1N1 (A/California/7/2009)	11%	25%	67%	63%	0.63
	Influenza B (B/Brisbane/60/2008)	0%	0%	56%	63%	0.58
non-vaccine strains	H3N2 (A/Brisbane/10/2007)	22%	50%	78%	75%	0.66
	H3N2 (A/Victoria/361/2011)	0%	0%	11%	13%	0.74
	H1N1 (A/Brisbane/59/2007)	0%	25%	0%	25%	0.21
	H5N1 (rgA/turkey/Turkey/1/2005)	0%	0%	0%	0%	1.00
	Influenza B (B/Wisconsin/1/2010)	0%	0%	22%	13%	0.55

Abbreviations: TIV, trivalent inactivated influenza vaccine. P values relate to the differences in seroprotection at day 21.
HAI assays were performed at the laboratories of the Health Protection Agency/Public Health England by Claudia Rosenow, Surita Gangar and Janice Baldevarona.

Table 4-5; HI titres expressed as seroconversion between day 0 and day 21

		Co-administration group (TIV + MVA-NP+M1)	Control group (TIV + placebo)	P value
vaccine strains	H3N2 (A/Perth/16/2009)	100%	75%	0.21
	H1N1 (A/California/7/2009)	56%	63%	0.58
	Influenza B (B/Brisbane/60/2008)	56%	63%	0.58
non-vaccine strains	H3N2 (A/Brisbane/10/2007)	78%	63%	0.44
	H3N2 (A/Victoria/361/2011)	11%	13%	0.74
	H1N1 (A/Brisbane/59/2007)	0%	0%	1.00
	H5N1 (rgA/turkey/Turkey/1/2005)	0%	0%	1.00
	Influenza B (B/Wisconsin/1/2010)	22%	0%	0.26

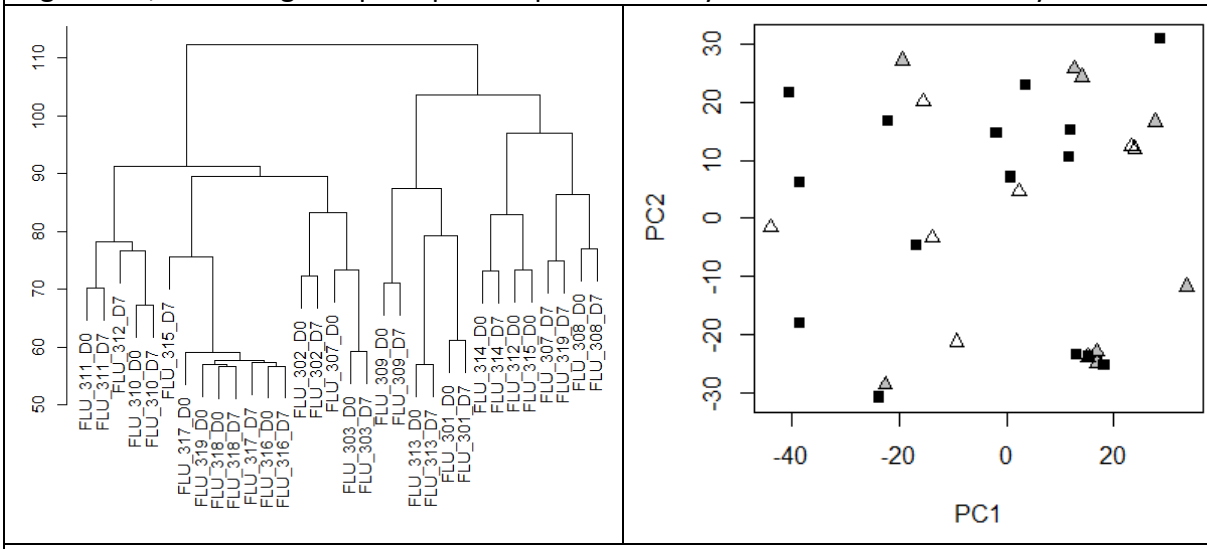
Abbreviations: TIV, trivalent inactivated influenza vaccine.
HAI assays were performed at the laboratories of the Health Protection Agency/Public Health England by Claudia Rosenow, Surita Gangar and Janice Baldevarona.

4.5 WHOLE BLOOD GENE EXPRESSION PROFILING FOLLOWING VACCINATION WITH EITHER TIV OR TIV/MVA-NP+M1 CO-ADMINISTRATION

Bucasas et al. have previously demonstrated that three distinct patterns of gene expression occur following vaccination with TIV (Bucasas et al., 2011). These expression patterns were detected using similar methodologies to those presented here, with RNA being extracted from whole blood collected into PAXgene tubes. They reported that while the maximal changes in gene expression were from the up-regulation of genes in the first 24 hours post vaccination, two other patterns were identifiable: Some 1,867 genes were up-regulated at 3 days post vaccination, and then maintained higher expression out to day 14. A final group of genes were down-regulated early post-vaccination but then remained under expressed through day 3 and beyond to day 14.

After normalisation of the data, clustering analysis and then principal components analysis was performed to explore whether the samples clustered into separate groups. Of particular interest was whether the day 0 and day 7 samples would segregate, and additionally whether the day 7 samples would segregate into two groups according to vaccination status. Figure 4-4 demonstrates that no segregation of the samples into any of these groups was evident.

Figure 4-4; Clustering and principal components analysis for FLU003 microarray data



This figure shows two visual representations of signal data generated from gene expression microarrays from samples taken in the FLU003 clinical trial. RNA was extracted from whole blood, which was collected both prior to vaccination (day 0, D0) and one week post-vaccination (day 7, D7). The left hand panel is a clustering analysis. The right hand panel is a principal components analysis (PCA). For the PCA the first and second principal components are shown on the x-axis and y-axis respectively. For the PCA, pre-vaccination samples are shown by black squares, whereas post-vaccination samples are shown by triangles shaded white for the control group, and shaded grey for the co-administration group. Neither method shows segregation of samples according to group or time point.

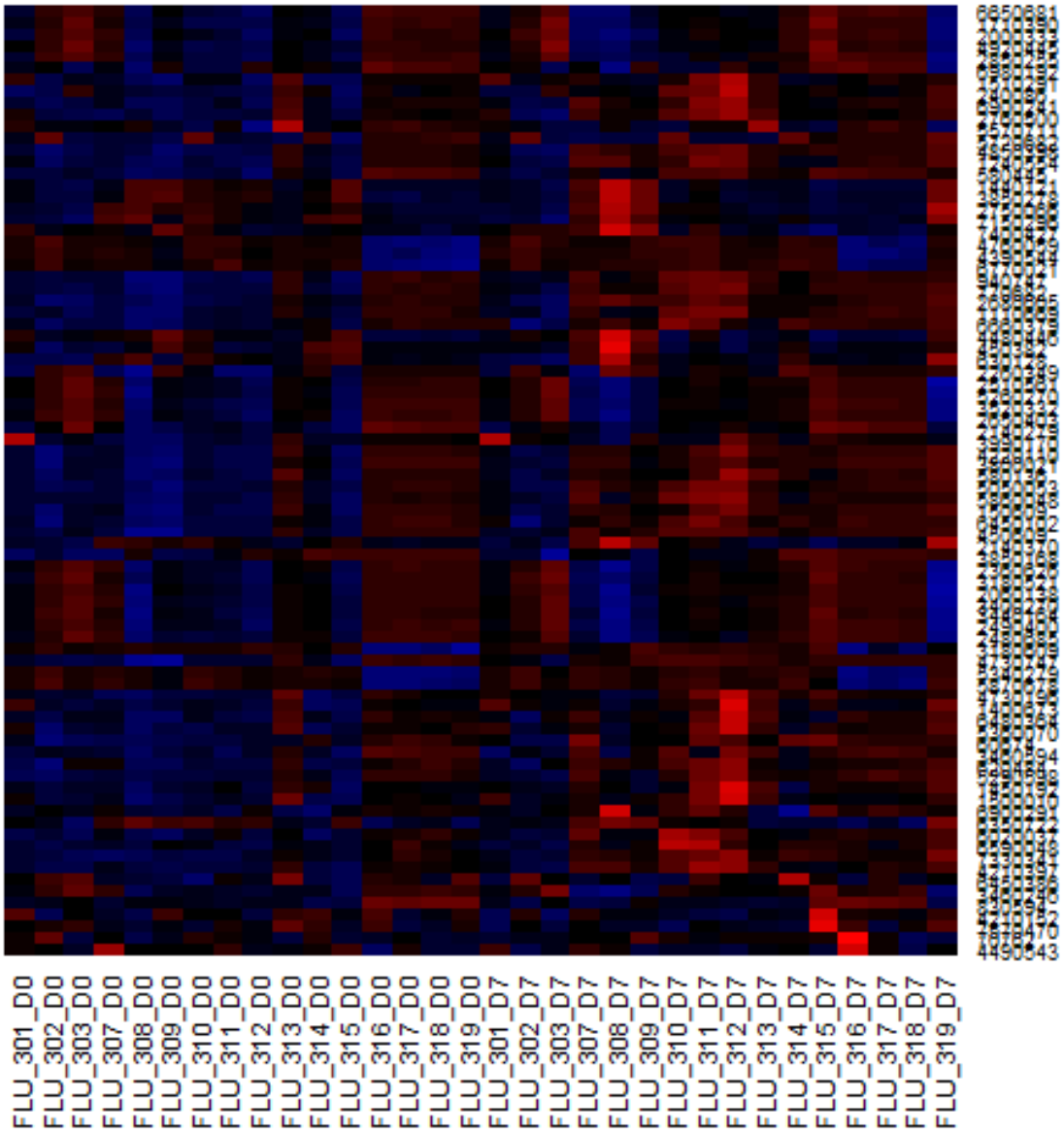
Analysis was then performed using Limma, to determine whether there were any significantly differentially expressed genes between day 0 samples and day 7 samples. This analysis was performed for all samples combined (as they had all received TIV) as well as separately according to treatment group (i.e. TIV only versus vaccine co-administration). For all three variations, no differentially expressed genes were identified.

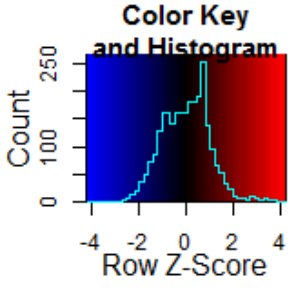
One explanation for the lack of differential gene expression could be the small sample size in the study population, and the large number of probes (36,748) in the data set. The large number of probes reduces the likelihood of finding significant differences due to the fact that multiple comparisons require adjustment to p values. In contrast to this small subject

population the Bucasas et al. data set had the advantage of access to 368 arrays from 92 individuals. Therefore in an attempt to focus in on the most significant genes, and reduce the number of comparisons, the FLU003 data set was reduced to 3,053 probes. These probes were selected based on the genes that were reported as being differentially expressed at day 3 and day 14 by Bucasas. Again, a Limma analysis was performed to look for differential expression between day 0 and day 7 but no significant changes were identified.

Exploration of the data set further confirmed that very few genes had substantial fold change differences between day 0 and day 7. A number of probes (n=82) were then selected based on whether at least one subject has a change of expression for that probe that was >2 (log2 scale). A heatmap was then generated to identify whether subgroups of individuals displayed similar patterns of gene expression. Figure 4-5 shows that expression patterns for volunteers 310, 311 and 312 were similar, and that expression patterns for volunteers 308 and 319 were similar. These appearances were confirmed by subsequent Limma analysis, revealing 50 and 28 significantly differentially expressed genes respectively. However with no clear phenotypic association within these subgroups of subjects, the functional significance of these gene lists is doubtful.

Figure 4-5; Heatmap showing probes with greatest change of expression



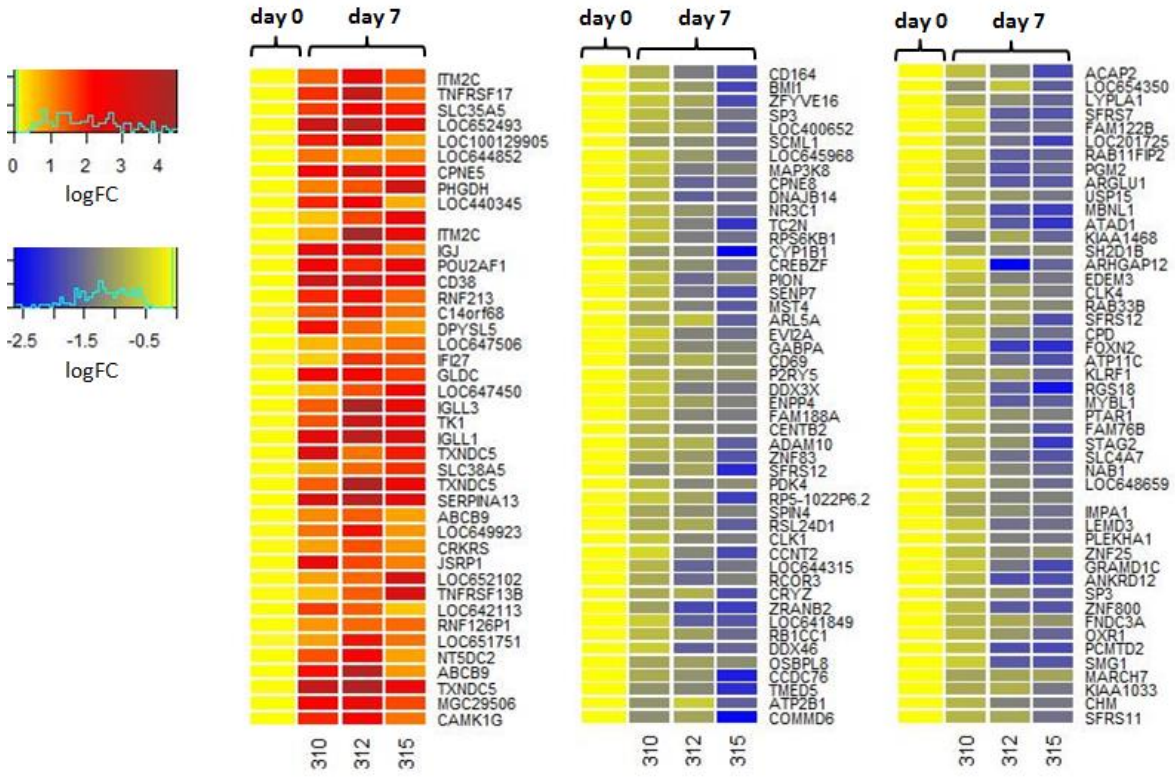


This heatmap represents gene expression before and after vaccination in the FLU003 clinical trial. Volunteers 301, 307, 308, 310, 311, 313, 314 and 318 were vaccinated with TIV and a saline placebo. Volunteers 302, 303, 309, 312, 315, 316, 317 and 319 received vaccine co-administration (MVA-NP+M1 and TIV). The subjects are arranged in columns with probes by rows. Red colours indicate greater than average expression and blue colours indicate lower than average expression.

A final analysis was performed to examine whether significant gene expression patterns were restricted to those volunteers who had the greatest immune response to vaccination. Using

this approach three volunteers (310, 312 and 315) were identified which had 138 genes with significant changes in expression between day 0 and day 7 (Figure 4-6). These three volunteers were among the top four highest responders to NP and M1 (in terms of fold change ELISpot assay response) and all seroconverted to at least one strain of influenza virus in the TIV. Within the list of up-regulated genes there were several which were indicative of an activated immune response, including the interferon-inducible protein IFI27, two members of the TNF receptor superfamily and three different immunoglobulin polypeptides.

Figure 4-6; Genes with significant changes in expression following vaccination in three subjects



Subjects 310, 312 and 315 were found to have 138 genes with significant differential expression between day 0 and day 7. The three heatmaps show genes (probes) by rows. Red colours indicate an increase in log fold change expression and blue colours indicate a decrease. Volunteer 310 was vaccinated with TIV and a saline placebo, whereas volunteers 312 and 315 received vaccine co-administration (MVA-NP+M1 and TIV).

4.6 DISCUSSION

Increased reactogenicity is often a concern for co-administration vaccination regimens, where for example increases in pain and other local adverse events have been observed with combined DTP-HBV-HIB vaccines (Bar-On et al., 2012). The split virion unadjuvanted seasonal influenza vaccine used in the FLU003 trial has a mild pattern of local and systemic adverse events as described in the summary of product characteristics. In contrast, the typical pattern of adverse events experienced from MVA-NP+M1 is more pronounced (as described in the Investigator's Brochure). Therefore, the fact that the vaccine co-administration group had a more severe pattern of adverse events than the control group was to be expected. However, the fact that vaccine co-administration was similar in reactogenicity to vaccination with MVA-NP+M1 alone was a novel finding which will encourage similar vaccination combinations to be explored in the related areas of TB and malaria vaccinology.

T cell responses to NP and M1 were significantly boosted by vaccine co-administration. In keeping with other MVA studies the post-vaccination peak response occurred 1 week after boost in the majority (89%) of subjects. In the co-administration group, the differences between day 0 responses and day 21 responses, and the differences between day 0 responses and day 182 responses were both significant.

The peak ELISpot assay response in the co-administration group was similar to that observed in the FLU001 extension trial when MVA-NP+M1 was administered alone. This was a reassuring finding as previous trials have shown that the co-administration of two vaccines may result in immune interference, leading to a less pronounced peak ELISpot assay response to the transgene: Ota et al. for example examined responses to MVA85A in infants (Ota et al.,

2011), and found that co-administration of the viral vectored vaccine with EPI vaccines led to a significant reduction in MVA85A immunogenicity (although responses to EPI vaccines were unaffected). The factors that determine immune interference between different vaccine combinations are not well understood. For example when using DNA vaccines targeting the H5N1 influenza virus, Patel et al. found that combining four different antigens (HA, NA, NP and M2) at a single site did not improve protection against homologous influenza virus challenge compared to using HA antigen alone. Combining the four antigens at the same site lead to a reduction in T cell responses and while this could be restored by vaccinating at different sites, this did not improve protection against challenge (Patel et al., 2012).

In the FLU003 study ELISpot assay responses in the co-administration group did suffer a more rapid decline that those previously observed with MVA-NP+M1 alone. However, this decline was not as severe as that observed in the TIV only group. The median fold increase in ELISpot assay responses was 3.7 in the TIV only group (with the fold change being 16.3 in one individual), and it was perhaps surprising to find such easily detectable responses. However, these T cell responses were not durable, and by 3 weeks post-vaccination they were no longer significantly elevated above day 0 ($p=0.495$). In the co-administration group it is possible that the T cells generated were heterogenous, with some short-lived T cells being stimulated by TIV and some longer lived T cells being stimulated by MVA-NP+M1.

The quantity of NP and M1 in the TIV used here is not known, however it is likely that with a split virion vaccine these proteins will be contained in the final preparation (García-Cañas et al., 2007) which has undergone only limited purification.

It is also likely that the phenotype of T cells that are induced following TIV will be different to that induced by MVA-NP+M1. Vaccination with TIV is more likely to induce CD4 T cells, because the protein antigens present in extracellular spaces will have been processed and presented via the MHC class II pathway. MVA-NP+M1 is able to produce antigen intracellularly, and therefore influenza virus peptides may be presented on MHC class I molecules, favouring a CD8 population. From earlier studies with young individuals, CD8 responses appear to dominate over CD4 responses. However, the data presented in Chapter 3 show that CD4 and CD8 responses are equally represented in older adults vaccinated with MVA-NP+M1. Viral vectored vaccines will not exclusively induce CD8 T cells because infected host cells will die (either by apoptosis or necrosis) and influenza virus proteins will be released into extracellular compartment for processing as described above.

The factors that govern T cell contraction following pathogen challenge are not well known (McKinstry et al., 2010), and neither are they well known following vaccination. There may be phenotypic markers which identify the durability of the T cell response and this could be further explored with flow cytometric analysis of frozen PBMC samples from FLU003 and FLU001 extension subjects.

Anti-TIV ELISAs were first used to assess the antibody responses in the FLU003 subjects. In keeping with the HI titre data (discussed below) there were baseline differences in antibody responses between the two groups. This was disappointing given that I had attempted to minimise differences between the two groups by randomisation. However randomisation does not guarantee the even distribution of any given biological parameter, and was more likely to have occurred when the sample size was as small as this. Randomisation was

performed on the day of vaccination, and primarily ensured that the two groups were vaccinated equally through the influenza season. It was not possible to randomise on the basis of baseline serology as samples were typically collected just 30-60 minutes prior to vaccination.

Given the fact that there were baseline differences in anti-TIV ELISA responses between the two groups, I went on to calculate fold change in ELISA units. However, although there was a trend towards increased responses in the co-administration group, these did not reach statistical significance. One disadvantage of the anti-TIV ELISA is that it does not distinguish the relative contributions made by antibody responses to the three different strains of influenza virus. If antibody responses were increased to one or two strains, but unchanged in the remaining strains then the positive effect would have been diluted. Thus, to assess strain-specific antibody responses, sera were assayed using the haemagglutinin inhibition assay.

Using the HAI assay, I was unable to demonstrate a significant increase in HI titres when using either seroprotection or seroconversion as the final outcome. Given the small numbers involved in this trial this is not unexpected. Unlike the ELISA data, HI titre data is ordinal and seroconversion /seroprotection percentages are calculated by dichotomising the data either side of a specific cut-point. This has previously been shown to result in a loss of information and statistical power (Capuano et al., 2007, Scott et al., 1997). Seroprotection has the disadvantage of not being able to take into account baseline differences between the two groups, and neither seroprotection nor seroconversion can account for a greater magnitude of response above the specified threshold of 1:40.

Expressing data as geometric mean titres allowed me to address these problems with the analysis. Firstly, it did not require me to dichotomise the data and secondly, by calculating the ratio (GMR) between pre-vaccination and post-vaccination time points it allowed me to compensate for unanticipated differences in serology between the two groups of interest. However the issue of the source data (from HI titres) being ordinal when antibody concentrations are fundamentally a continuous variable remains. This is just one of the reasons why the influenza virus research community needs better methodologies to determine the serological evidence of successful vaccination or infection.

In the vaccine co-administration group, subjects were shown to have a significant increase in GMR for the H3N2 component of TIV. An increase in GMR was also observed for the H1N1 component, although this was not statistically significant. The GMRs for influenza B viruses were similar between the co-administration group and the control group.

The reasons why an increase in HI titres should be specific for the H3N2 component of TIV are not clear, however it is interesting to speculate on a hypothesis given that the NP+M1 insert contained in MVA-NP+M1 is derived from H3N2: During an in vivo infection with H3N2, a naive B cell specific for H3 may internalise a virion. The associated NP and M1 proteins may then be processed and presented on MHC class II. Thus, B cells specific for antigenically unique influenza virus proteins can receive help from T cells specific for antigenically conserved regions of the influenza virus genome. If any NP or M1 were present in TIV, and if this were bound to H3 molecules, then this might provide a rationale for the H3N2 specific effect because these B cells may be more likely to receive stimulatory help from T cells.

There are however, at least two arguments against this hypothesis. Firstly, while there is some NP/M1 in TIV, there is no evidence that this is bound to H3 in a split virion vaccine (although

this would be more plausible for a whole virion vaccine). Secondly, the NP and M1 regions are well conserved between different subtypes and therefore T cells may equally be able to provide help to B cells specific for H1. Interestingly however there was an increase seen in H1 titres and it was the influenza B virus titres that were not affected by vaccine co-administration.

Overall, these findings have several important implications. Firstly they demonstrate the principal that two influenza vaccines with different mechanisms of action can be safely delivered at the same injection site without leading to a synergy of adverse reactions. In future pandemics it may be necessary to deliver both a seasonal influenza vaccine and a pandemic influenza vaccine to the same population at the same point in time. This situation occurred during the 2009 H1N1 pandemic, when seasonal influenza vaccine manufacture was already underway at the point when the outbreak occurred. In the UK, the NHS began its pandemic vaccine administration programme on 21st October 2009. If MVA-NP+M1 (or another MVA-vectored vaccine) were to be licensed as a pandemic influenza vaccine then it is very relevant to know that it can be safely and effectively delivered alongside that season's trivalent inactivated influenza vaccine.

Secondly, these data provide a rationale for future studies with similar combinations of vaccines. An ideal universal vaccine would induce both humoral and cellular arms of the adaptive immune system, but this may not be possible without utilising vaccines with different mechanisms of action. Similar studies could be performed with either recombinant haemagglutinin or monovalent inactivated H5/H7 influenza vaccines alongside an MVA-vectored vaccine. This would have the benefit of inducing cross-reactive T cells, and may be able to enhance the antibodies produced against protein antigens which are typically poorly immunogenic.

CHAPTER 5: CHARACTERISING THE HOST RESPONSE TO INFLUENZA VIRUS CHALLENGE

5.1 INTRODUCTION

Having proven that a candidate vaccine is immunogenic there are multiple strategies available for testing vaccine efficacy. One option is to perform field studies where subjects receive either the candidate vaccine or a comparator. Subjects then enter a period of follow up where they self-report any symptoms consistent with influenza. Many different pathogens can cause an influenza-like illness (ILI) and therefore laboratory confirmation should be used to define cases. In field studies, subjects can collect and post self-taken swabs to a diagnostic laboratory. Viral culture or PCR methods are preferable to serological methods for defining a case of laboratory confirmed influenza (LCI). For conventional vaccines like TIV, serological methods can introduce bias (Osterholm et al., 2012) because vaccinated individuals will be less likely to demonstrate seroconversion (i.e. a 4-fold increase in HI titre).

However for a vaccine such as MVA-NP+M1, the most appropriate phase II trial to perform was a challenge study, performed in quarantine facilities. One theoretical problem had been the possibility of subjects developing immunopathology. This phenomenon, seen in some animal models, occurs when infection takes place in the context of strong, pre-existing host T cell responses to the pathogen. Performing a clinical trial in quarantine, and with fewer subjects, provides an environment where such potential complications can be rapidly detected.

The challenge study was performed in 2009 as previously described (Lillie et al., 2012). During the trial, samples were taken for subsequent analysis of gene transcription. Samples from the

day prior to challenge and then 12 hours, 24 hours and 48 hour post-challenge were collected into PAXgene tubes and frozen.

There were several questions that I wanted to address in the analysis of this data. Because this was a vaccine efficacy study I was interested to know how the host response to influenza virus challenge was affected by vaccination with MVA-NP+M1. However this question could only be addressed once I had explored what constituted a "normal" host responses to influenza virus challenge. Additionally, one would expect to see variation in the host response to influenza virus challenge, and it was of interest to explore the connection between response phenotype (i.e. clinical outcome) and different patterns of gene expression. Given that there might be expected to be variations in host response, could differences in the pattern of gene expression following influenza virus challenge be used to better categorise the biological outcome of subjects in a challenge experiment?

5.2 FLU002 CHALLENGE OUTCOMES

The clinical outcomes of the FLU002 challenge have been previously published (Lillie et al., 2012). Briefly, 22 subjects (11 vaccinees and 11 controls) entered quarantine facilities and were challenged with A/Wisconsin/67/2005. Two of the 11 vaccinees (18%) and 5 of the 11 control subjects (45%) developed laboratory-confirmed influenza. While these differences were themselves not statistically significant, there was a significant reduction ($p = 0.036$) in the duration of virus shedding in those vaccinees who developed influenza (Lillie et al., 2012).

I have represented the combined clinical and virological outcomes of the FLU002 study in the Figure 5-1. Although the correlation between shedding of virus and seroconversion was poor, it is interesting to note the distribution pattern of subjects in the grid. Subjects clustered along the diagonal from top left to bottom right and no subjects occupied the other corners. Thus, no subjects who had moderate/severe symptoms failed to either seroconvert or shed virus, and there were no subjects who both seroconverted and shed virus that had no symptoms at all.

Figure 5-1; Summary of clinical outcomes of the FLU002 challenge study

		Symptoms			
		None	Mild	Mod/Sev	
Shedding	-	76 108	58 32 96		-
	-	79 70 19 86 41 93	37 109	72	+
	+	80	81 95		-
	+		64	39 87 84 100	+

Seroconversion

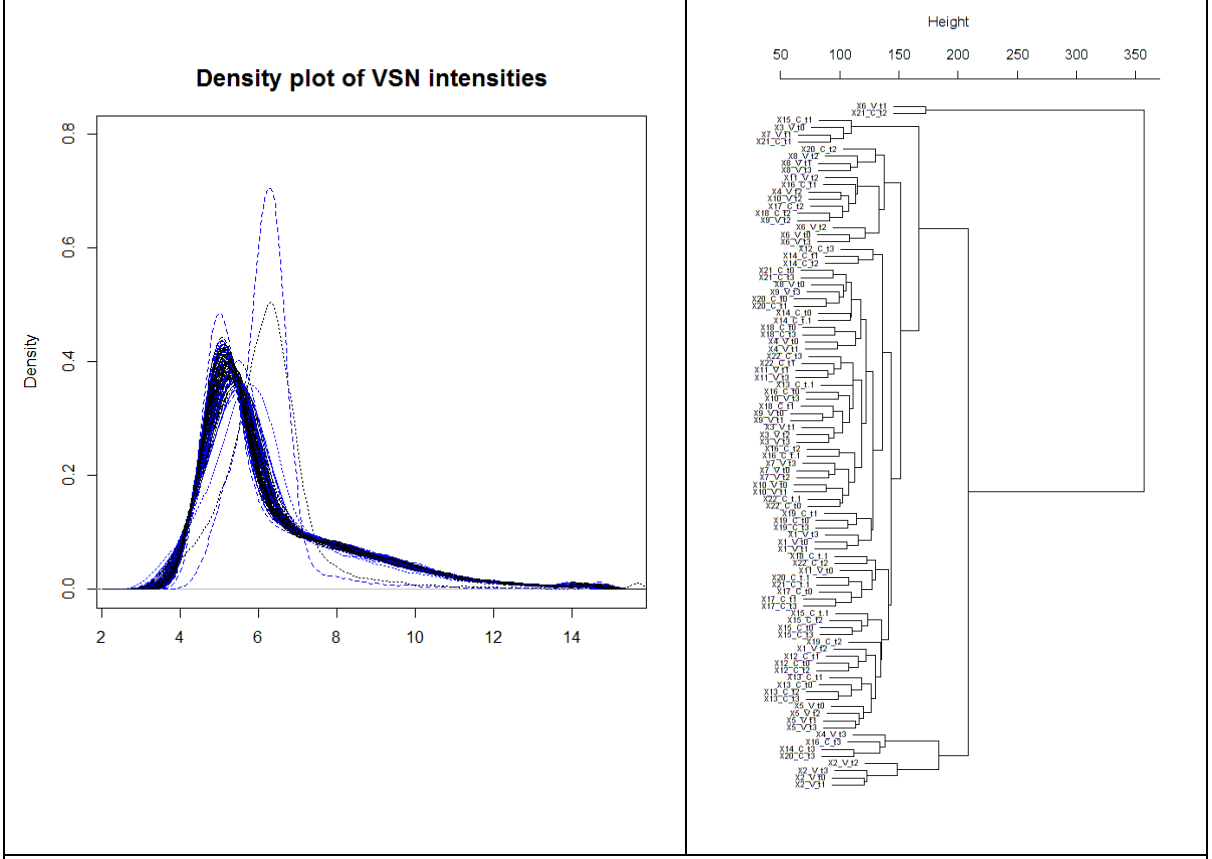
This figure shows the classification of subjects according to their symptoms, whether they had detectable shedding of influenza virus and whether there was post-challenge evidence of seroconversion. The shaded area represents those subjects that were defined (according to the clinical trial protocol) as having laboratory confirmed influenza (LCI). Individuals are referred to by subject number; red font indicates that the subject was a vaccinee and black font indicates a control. The figure serves to highlight the discordance between seroconversion and viral shedding, as there are more volunteers in the middle two rows than there are in the top and bottom rows.

5.3 ANALYSIS OF MICROARRAY DATA

5.3.1 IDENTIFICATION AND REMOVAL OF OUTLYING SAMPLES

The data was filtered and normalised as described in Section 2.5.5 Following this the data was inspected to determine whether any individual samples had clear abnormalities in the distribution of gene expression data. Figure 5-2 shows a density plot and a cluster dendrogram for the 96 samples. From these it can be seen that two samples are outliers.

Figure 5-2; Density plot and cluster dendrogram of normalised gene expression data

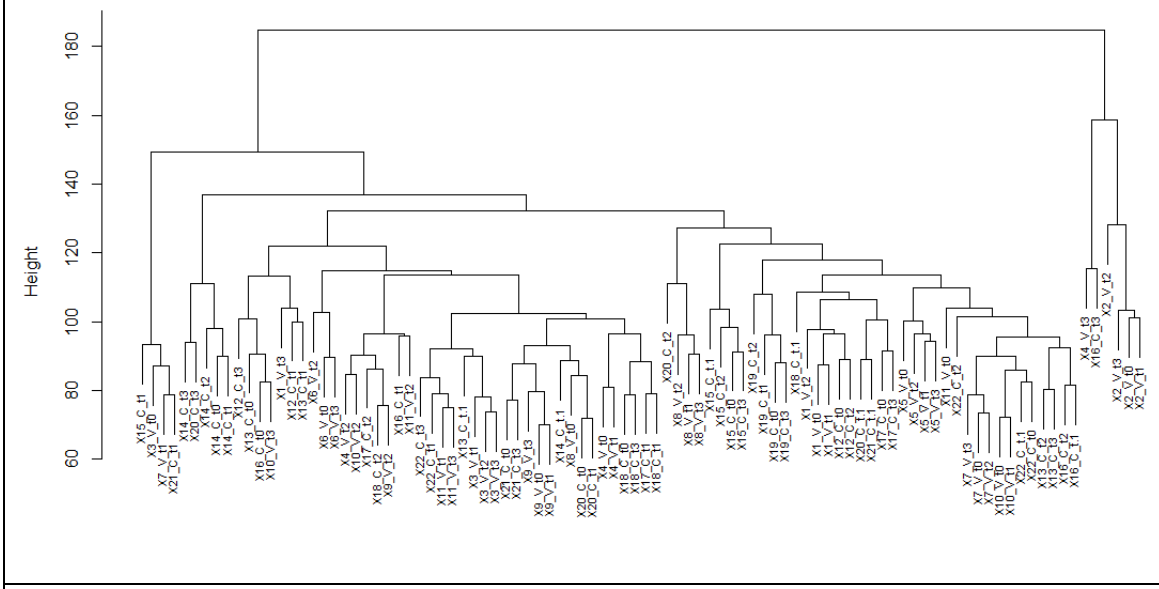


This figure shows two visual representations of signal data generated from gene expression microarrays from samples taken in the FLU002 clinical trial. RNA was extracted from whole blood, which was collected both prior to influenza virus challenge and then 12 hours, 24 hours and 48 hours post-challenge. The density plot (left panel) has signal intensity (arbitrary units) on the x-axis (log2 scale). The right hand panel is a cluster dendrogram generated using unsupervised hierarchical clustering. These figures show how the two outlying samples were identified and excluded.

- One excluded sample came from volunteer 058, a vaccinee who went on to develop mild symptoms but did not shed any virus and so did not fulfil the definition of laboratory confirmed influenza. This sample was from the first post-challenge time point (i.e. 12 hours post challenge).
- The second excluded samples came from volunteer 108, a control subject who had a symptom score of zero and did not shed virus. This sample was from the second post-challenge time point (i.e. 24 hours post challenge).

All subsequent analysis described is performed with these two samples excluded.

Figure 5-3; Cluster dendrogram for 94 filtered and normalised samples



This figure is a representation of gene expression microarray data from the 22 subjects in the FLU002 study, comprising 94 filtered and normalised samples. The dendrogram highlights the clustering of 6 samples on the right hand side of the figure. Four of these samples relate to the same volunteer, and the other two samples are from two volunteers with laboratory confirmed influenza taken 48 hours post-challenge.

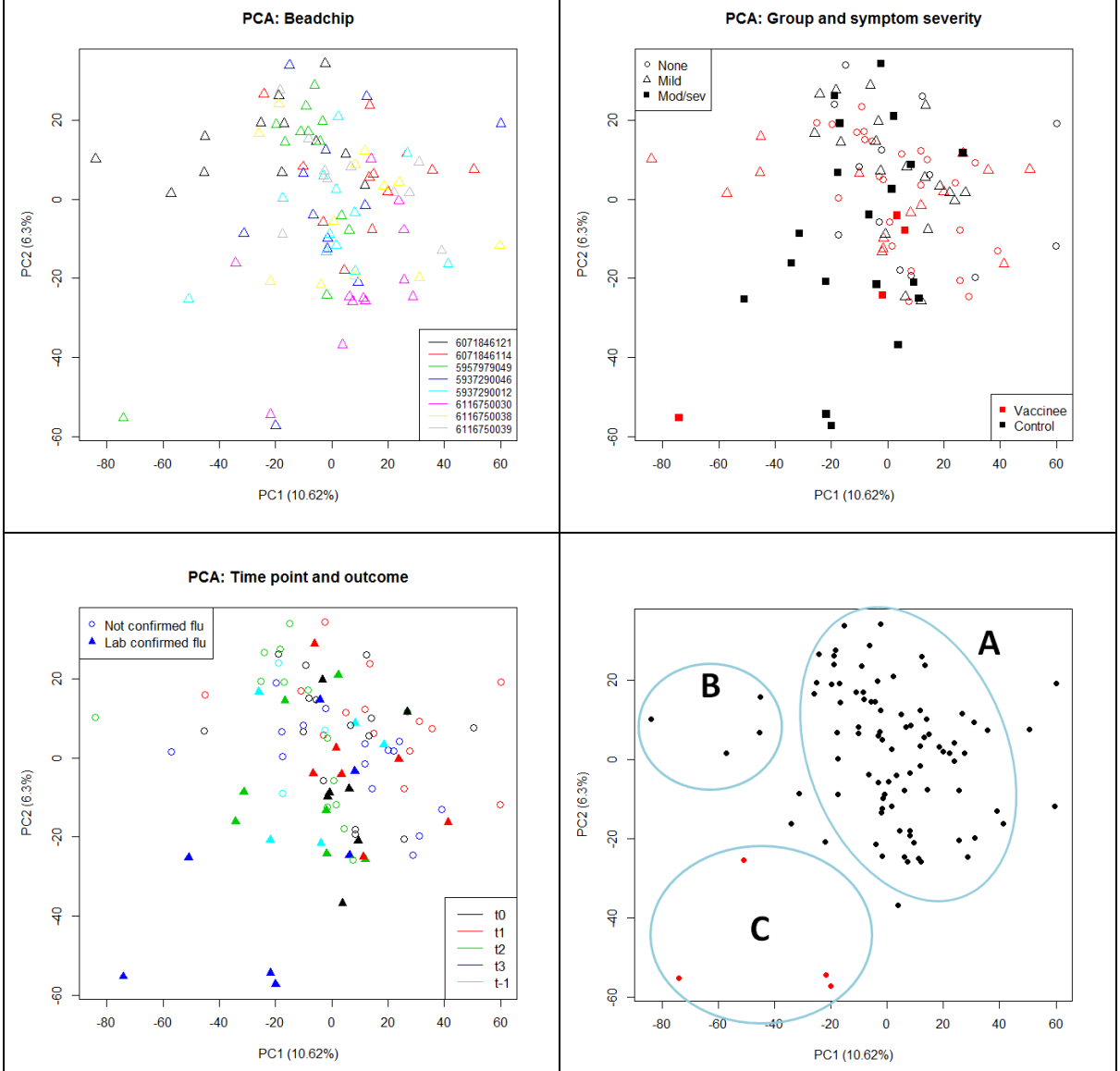
5.3.2 CLUSTERING ANALYSIS

Unsupervised hierarchical clustering was used to try and identify associations within the data set. In Figure 5-3 a dendrogram is shown which reveals 6 samples clustering on the right hand side. Four of these six samples relate to the same volunteer. The remaining two samples are both from subjects with laboratory confirmed influenza which were taken 48 hours post challenge.

5.3.3 PRINCIPAL COMPONENTS ANALYSIS

Principal components analysis is a method of exploring variation in a complex data set. Variation within the data set can be mathematically grouped into a series of components, representing combinations of genes. These components are then ranked according to how much of the total variation in the data set is explained by each individual components. By plotting any two components against one another, it allows an investigator to visualise data that previously existed in a multi-dimensional form. By labelling data points with phenotypic information about the samples in the data set, meaningful associations between the clinical outcomes and gene expression data can be inferred. I applied this analytical technique to the FLU002 data and display the results in Figure 5-4.

Figure 5-4; Principal components analysis of FLU002 microarray data

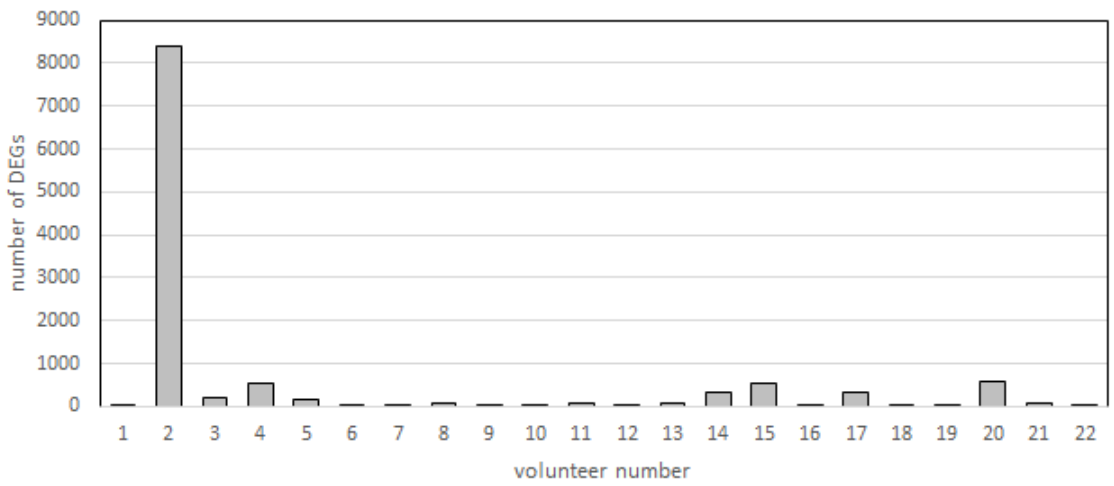


The four panels represent the same principal components analysis labelled with different information about the 94 included samples. The top left shows each data point as a triangle, with different colours indicating the 8 bead chips that were used in the microarray experiment. The top right panel shows control subjects in black and vaccinated subjects in red. The symptoms experienced by each subject is denoted by symbols: Open circles indicate no symptoms, open triangles indicate mild symptoms, and filled squares indicate moderate-severe symptoms. The bottom left panel shows samples from subjects with laboratory confirmed influenza as filled triangles, with open circles indicating no confirmed influenza. Colours represent the different time points at which samples were collected. The bottom right panel represents my interpretation of the previous three plots: A, is main body of data points, where there is no clear pattern according to chip, vaccination status, symptoms, time point or influenza disease. B, represents four samples from the same volunteer (also identified by the cluster dendrogram in Figure 5-3). C, is a cluster of four samples, each from a different volunteer, each taken at 48 hours post-challenge, each with laboratory confirmed influenza and each with moderate-severe rather than mild symptoms. This shared clinical phenotype was also represented by the lowest right hand box in Figure 5-1.

5.3.4 VOLUNTEER 032 HAS A DISTINCT TRANSCRIPTIONAL SIGNATURE

Principal components analysis highlighted that one individual had substantial differences in gene expression compared to the other trial subjects. These differences in gene expression were quantitated using Limma. A simple group analysis was run to compare gene expression from these four samples to the remaining data set. Differential expression was found in 8,388 probes (q value 0.05). It is likely that most individuals will have some differential expression in this type of analysis, therefore the same analysis was repeated for each volunteer and the number of probes identified is shown in Figure 5-5 below.

Figure 5-5; Differentially expressed genes between volunteers



The number of differentially expressed genes (probes) is shown on the vertical axis for each volunteer compared to the remaining subjects ($q < 0.05$). Volunteer 032 is represented by the second column in the bar chart (number 2). There is some natural variation in constitutive gene expression between the volunteers. Thus up to 1,000 probes can be found that are differentially expressed in any one individual compared to the rest of the group. However, volunteer 032 has substantially more genes that are being differentially expressed.

To explore the reasons behind this large number of differentially expressed genes, the functional annotation tool DAVID was used (Section 2.5.5). The gene list was converted into 7,933 Entrez gene IDs which corresponded to 6,205 DAVID IDs. The most significantly

enriched gene ontology terms were RNA processing, translation and splicing as well as mitosis, nuclear division and cell cycle processes. The majority of the most significant differentially expressed genes were under expressed in volunteer 2, suggesting that the cells present in the samples taken were less active. Furthermore, amongst the most significant DEGs were CD160 and CTLA4 – possibly suggesting that the volunteer may have had reduced numbers of certain lymphocytes (e.g. NK cells).

Because this volunteer had an abnormally high degree of differential expression (independent of time point) then this may impair subsequent analyses. The exclusion of these samples from the dataset might therefore allow more subtle differences to be identified and this is stated where it has been performed.

5.3.5 VOLUNTEERS WITH LABORATORY CONFIMED INFLUENZA AND WHO HAVE MODERATE/SEVERE SYMPTOMS DEVELOP A CHARACTERISITIC SIGNATURE OF GENE EXPRESSION

From the principal components analysis it appeared that four clinical samples shared a common profile of gene expression. These four samples also shared a unique clinical phenotype. They were from the four subjects with laboratory confirmed influenza (LCI) who had moderate-severe symptoms, and they were all samples from the final time point. It is perhaps not surprising that earlier samples from the same four volunteers did not share this gene expression profile, as the majority of subjects are asymptomatic in the first 24 hours post-challenge.

To assess the number of differentially expressed genes between the pre-challenge time point and the 48 hours post challenge time point a paired Limma analysis was conducted and revealed 2,651 differentially expressed genes in the four individuals with moderate-severe laboratory confirmed influenza. In comparison there were no differentially expressed genes between baseline and 48 hours post-challenge in either the mild LCI volunteers or non-LCI volunteers. There were also far fewer genes that were differentially expressed between baseline and either of the other two time points in all three groups (Table 5-1).

Table 5-1; Number of DEGs for three groups of volunteers

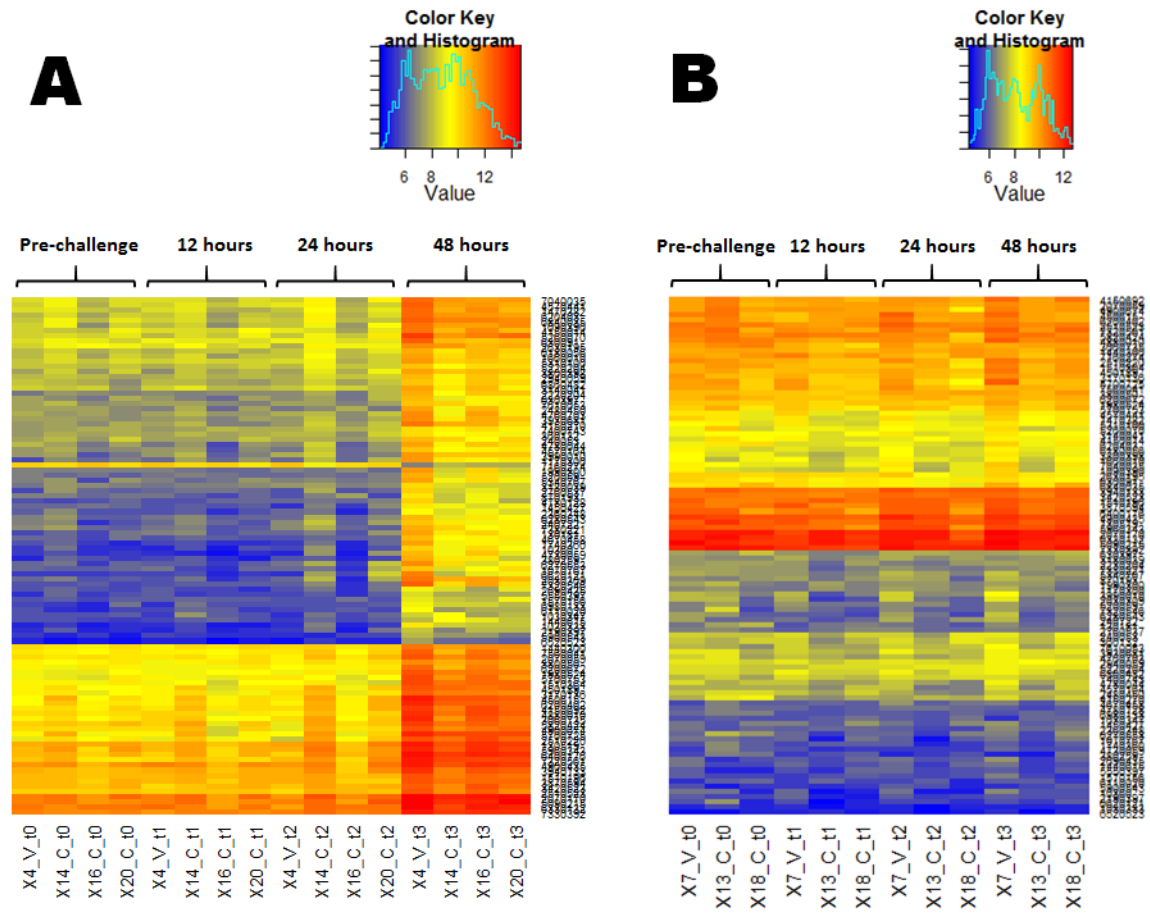
	t0 vs t1	t0 vs t2	t0 vs t3
Moderate-severe LCI (n=4)	0	0	2,651
Mild LCI (n=3)	0	0	0
Non-LCI (n=15)	133	854	0

Abbreviations: LCI = laboratory-confirmed influenza.
t0 = pre-challenge; t1 = 12 hours post-challenge;
t2 = 24 hours post-challenge; t3 = 48 hours post-challenge.

The 2,651 genes were reduced to 100 (top 3.8%) and the expression of these genes was compared between the three groups (Figure 5-6). From the heatmap it is clear that these top 100 genes are not differentially expressed by volunteers with laboratory confirmed influenza and mild symptoms.

To validate the methods used to select these top 100 genes equivalent analysis was performed using two separate software packages (maSigPro and timecourse). The overlap between the top 100 differentially expressed genes was 81% and 74% respectively.

Figure 5-6; Heatmaps showing differences between mild and moderate-severe LCI volunteers



The two heatmaps show the dynamic changes in expression for the same 100 genes (rows). These genes were selected as the most significant genes with differential expression between baseline and 48 hours post-challenge in the moderate-severe LCI group. Expression values are on a log base 2 scale with blue colours indicating low expression and red colours indicating high expression.

In A, there are a total of 16 samples (columns) which relate to four individuals with samples taken at four time points. The samples are arranged in chronological order first and then by volunteer. At the final time point there is a clear change in the colours, representing the enhanced expression of the majority of these genes.

In B, there are a total of 12 samples (columns) which relate to three individuals with mild LCI from the same four time points. As with A, the samples are arranged in chronological order first and then by volunteer. Across the four time points the expression of these same 100 genes does not appreciably change.

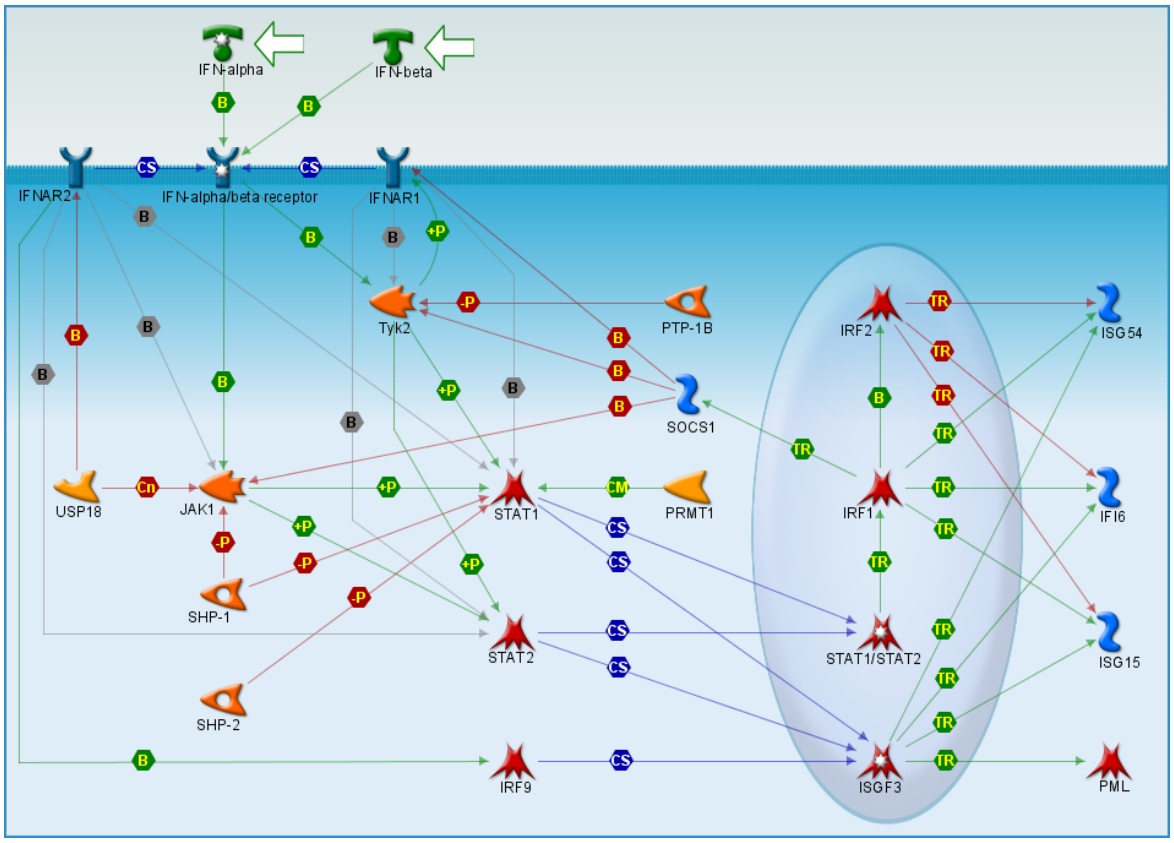
Having established that a large number of genes were differentially expressed in this group of individuals I wanted to establish which biological pathways connected these genes. I used the online bioinformatics resource DAVID to identify gene ontology terms (relating to biological processes) that were over-represented in the genes that were differentially expressed. Nine terms were significantly enriched ($q < 0.05$ FDR method), seven of which related to the immune system (including innate immune responses, host response to viruses and regulation of immune responses). The remaining two terms were related to translation and translational elongation.

A large number of the differentially expressed genes (DEGs) were anti-viral effector proteins that are known to be induced by type I interferons. Some DEGs were involved in the pathways that sensed viral infection and lead to the production of type I interferons, and other DEGs were involved in the signalling processes that link type I interferon receptors to the transcription of interferon response genes (as shown in Figure 5-7 below).

Co-ordination of the type I interferon response to viruses is assisted by a molecular mechanism that is similar to protein ubiquitylation. The protein ISG15 is an ubiquitin homolog and can be added (ISGylation) or removed (deISGylated) from transcription factors, enzymes or anti-viral effector proteins to modulate their function (either enhancing or inhibiting). HERC5 is a ligase which can join ISG15 to protein substrates and USP18 is one of the proteases which can remove ISG15 molecules from protein targets. Activation of ISG15 occurs via ubiquitin-activating enzyme E1-like (UBE1L), and UBE2L6 (also called UBCH8) acts as a carrier for ISG15. The actions of these proteins are depicted in Figure 5-8 (Sadler and Williams, 2008) and all the genes which encode these five proteins (ISG15, HERC5, USP18, UBA7 (which

encodes UBE1L) and UBE2L6) are significantly up-regulated in the FLU002 volunteers with moderate-severe laboratory confirmed influenza. Figure 5-7 also shows USP18 interacting with JAK1 as it transduces interferon receptor binding from the cell surface to the nucleus.

Figure 5-7; Type I interferon signalling pathway (produced by MetaCore™)



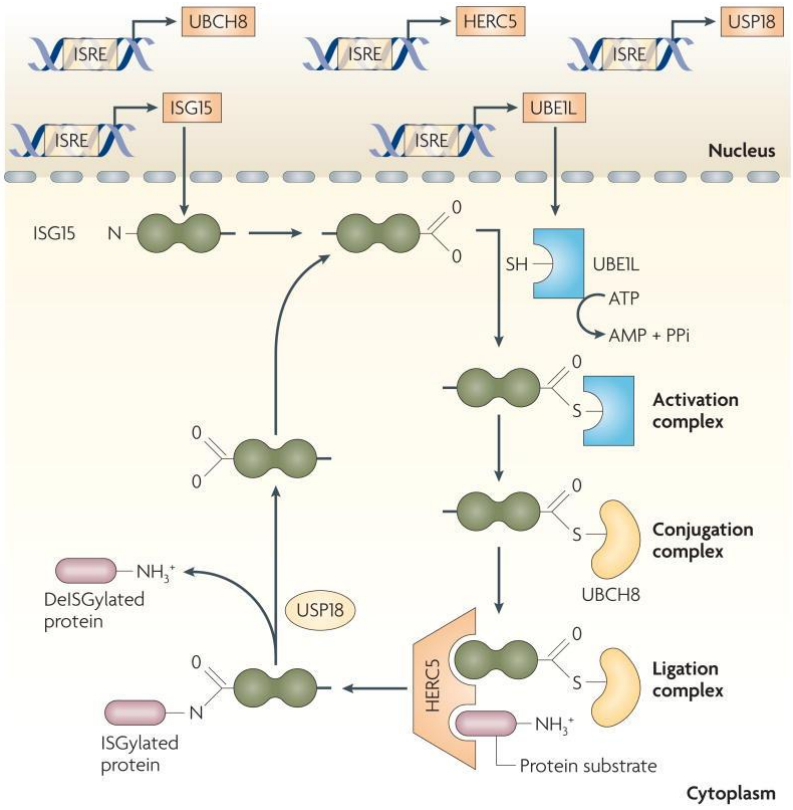
In volunteers with moderate-severe LCI STAT1, STAT2 and IRF9 are all significantly up-regulated. STAT1 and STAT2 are tyrosine phosphorylated by upstream kinases and combine with IRF9 to form ISGF3, which is capable of binding IFN-stimulated response elements (ISREs).
The downstream products of activated STAT1/STAT2 +/- IRF9 include IRF1, IRF2, IFIT2 (also known as ISG54), IFI6, ISG15 and PML. All these gene are also significantly up-regulated in moderate-severe LCI.

Key:
Green arrows and hexagons indicate positive effects or activation
Red arrows and hexagons indicate negative effects or inhibition
"CS" (with blue hexagons and arrows) indicate formation of a complex from multiple subunits
"+P"/"-P" indicates phosphorylation and dephosphorylation respectively
"TR" indicates transcriptional regulation of the linked protein

Image reproduced with permission from http://lsresearch.thomsonreuters.com/maps/429/

One family of genes that was most consistently up-regulated in volunteers with moderate-severe LCI was the OAS family. Twelve different probes each relating to one of the four OAS family members (OAS1, OAS2, OAS3 and OASL) were amongst the top ~1200 probes that were differentially expressed. These genes encode enzymes called 2',5'-oligoadenylate-synthetases which act in concert with RNaseL to decay viral RNA. The relevance of this pathway to influenza virus has been demonstrated by the susceptibility of RNaseL deficient mice to influenza virus challenge.

Figure 5-8; The role of the ubiquitin homolog USG15 in type I interferon responses



The co-ordination of the host response to type I interferon is achieved via the tagging and untagging of the ubiquitin like molecule ISG15 to target proteins. The genes that encode UBCH8, HERC5, USP18, ISG15 and UBE1L were all up-regulated in volunteers with moderate-severe LCI.
Figure reproduced with permission from Sadler and Williams, 2008.

A second important group of anti-viral effectors are encoded by the genes MX1 and MX2. The products MxA and MxB are guanine-hydrolysing proteins with a central interacting domain and leucine zipper domain. MxA can recognise some viral nucleocapsid like structures and viral polymerase enzymes. The binding to polymerases prevents the replication of viral nucleic acids and the sequestration of structural proteins prevents the assembly of viral particles. Both of these genes were also significantly up-regulated in volunteers with moderate-severe LCI. Interestingly there are several other families of guanine-hydrolysing proteins and the guanylate binding proteins GBP1, GBP2, GBP3, GBP4, GBP5 and GBP6 were all found to be significantly up-regulated amongst the same 4 volunteers.

Another key element of host defence is for infected cells to be able to reduce translation and thereby reduce the rate at which new viral particles are generated. The enzyme PKR, produced by the EIF2AK2 gene is a serine-threonine kinase which phosphorylates the initiation factor eIF2 (Janeway et al., 2008). Following phosphorylation of its alpha-subunit, eIF2 binds to and sequesters EIF2β (a guanine nucleotide exchange factor), which prevents the recycling of GDP and stops translation (Sadler and Williams, 2008). The EIF2AK2 gene was significantly up-regulated in the four volunteers with moderate-severe LCI, however interestingly the gene EIF2AK3 was significantly down-regulated. This gene encodes a second enzyme (PERK) which functions in a similar manner to halt translation, however PERK is activated in response to endoplasmic reticulum stress rather than by the presence of viral nucleic acids.

Multiple significant DEGs were found that had roles in the detection of viruses. The two principal examples were DDX58 (also known as RIG-I) and IFIH1 (also known as MDA-5). RIG-

I and MDA-5 are cytoplasmic proteins that contain RNA helicase domains. They function as pattern recognition receptors by binding to the long RNA molecules that characterise viral genomes as opposed to host RNA molecules which are comparatively short (Janeway et al., 2008). Further examples included toll-like receptor 7 (TLR-7) which also senses viral RNA, and UNC93B1 which has an important role in localising TLR-3, TLR-7 and TLR-9 to the appropriate intracellular compartments. The proteins TAP-1 and TAP-2 were both found to be up-regulated in volunteers with moderate-severe LCI. These proteins come together to form a heterodimeric complex which transports peptide fragments generated by the proteasome into the endoplasmic reticulum. By up-regulating these proteins, antigen processing and presentation is increased.

Seven probes were significantly differentially expressed which encoded the four family members of the IFIT proteins (interferon-induced proteins with tetratricopeptide repeats). IFIT1 is a protein which, similarly to RIG-I, can detect unmodified 5'-triphosphorylated RNA (Ablasser and Hornung, 2011). IFIT1, IFIT2, and IFIT3 then combine to form a polyprotein complex, which can sequester viral RNA and thus inhibit replication (Pichlmair et al., 2011).

5.3.6 DIFFERENCES BETWEEN VACCINEES AND CONTROLS

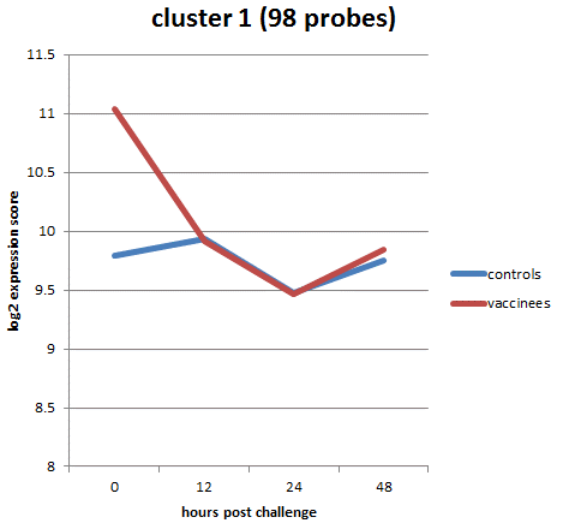
Baseline differences between vaccinees and controls were first analysed using the Limma package, however no differentially expressed genes were identified ($q < 0.05$). This finding was consistent whether or not volunteer 032 was included, and whether only t0 samples were analysed, or whether t0 and t-1 samples were analysed.

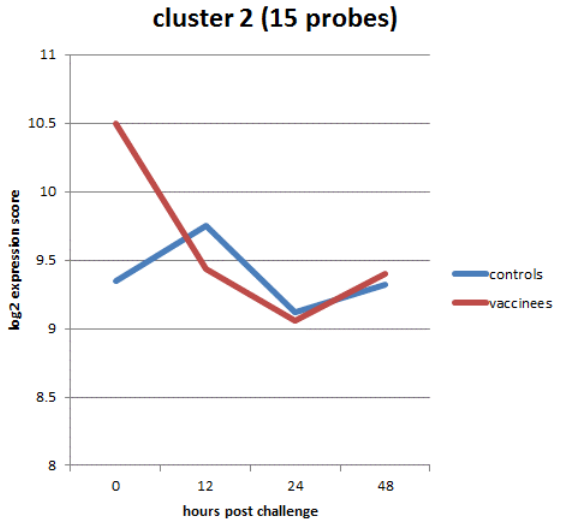
I then applied Limma with a factorial design, which looks at time course differences across each group of interest (vaccinees and controls), and then additionally compared the differences in response between these two groups. Each group had a number of differentially

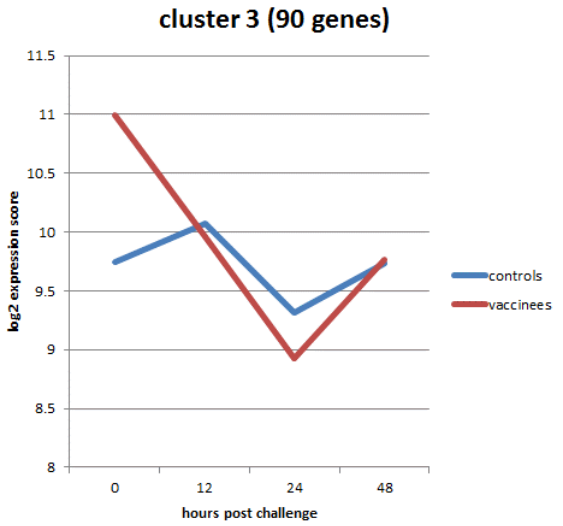
expressed genes across the course of the challenge, however, there were no significant differences between the two groups in how they responded.

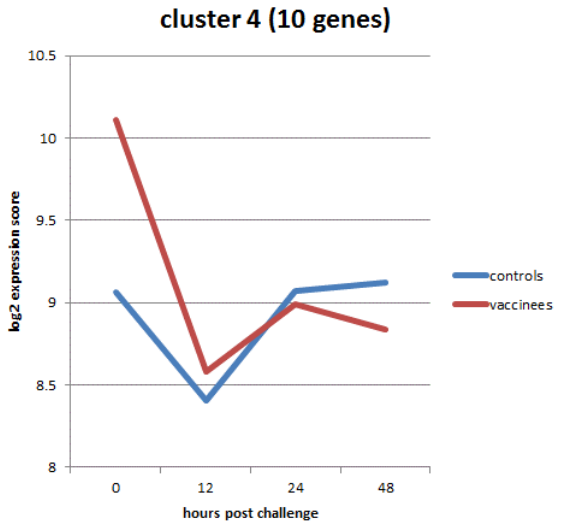
Previously I used the maSigPro package to confirm the large number of differentially expressed genes in volunteers with moderate-severe LCI following challenge. I therefore applied the maSigPro package to identify differentially expressed genes between vaccinees and controls. When all volunteers were included in an initial analysis the number of transcripts with differential expression was 903. However, by excluding t3 samples from volunteers with severe influenza this number decreased to 775. Finally, by excluding volunteer 032 from the analysis the number of transcripts was 579 (q value 0.05). A subset of these genes was selected (218 genes at $q < 0.01$) and found to cluster into 5 groups, as indicated in Figure 5-9.

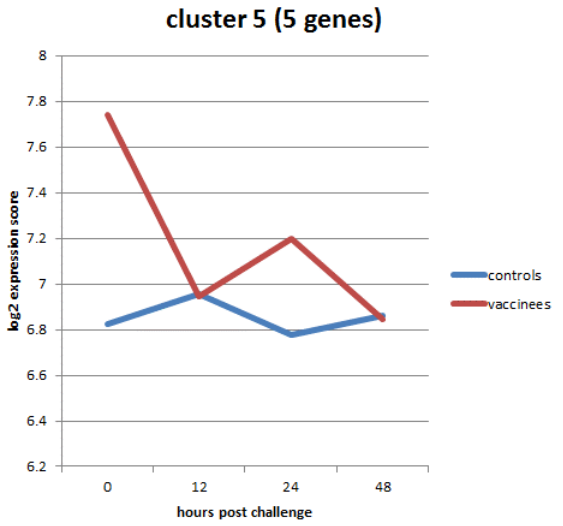
Figure 5-9; maSigPro analysis of DEGs between vaccinees and control subjects











Differentially expressed genes (218, q < 0.05) as determined by maSigPro and grouped into 5 clusters. For all of the five clusters the mean differences in expression were greatest at the pre-challenge time point and then almost identical at the final time point. This suggests that these genes are not differentially expressed in response to influenza virus challenge, but instead that they were differentially expressed prior to challenge.

These genes may have been differentially expressed as a result of vaccination, however this finding is in contrast to the results obtained via the Limma analysis.

5.4 USING GENE EXPRESSION DATA TO CLASSIFY OUTCOMES IN AN INFLUENZA VIRUS CHALLENGE MODEL

While I found that over 1,000 probes were differentially expressed between my 4 cases of moderate/severe LCI and the remaining samples, it is clear that within this list, some genes will show greater differences than others. The genes which show the greatest differences between the two groups can be identified using different methods – for example by their fold change differences or by ranking them according to p value after adjustment for multiple comparisons. These genes can also be identified using specialist software packages, as I will further describe in this section.

The R package "predication analysis for microarrays" (pamr) is available to identify a small number of DEGs, whose variation can then form the basis for the classification of samples. The statistical methodology used has been termed *nearest shrunken centroids* and is described by Tibshirani et al. (Tibshirani et al., 2002). Essentially, a set of training data is analysed by nearest-centroid classification. A centroid is the geometric centre of a two-dimensional region, but the term can be extended to refer to the centre of any object with *n* dimensions. For the training set, the class to which each sample belongs has been determined by the operator, and each class has its own centroid. Then for each of the test samples the gene expression profile is analysed and the centroid for that sample is calculated. The distance (squared distance) between the sample centroid and each of the classification centroids are then computed and compared. The predicted class of the new sample is the one whose centroid is the closest match (Tibshirani et al., 2002). The use of shrunken centroids helps to reduce noise from the data and is able to identify a small subset of genes can that succinctly characterise each class (Tibshirani et al., 2002).

Training was performed using 44 samples from the FLU002 data set. These samples included all 22 samples taken immediately prior to challenge (t0) and all 22 samples taken 48 hours post-challenge (t3). The amount of shrinkage is determined by the operator and is selected based on where the test errors and cross-validated errors are minimised. For the FLU002 data set (the training data) the shrinkage parameter was optimal at 8, where there were only 6 active genes. These genes are described in the table below.

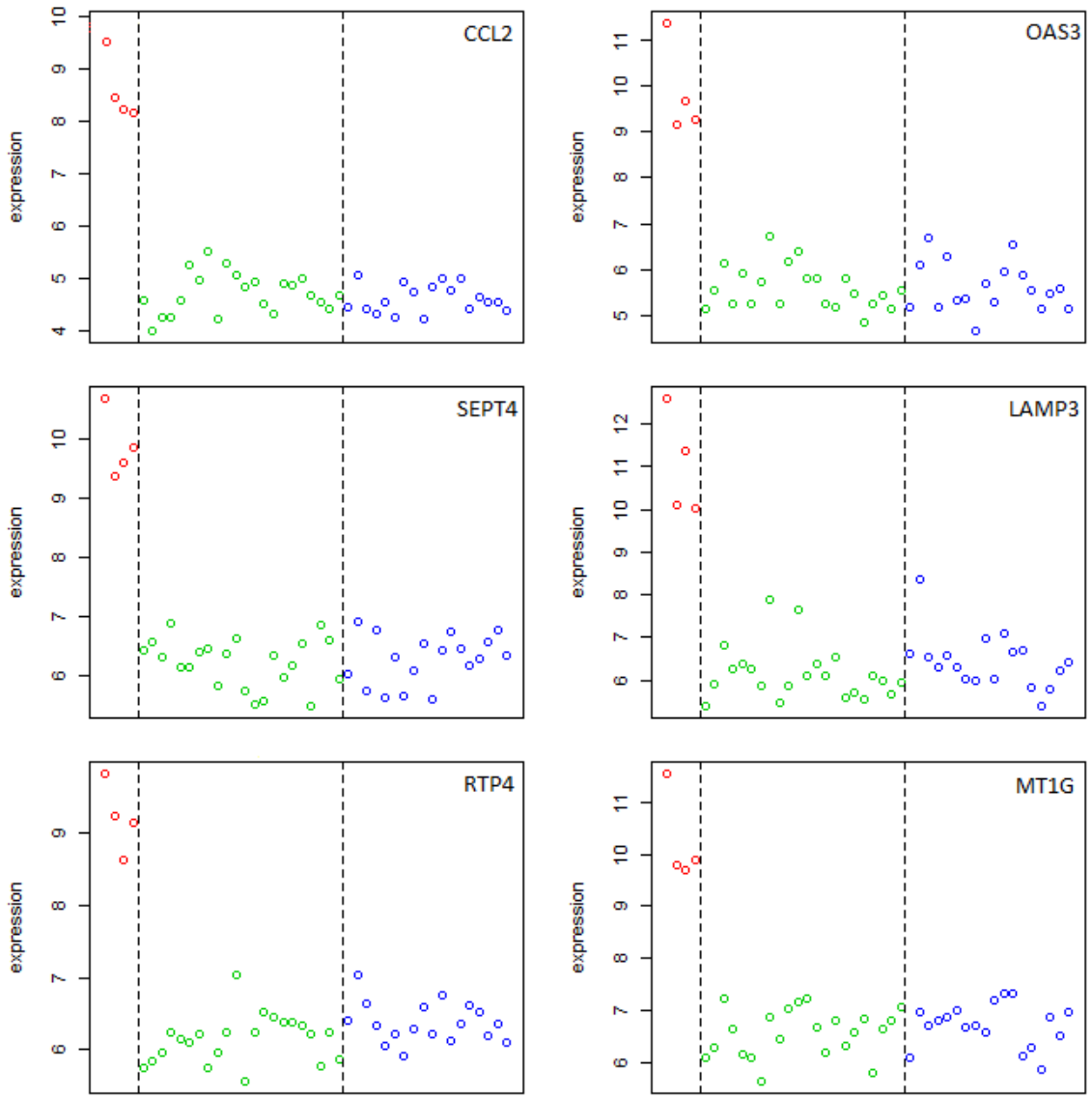
Table 5-2; List of 6 genes that succinctly describe variation in the FLU002 data set

Gene	**Summary of encoded protein**	**Possible role in host response to influenza viruses**
CCL2	Also known as monocyte chemotactic protein. Secreted by activated monocytes, macrophages and DCs.	Recruitment of monocytes to the site of infection. Identified as a prognostic marker in cases of H1N1 influenza. (Zhang et al., 2012)
SEPT4	A highly conserved GTP protein which acts as a molecular scaffold. (Liu, 2012)	Important roles in cell proliferation and apoptosis but no reported role in influenza virus or other viral infections.
LAMP3	Lysosomal-associated membrane protein 3. Expressed by activated DCs. (de Saint-Vis et al., 1998)	Significantly induced in human lung epithelial cells (A549) following influenza virus infection *in vitro*. (Zhou et al., 2011)
RTP4	Receptor transporter protein 4. Known to be secreted by monocytes. (Mehraj et al., 2013)	Known to be induced by type I interferons and have targeted anti-viral actions. (Schoggins et al., 2011)
MT1G	Encodes a metallothionein isoform: A low molecular weight, cysteine rich protein which binds zinc.	Also known to be inducible in monocytes. (Pauwels et al., 1994)
OAS3	2',5'-oligoadenylate-synthetase 3 is one of the four OAS family members, which can be induced and expressed separately, but have similar actions.	OAS acts in concert with RNaseL to decay viral RNA. (Sadler and Williams, 2008) Known to have a protective role against influenza viruses in mice.

Using data from only these 6 genes pamr was able to correctly classify all 4 t3 samples from the moderate/severe LCI group and none of the other samples were misclassified in this group. For the 22 t0 samples and the remaining 18 t3 samples pamr was unable to classify them into two separate classes. However, this finding was entirely consistent with previous results described in earlier sections. The expression values for each of the 6 genes for all 44 samples is shown in Figure 5-10.

To validate these findings I required access to a test set of data. For this I chose to use data from a microarray experiment published by Huang et al. (Huang et al., 2011). The Huang study was an influenza virus challenge similarly conducted by Retroscreen using influenza A/Wisconsin/67/2005 virus. The investigators had similarly collected whole blood into PAXgene tubes and after RNA extraction had performed a globin mRNA depletion procedure. Normalised gene expression data were available to download from GEO (GSE30550).

Figure 5-10; Expression of 6 genes which can classify moderate-severe LCI



The 22 pre-challenge samples are represented by green circles. The 22 samples taken at 48 hours post-challenge are shown as either red or blue circles. The red circles are the 4 volunteers who had laboratory confirmed influenza (LCI) with moderate-severe symptoms, and the blue samples are the remaining 18 volunteers. Log2 expression values are higher for all 6 genes in the moderate-severe LCI volunteers whereas they are indistinguishable for the remaining volunteers.

Seventeen subjects were described and successful inoculation was reported for all subjects. However the definition of successful inoculation was broad, and included subjects whose only evidence of influenza virus infection was an elevation of HI titre but who were neither symptomatic nor shed virus. Huang divided his subjects into “symptomatic infection” and “asymptomatic infection” based on a modified Jackson score collected over the first five days following challenge. Those with a symptom score of 0-5 were called asymptomatic and those with a score of 6 or over were described as symptomatic.

I then used pamr to test a series of samples from the Huang study. If the symptomatic subjects from the Huang study had the same biological phenotype as the subjects with moderate-severe symptomatic laboratory confirmed influenza from the FLU002 study then pamr should be able to predict the class to which each sample from the Huang study should belong. The threshold (degree of shrinkage) remained at 8, so only data from the above 6 genes was used to make the predictions. Pamr successfully identified all (8/8) of the “asymptomatic infection” subjects and successfully identified all but one (8/9) of the “symptomatic infection” subjects (Figure 5-11).

Interestingly the one subject that was not successfully identified was borderline between the two definitions. Subject 15 was classified as symptomatic by Huang et al. based on a symptom score of 8 over the first five days. Not only was this only just above the threshold of 6, but the symptoms were intermittent rather than monophasic, the latter being much more typical of the rest of the symptomatic group.

Figure 5-11; Classification of test set data

		Individual sample predications by pamr						
Huang I.D.	Huang phenotype	Baseline sample	Hour 0	Hour 45	Hour 53	Hour 60	Hour 69	Group predicted
1	Sx	n	n	S	S	n	n	YES
2	Asx	n	n	n	n	n	n	YES
3	Asx	n	n	n	n	n	n	YES
4	Asx	n	n	n	n	n	n	YES
5	Sx	n	n	S	S	S	S	YES
6	Sx	n	n	S	S	S	S	YES
7	Sx	n	n	S	S	S	n	YES
8	Sx	n	n	n	n	S	S	YES
9	Asx	n	n	n	n	n	n	YES
10	Sx	n	n	n	n	n	S	YES
11	Asx	n	n	n	n	n	n	YES
12	Sx	n	n	S	S	S	S	YES
13	Sx	-	n	n	n	S	S	YES
14	Asx	n	n	n	n	n	n	YES
15	Sx	n	n	n	n	n	n	NO
16	Asx	n	n	n	n	n	n	YES
17	Asx	n	n	n	n	n	n	YES

Data from the 17 subjects described by Huang et al. were used to validate the predictive power of the six genes CCL2, SEPT4, LAMP3, RTP4, MT1G and OAS3. The subject number and clinical status of each subject is listed: “Sx” indicates that the subject was symptomatic and “Asx” indicates that the subject was asymptomatic. For each subject samples were tested at six different time points. Two pre-challenge samples were tested to demonstrate that the changes in gene expression were dynamic. Four consecutive post-challenge samples were tested to cover the range over which volunteers became symptomatic. An “S” indicates that the test set sample would be classified with the moderate-severe FLU002 samples, and an “n” indicates that the samples would be classified as either a pre-challenge sample or having none/mild symptoms. With the exception of subject 15 all symptomatic subjects were correctly identified in at least one post-challenge sample.

5.5 DISCUSSION

The primary objective of the FLU002 study was to determine the number of vaccinees and the number of controls that developed laboratory-confirmed influenza (LCI) following challenge. LCI was defined as the detection of viral shedding in a subject with at least mild symptoms of influenza (as described in Section 2.2.2). To perform a robust and objective clinical trial it is important to have a clear case definition for influenza. However, which definition to select is less obvious and different groups have used different definitions as highlighted in Table 5-3 below:

Table 5-3; Published reports using different definitions of influenza following challenge

Definition of infection	Study design	Reference
"Evidence of virus shedding or seroconversion by day 28"	H1N1 challenge and H3N2 challenge	(Wilkinson et al., 2012)
"Symptomatic infection" corroborated by PCR analysis of nasopharyngeal washes. "Asymptomatic infection" corroborated by "multimodal" data including serological analysis	H3N2 challenge Jackson score cut offs: Asymptomatic: 0-5 Symptomatic: 6+	(Huang et al., 2011)
Symptomatic infection defined as a Jackson score >5 and two consecutive days of viral shedding (either by culture or by PCR)	H1N1 challenge and H3N2 challenge	(Woods et al., 2013)
Jackson score >3, with any evidence of shedding into nasopharyngeal washes by viral culture	H3N2 challenge Vaccine efficacy study	(Lillie et al., 2012)
Laboratory-confirmed case defined by ≥4-fold rise in either HI or MN titres between baseline and the day 28 serum specimen, or a positive test by either viral culture or PCR	H3N2 challenge Transmission study	(Killingley et al., 2012)

In the Huang study, all subjects were reported as having had successful inoculation. However, half of the subjects were not symptomatic and did not shed virus. The only evidence of influenza infection was a raise in HI titres at the end of the study, but it is questionable

whether this evidence is sufficient to support influenza infection. The definition of infection is the invasion and multiplication of microorganisms within host tissues. Support for this can only come from either symptoms of infection, or evidence of multiplication in the form of shedding. If challenge had been performed with non-viable virus, then this would still act as a stimulant to the immune system and would theoretically be sufficient to cause a rise in HI titres. This would more closely fit the definition of vaccination; i.e. the inoculation of immunogenic material to stimulate a healthy individual's immune system to develop protective immunity to a pathogen (Janeway et al., 2008) .

One of the difficulties with selecting the best case definition is that there is typically a lack of concordance between different virological outcomes. For example, a subject may have had symptoms of influenza, several days of viral shedding and a strong cell mediated response to influenza virus, but may not have seroconverted. Undoubtedly such a subject will have had influenza, but the reason for the individual absence in serological response is not clear. This may, for example, reflect a laboratory aberration rather than a biological phenomenon. Alternatively a person may have had viral shedding and have seroconverted, but not had significant symptoms. This might be explained by the inter-individual variation in reporting of symptoms, despite the use of standardised tools. Previous groups have also highlighted these grey areas in clinical phenotyping. Woods et al. defined "symptomatic infected individuals" and "asymptomatic non-infected individuals" with ease. However they also recognised the existence of subjects with indeterminate phenotypes, which they termed "asymptomatic viral shedders" and "symptomatic non-viral shedders" (Woods et al., 2013).

I propose that future challenge studies would benefit from a categorisation of subject outcomes which also included information on the expression of specific host genes. This additional information might help to counteract some of the inter-subject variability in the reporting of symptoms, which is particularly significant for individuals with relatively few symptoms who are borderline around the arbitrary thresholds for being symptomatic or asymptomatic.

Infection with influenza virus causes a range of symptoms, but broadly these consist of upper respiratory tract symptoms and the systemic symptoms of malaise, headache, feverishness etc. It may be the case that the gene expression signatures described herein associate more closely with systemic symptoms rather than local symptoms. These questions could be addressed in future studies if the different categories of symptoms were recorded and reported separately.

The use of such host gene responses in defining cases of influenza would also have disadvantages. One such disadvantage is that the described host gene response occurs infrequently during influenza virus challenge. In the FLU002 study it only occurred in 18% of subjects (4/22) and in the Huang study it only occurred in 47% of subjects (8/17). Vaccine efficacy studies require a high rate of infection in control subjects in order to demonstrate protective efficacy. However it is unfortunately not simply a case of increasing the dose of the challenge virus in order to increase the rate of productive infection. Nevertheless if the issue of low attack rates cannot be solved then influenza virus challenge models become less feasible and the progress of experimental vaccines through the stages of clinical development will be impaired.

How then can we improve the conduct of future influenza virus challenge studies and are there any changes that we can make to the screening and recruitment process to help provide meaningful results with small sample sizes? Achieving higher attack rates is important and the reliance upon undetectable HI titres as indicating susceptibility is inadequate. One option would be to extend antibody testing to include results of microneutralisation titres, and only include subjects who are negative on both assays. A second option would be to pre-screen for T cell responses to influenza viruses (for example by ELISpot assay) and exclude subjects with high responses. Unfortunately when evaluating a T cell vaccine this approach has significant disadvantages: By selecting individuals with low responses, optimum responses to vaccination may not be achieved. This may affect the efficacy of the vaccine and may make the results less generalizable.

There may be host genetic factors that determine susceptibility to infection following challenge, and by combining FLU002 data with other challenge data this hypothesis could be explored. It has already been established that single nucleotide polymorphisms in IFITM3 can profoundly affect the course influenza virus infection in humans (Everitt et al., 2012), and therefore it is not a large jump to anticipate that other polymorphisms will associate with susceptibility to challenge. Should these studies be productive then not only would they help improve influenza virus challenge studies but they would provide valuable insights into host-pathogen interactions with influenza viruses.

I have already described the large number of biological pathways that are activated in individuals with moderate-severe laboratory confirmed influenza, and shown that many of these are linked by being effector mechanisms of type I interferons. However I have also shown that the gene expression signature of symptomatic influenza can be reduced to

variation in 6 genes. Further examination of these genes suggest that some of them are likely to have a biologically relevant role in the host response to influenza viruses:

- CCL2 is a chemotactic protein which act on monocytes (Janeway et al., 2008) drawing them to the site of infection. It may be secreted by activated monocytes, dendritic cells or macrophages. It has also been identified as a prognostic indicator in H1N1 influenza (Zhang et al., 2012) indicating that the degree of up-regulation is related to the clinical severity of the case.
- SEPT4 is a highly conserved GTP binding protein. It acts as a molecular scaffold and has important roles in cell proliferation and apoptosis (Liu, 2012), however it has no reported role in either influenza or other infectious diseases.
- LAMP3 is a member of the *LAMP* family of lysosomal membrane glycoproteins. Before acquiring its current name it was referred to in the literature by the names DC-LAMP, TSC403 or CD208 (Kanao et al., 2005). It is homologous to CD68 (a macrophage/monocyte glycoprotein that binds LDL) and is expressed by DCs, becoming sharply up-regulated when DCs are activated by LPS (de Saint-Vis et al., 1998). It is also known to be expressed in lung tissues and overexpressed in several different forms of solid tissue cancer (Kanao et al., 2005). LAMP3 has already been shown to have a role in influenza virus infection in an *in vitro* model. Zhou et al. infected human lung epithelial (A549) cells with influenza A/PR/8 virus, and demonstrated a 35-45 fold increase in expression (Zhou et al., 2011). The authors also suggest that as expression was blunted in IFN-deficient Vero cells, the induction of LAMP3 may be interferon dependent . The finding that *LAMP3* expression is up-

regulated in whole blood samples following influenza virus challenge suggests that LAMP3 could have an important role in the response to influenza virus by DCs.

- Receptor transporter protein 4 (RTP4) is known to be induced by type I interferons and known to be secreted by monocytes (Mehraj et al., 2013). Recent studies have demonstrated it to have dose-dependent effect on viral inhibition in some in vitro models of infection (Schoggins et al., 2011).
- The MT1G gene encodes a metallothionein isoform (1G). This product is a low molecular weight cysteine rich protein which binds zinc. There are very few specific publications on this isoform and therefore it is not known whether it is inducible by interferon. However it is known to be expressed in monocytes (Pauwels et al., 1994).
- The 2',5'-oligoadenylate-synthetase (OAS) enzymes consist of four family members called OAS1, OAS2, OAS3 and OASL. They are reported as being capable of separate induction and expression (Sadler and Williams, 2008) but have similar actions. OAS3 was the 6th gene selected by pamr, but all four family members were amongst the top 100 differentially expressed genes, and their presence in these gene lists was often confirmed by the presence of multiple probes which corresponded to alternative transcripts of the same gene.

It is an interesting observation that these 6 genes do not appear to be immediately connected in the same molecular pathway. This might reflect the fact that in order to describe variation in a group of samples with the fewest number of genes, it is necessary to select genes which are not immediately dependent on one another, but instead represent distinct networks of molecular signals.

I used the online functional annotation tool DAVID to interrogate gene lists and identify gene ontology terms that were significantly enriched. DAVID is a free and highly usable tool, and has the advantages that it can visually display genes according to BioCarta and KEGG pathway maps and that it can convert gene identifiers from one type to another. However DAVID is by no means the only tool available, and the application of different tools to the same set of test genes can produce "variations of exceptional magnitude" (Tieri and Nardini, 2013). It is therefore important to use a tool whose purpose most closely matches the type of analysis that the user wishes to perform. For example, if the specific analysis of innate immune system pathways was desired then the InnateBD platform would have been a good candidate (Breuer et al., 2013). InnateDB has the advantage of a team of curators that manually review the literature and map molecular interactions according to a defined set of criteria.

In looking at the effect of vaccination, I have demonstrated that (in whole blood) there were no significant differentially expressed genes between the vaccine group and the control group prior to challenge. This was not an altogether unexpected finding, as I have previously shown in Chapter 4 and will go on to show in Chapter 7, that the gene expression signature following MVA is no longer detectable one week following vaccination. Similar findings have also been shown with other MVA vaccines, notably the tuberculosis vaccine MVA85A (Matsumiya et al., 2014).

I used two different methods to identify differences in gene expression between vaccinees and controls across time, and these had different outcomes. The Limma package found no differentially expressed genes whereas the maSigPro package was able to identify a small number of transcripts. Examining the expression profile of these probes, it is clear that almost all of them have greater expression at baseline in the vaccinees compared to the controls.

The differences in mean expression levels are much less at the other time points. Arguably therefore these findings are baseline differences between the two groups rather than reflecting differential response to challenge.

In conclusion I have shown that there is a striking signature of gene expression detected in whole blood in subjects who have moderate-severe symptomatic influenza following challenge. It has been surprising to observe that an attenuated form of the same signature was not seen amongst influenza subjects with fewer symptoms. In Chapter 7 I will use the same techniques to examine the host transcriptional response to viral vectored vaccines. It will be of interest to explore whether there is a similarly striking signature of gene expression, and whether it will vary according to the extent of symptoms experienced, the individual immunogenicity achieved or the type of vector used.

CHAPTER 6: SAFETY AND IMMUNOGENICITY OF A NOVEL COMBINATION OF HEPATITIS C VIRUS VACCINES

6.1 INTRODUCTION

In Section 1.2.2 I described the evidence for T cells having an essential role in the prevention of acute HCV from establishing a chronic infection. This provides the scientific rationale for using a T cell generating vaccine for the prevention (and potentially for the treatment) of HCV. There are several vaccine platforms that act to stimulate an immune response dominated by T cells, and these have been described in Section 1.1.4 and Section 1.4.

In the context of HCV, most vaccines candidates have been initially developed as therapeutic rather than prophylactic vaccines. This focus probably reflects the economic incentives to finding an effective biological treatment for chronic hepatitis C. With over 150 million people chronically infected around the world, and with either cost or effectiveness of current therapies limiting the successful treatment of patients, there is a clear market for an effective therapeutic vaccine. On the other hand, prophylactic vaccines would require larger phase III clinical trials to establish their efficacy, and therefore they would be more expensive to develop. Furthermore their adoption into routine vaccination regimens would be dependent on the incidence of hepatitis C in local populations, and therefore the scope for rolling out a prophylactic vaccine for HCV would not be guaranteed, even if it were highly effective. Few vaccines candidates for HCV have been taken into clinical trials, but those where data has been published are described below:

The peptide vaccine IC41 has been administered to 54 healthy volunteers in a Phase I clinical trial in Austria (Firbas et al., 2010). Unfortunately peptide vaccines are not intrinsically

immunogenic, and therefore at least 8 separate doses of vaccine needed to be administered. Furthermore the vaccine consisted of only five synthetic peptides containing eight T cell epitopes. Other vaccine methods would have the potential to activate T cells against a much broader range of epitopes.

One mechanism for increasing immunogenicity is to present peptides in the form of a virosome. A clinical trial using synthetic HCV peptides presented in a virosome has been performed in 30 healthy adults in Switzerland (ClinicalTrials.gov Identifier: NCT00445419). Although this trial was declared complete in 2008 data has not yet been published.

A DNA vaccine has been developed for HCV called ChronVac-C. Although this has been tested in a small numbers (n=12) of HCV-infected patients (Weiland et al., 2013), no data has been published regarding immunogenicity in healthy volunteers.

T cell responses to HCV have also been induced by vaccines intended to primarily intended to induce neutralising antibodies to HCV surface glycoproteins (Frey et al., 2010).

Viral vectored vaccines are particularly effective at inducing high numbers of circulating T cells, and adenoviruses have been particularly successful in this. The advantages of using adenoviruses as vectors are described in Section 1.4.2. However, adenoviruses are a natural human pathogen, and a substantial proportion of the population will have anti-vector immunity. This anti-vector immunity severely mitigates the response than can be generated to the antigenic insert. It would therefore be an advantage to use adenoviruses which have low prevalence of anti-vector immunity in the population. Adenoviruses also circulate in non-

human primates, and the adenoviruses that circulate in chimpanzees are phylogenetically indistinguishable from those that infect humans.

Previous research has therefore established a chimpanzee adenoviral (ChAd) vectored vaccine which has been recombineered to express a genetically conserved portion of the HCV genome (Colloca et al., 2012). This vaccine (ChAd3-NSmut) was excellent at priming T cells in healthy young adults in a clinical trial (HCV001) in Oxford (Barnes et al., 2012). Regimens that utilised either a single priming vaccination or a double priming vaccination were compared, and a double prime regimen was found to offer little additional benefit. Despite achieving an excellent post-prime peak response, there was a rapid decline in antigen-specific T cells over 2-6 weeks post-peak. A boosting vaccination is needed if these responses are to be maintained and therefore to be of potential clinical utility.

Boosting with the same ChAd3-NSmut vaccine would be unlikely to be successful due to the establishment of anti-vector immunity as I have described above. However, an alternative (heterologous) adenoviral vector might be capable of boosting these T cell responses and have sufficiently divergent sequence to prevent cross-reactivity of anti-vector immune responses. Data from the HCV001 clinical trial demonstrated that while these T cell responses can be boosted by a heterologous adenoviral vectored vaccine, the post-boost responses do not reach a greater peak than the post-prime level.

In other areas of vaccinology a successful approach has been to utilise a heterologous prime-boost strategy, comprising of an adenovirus prime and an MVA-vectored vaccine to boost (Reyes-Sandoval et al., 2010, Hill et al., 2010). During 2010 a recombinant MVA encoding the same NSmut insert was manufactured to GMP by IDT Biologika, Germany. The HCV003 clinical trial was approved as a first in man study of MVA-NSmut, and its use in a prime-boost regimen with ChAd3-NSmut.

6.2 PRIME-BOOST VACCINATION WITH ChAd3-NSmut AND MVA-NSmut IS SAFE IN HEALTHY YOUNG ADULTS

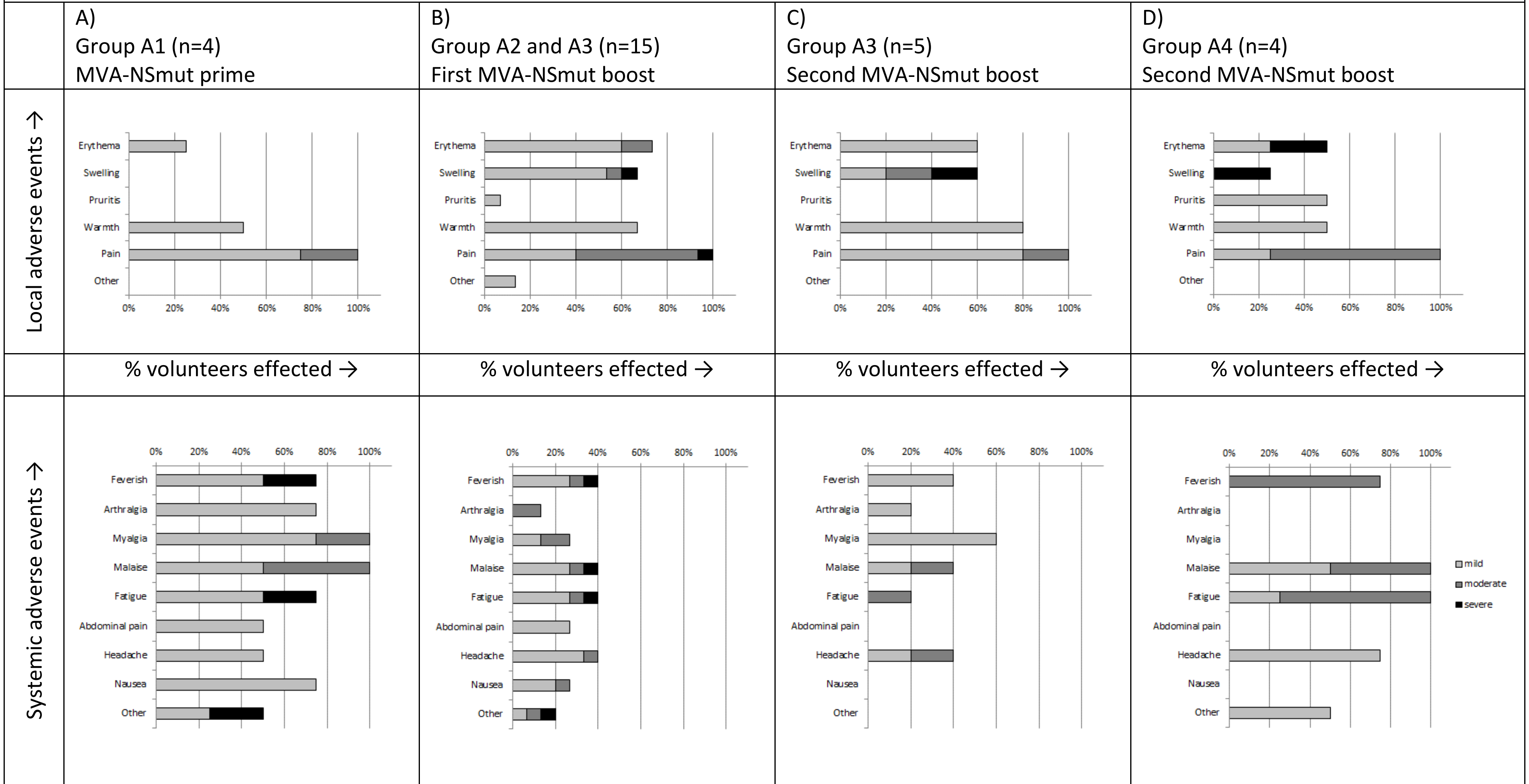
Volunteers were enrolled sequentially into different groups as outlined in Table 6-1 (below). Four volunteers were initially vaccinated with MVA-NSmut at a dose of 2 x10^8 pfu (group A1). Only after vaccine safety had been established in this group were subsequent groups (A2, A3 and A4) enrolled. The same dose of MVA-NSmut was administered to all volunteers across the four groups, and ChAd3-NSmut was given at a dose of 2.5 x10^{10} vp in groups A2, A3 and A4.

	Table 6-1; Vaccine regimens for the different groups in the HCV003 study				
Group	Number of volunteers	First vaccine (when given)	Second vaccine (when given)	Third vaccine (when given)	Fourth vaccine (when given)
A1	4	MVA-NSmut (week 0)	N/A	N/A	N/A
A2	10	ChAd3-NSmut (week 0)	MVA-NSmut (week 8)	N/A	N/A
A3	5	ChAd3-NSmut (week 0)	MVA-NSmut (week 8)	ChAd3-NSmut (week 16)	MVA-NSmut (week 24)
A4*	4	ChAd3-NSmut (week 0)	MVA-NSmut (week 8)	ChAd3-NSmut (week >21)	MVA-NSmut (week >21+8)
Doses: All doses of MVA-NSmut were 2 x 10^8 pfu. All doses of ChAd3-NSmut were 2.5 x 10^{10} vp. * Volunteers in group A4 were recruited from those individuals who had previously completed the vaccine schedule in group A2. The interval between the third vaccine and the first vaccine was a minimum of 3 months (but with no maximum limit). However the interval between the third vaccine and the fourth vaccine was fixed at 8 weeks.					

Volunteers were first reviewed 1-5 days post-vaccination and adverse events were recorded. Solicited adverse events were documented using a standard case report form. Additional (non-solicited) adverse events reported by volunteers were also recorded, both at interview and on a diary card provided to all volunteers. Severity was subsequently ascribed according to the clinical trial protocol. The diary cards allowed volunteers to document their symptoms, (including temperature and the size of any swelling or erythema at the vaccine site) on a daily basis to maintain accuracy. At either 1 week or 2 weeks post-vaccination, blood samples were sent for determination of full blood count and routine biochemical profiling.

For each adverse event, causality was attributed according to the trial protocol. Adverse events with possible, probable or definite causality are described here as being related to vaccination. The frequency of related adverse events (i.e. adverse reactions) is shown in Figures 6-1 and 6-2 below.

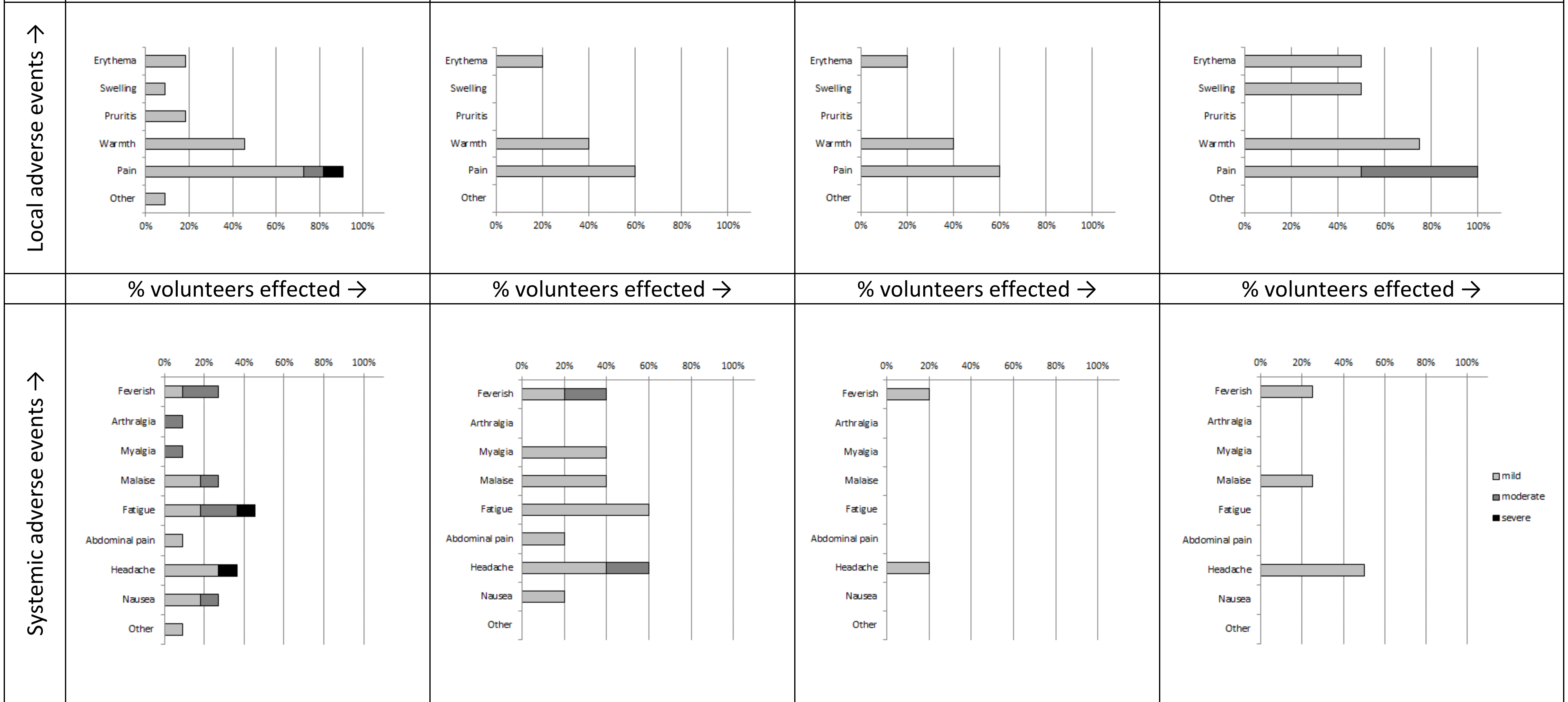
Figure 6-1; Safety profiles for MVA-NSmut in the HCV003 trial



The profile of adverse events is shown for MVA-NSmut. Adverse events with possible, probable or definite causation (i.e. adverse reactions) are shown. Local adverse events are shown in the top panels and systemic adverse events are shown in the bottom panels. From left to right, the top and bottom paired figures relate to: A) MVA-NSmut administered as a priming vaccine in Group A1 (n=4); B) MVA-NSmut administered as a boosting vaccine for the first time in Groups A2 and A3; C) MVA-NSmut administered as a boosting vaccine for the second time in Group A3; and D) MVA-NSmut administered as a boosting vaccine for the second time in Group A4

Figure 6-2; Safety profiles for ChAd3-NSmut in the HCV003 trial

A) Group A2 (n=11) ChAd3-NSmut prime	B) Group A3 (n=5) ChAd3-NSmut prime	C) Group A3 (n=5) Second ChAd3-NSmut	D) Group A4 (n=4) Second ChAd3-NSmut

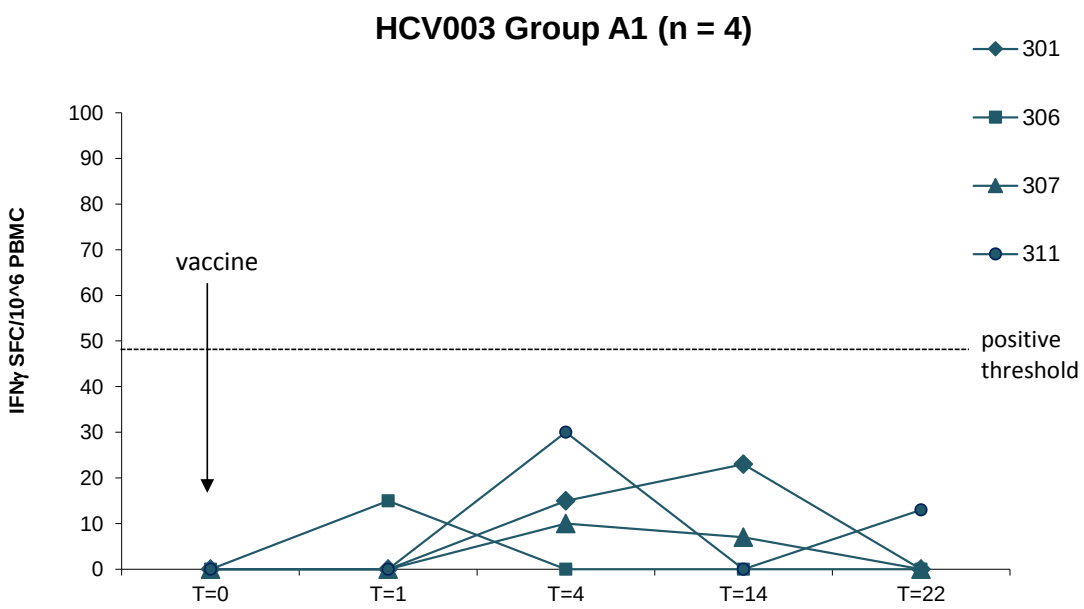


The profile of adverse events is shown for ChAd3-NSmut. Adverse events with possible, probable or definite causation (i.e. adverse reactions) are shown. Local adverse events are shown in the top panels and systemic adverse events are shown in the bottom panels. From left to right, the top and bottom paired figures relate to: A) ChAd3-NSmut administered as a priming vaccine in Group A2 (n=11); B) ChAd3-NSmut administered as a priming in Groups A3; C) ChAd3-NSmut administered for a second time in Group A3, 16 weeks after the initial prime; and D) ChAd3-NSmut administered for a second time in Group A4

6.3 MVA-NSmut ALONE DOES NOT EFFECTIVELY PRIME NAIVE T CELLS

Four subjects were vaccinated with MVA-NSmut as a priming vaccine (Figure 6-3). At 1 week or 4 weeks post-vaccination, summed IFN-γ ELISpot assay responses (minus background) were very weakly detectable (10 – 30 SFU/10^6 PBMC) for all four volunteers. However using the cut off rules outlined in Section 2.3.2.4, these responses would not be deemed positive as they do not pass the threshold of 3 standard deviations above the mean response from seronegative and unvaccinated volunteers.

Figure 6-3; IFN-γ ELISpot responses in Group A1 volunteers (MVA-NSmut prime)



T cell responses to vaccine immunogen as determined by IFN-γ ELISpot assay are shown on the γ-axis, with time post-vaccination in weeks on the x-axis. Line with different symbols represent the four volunteers in Group A1. The dashed line represents the threshold at which a result would be deemed positive. Administration of the vaccine MVA-NSmut is arrowed.
ELISpots were performed by Anthony Brown, Rachel Townsend and Leo Swadling.

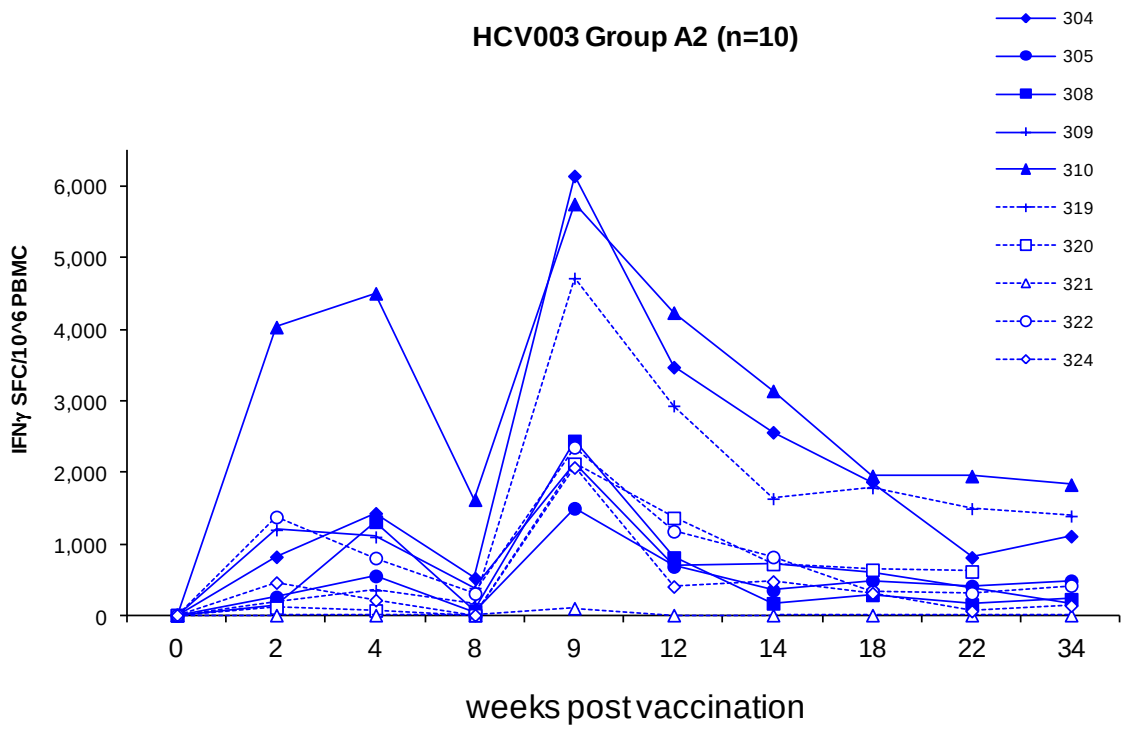
6.4 MVA-NSmut EFFECTIVELY BOOSTS T CELL RESPONSES PRIMED BY ChAd3-NSmut

6.4.1 Magnitude of IFN-γ ELISpot responses to NS peptides

Ten volunteers completed the prime-boost vaccination schedule in group A2. One additional volunteer received the priming vaccine but then withdrew from the study prior to receiving the boost vaccination. Of the ten volunteers that received both vaccines, 1 (10%) did not respond to priming vaccination (volunteer 321). However this volunteer had also received a slightly lower dose of vaccine (1.9 $x10^{10}$ vp) as the required volume could not be extracted from the vaccine vial. This volunteer remained in the study and subsequently received boosting vaccination (see below). Of the remaining 9 volunteers, 4 had a peak IFN-γ ELISpot assay response at 2 weeks post-vaccination and 5 had a peak IFN-γ ELISpot assay response at 4 weeks post-vaccination (Figure 6-4). Of the ten volunteers that received both vaccines the median peak IFN-γ ELISpot assay response post ChAd3-NSmut was 880 SFU/million PBMC.

All ten subjects saw a boost in their IFN-γ ELISpot assay responses following MVA-NSmut and in all cases the boost was maximal at study week 9 (i.e. 1 week post-boost vaccination). The subject who did not respond to ChAd3-NSmut, did respond to MVA-NSmut boost (albeit weakly at 107 SFU/million PBMC) suggesting that there may have been some effect from priming vaccination in this individual that was not detected by IFN-γ ELISpot assay. The median peak post-boost IFN-γ ELISpot assay response was significantly higher than that seen following ChAd3-NSmut (2,240 vs 880 SFU/million PBMC 2; p= 0.002, Wilcoxon signed rank test).

Figure 6-4; IFN-γ ELISpot responses in Group A2 volunteers



Each line with a different symbol represents one of the ten volunteers in Group A2 that received both vaccines of the prime-boost regimen. The responses to vaccination are shown over time, with the priming vaccination (ChAd3-NSmut) being given at week 0 and the boosting vaccination (MVA-NSmut) being given at week 8. The y-axis shows T cell responses to the vaccine immunogen as determined by IFN-γ ELISpot assay. These responses comprise the summed (background subtracted) responses from the six peptide pools, and are shown per million PBMC. Time in weeks is shown on the y-axis (not to scale), with each entry representing a study visit.
ELISpots were performed by Anthony Brown, Rachel Townsend and Leo Swadling.

6.4.2 Duration of ELISpot responses to NS peptides

An effective prophylactic vaccine would need to induce a durable immune response. Therefore I went on to analyse the maintenance of response to NS peptides over time. With

the exception of the volunteer who failed to respond to ChAd3-NSmut vaccination, all volunteers had detectable ELISpot assay responses to NS peptide 6 months post-boost.

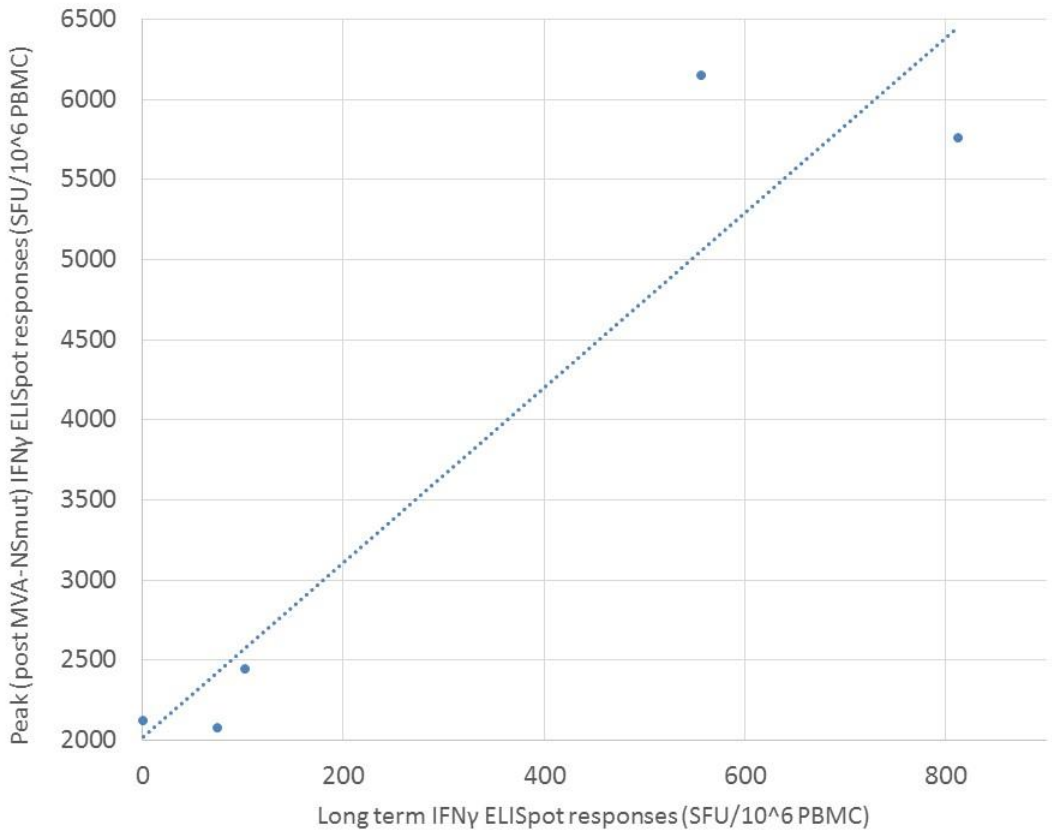
To examine the durability of ELISpot assay responses further, the HCV003 trial was amended to allow for additional late blood draws to be collected from volunteers in Group A2. This resulted in some (but not all) volunteers returning for further blood tests as described in the Table 6-2 (below).

Table 6-2; Persistence of T cell responses to NS peptides in HCV003 Group 2		
SUBJECT NUMBER	FINAL IFN-γ ELISPOT ASSAY RESULT (weeks post enrolment)	REASON IF FINAL IFN-γ ELISPOT ASSAY RESULT NOT AVAILABLE
304	557 SFU/million PBMC (92 weeks)	
305	n/a	Unable to visit Oxford
308	103 SFU/million PBMC (91 weeks)	
309	Undetectable (72 weeks)	
310	813 SFU/million PBMC (70 weeks)	
319	n/a	Entered Group A4
320	n/a	Unable to visit Oxford
321	n/a	Undetectable response at week 34
322	n/a	Entered Group A4
324	75 SFU/million PBMC (72 weeks)	
The persistence of vaccine responses in Group A2 from the HCV003 trial is shown above. Each volunteers was invited to donate one further blood sample and the outcomes are described. Where volunteers returned and provided a further blood sample, the IFN-γ ELISpot assay result is shown. One volunteer was not invited to participate (321) as IFN-γ ELISpot responses were already undetectable at previous study visits. Two volunteers were not able to participate as having completed group A2, they immediately entered group A4 (as described in Table 6-1)		

Of the five volunteers who were able to donate additional samples, responses were still detectable in 80% after a mean duration of follow up of 18 months. For the five subjects

where long term data was available it was evident that the two volunteers with the highest long term responses (both greater than 500 SFU/million PBMC) were also the two volunteers with the highest peak responses to vaccination (both greater than 5,000 SFU/million PBMC). A correlation between peak responses and the magnitude of long term responses is shown in Figure 6-5.

Figure 6-5; Correlation between peak responses and magnitude of long term responses



This figure shows vaccine responses as measured by IFN-γ ELISpot assay. Data is shown for five volunteers from the HCV003 study. These five volunteers are those described in Table 6-2, and are the only volunteers from which both data points are available. The correlation between peak responses to NS peptides and magnitude of the long term responses to NS peptide is shown. All peak responses occurred one week following boost vaccination with MVA-NSmut, and the long term responses were measured 70-92 weeks following priming vaccination with ChAd3-NSmut. This correlation is significant ($r^2 = 0.8919$, $p = 0.0156$).

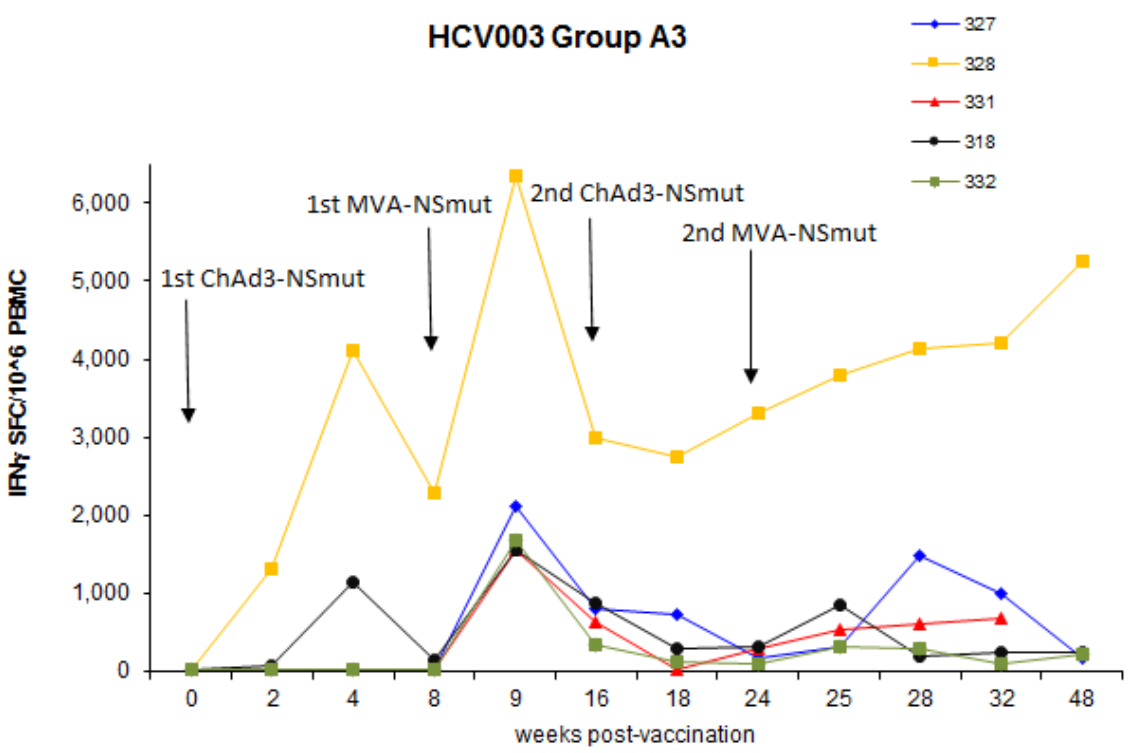
6.5 THE IMMUNOLOGICAL CONSEQUENCES OF REPEATING VACCINE ADMINISTRATION

6.5.1 RESPONSES TO A SECOND DOSE OF ChAd3-NSmut ARE DEPENDENT ON TIMING

Group A3 and Group A4 both received two doses of ChAd3-NSmut and two doses of MVA-NSmut. Group A3 (n=5) received their second dose of ChAd3-NSmut 16 weeks from the first dose and only 8 weeks after being boosted with MVA-NSmut, whereas Group A4 (n=4) received their second dose of ChAd3-NSmut at least 34 weeks from the first dose (see Table 6-1). For Group A3, all volunteers saw a fall in the magnitude of IFN-γ ELISpot assay responses between the day of their second ChAd3-NSmut vaccination and two weeks after this vaccination (Figure 6-6).

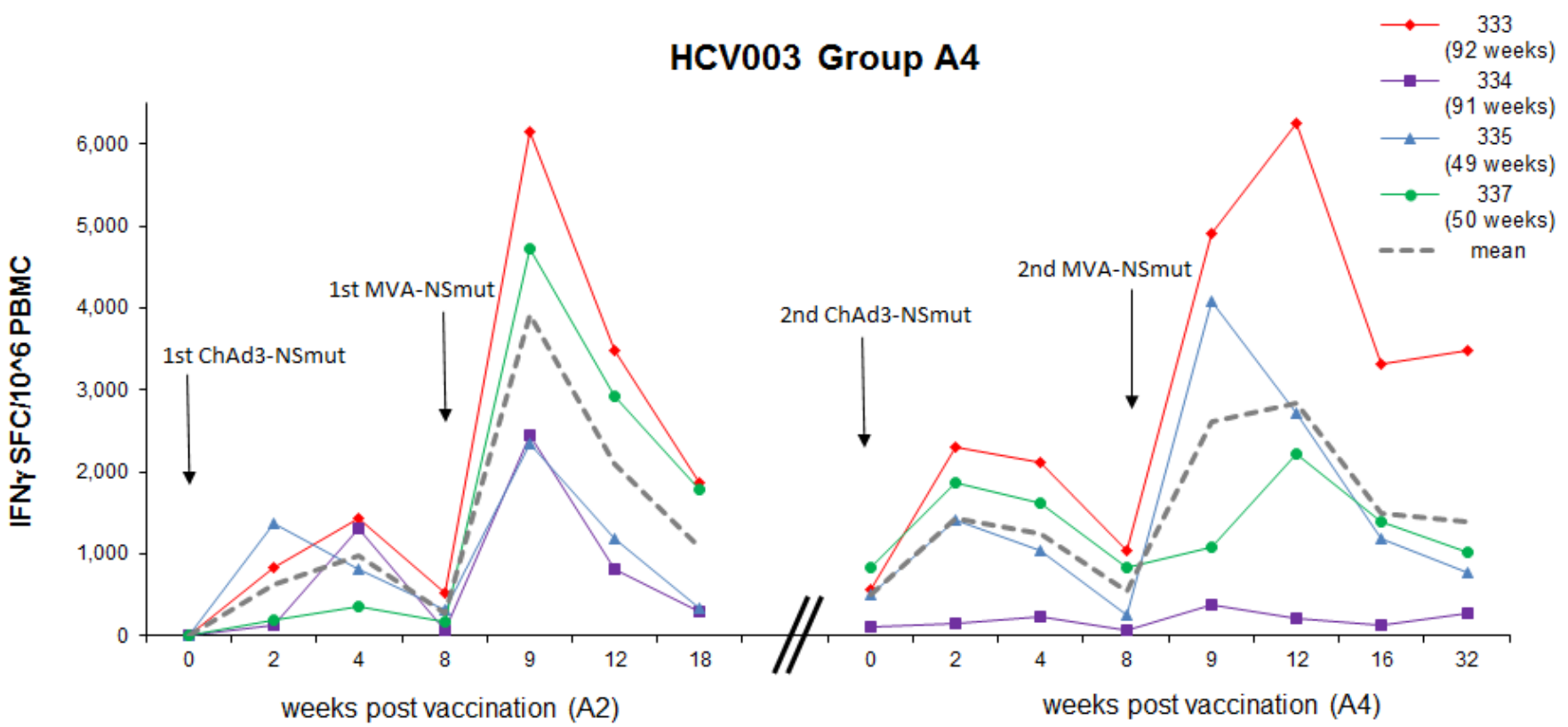
For Group A4 the opposite was true, with all volunteers seeing an increase in the magnitude of ELISpot assay responses between the day of vaccination and two weeks post-vaccination (Figure 6-7).

Figure 6-6; IFN-γ ELISpot responses in Group A3 volunteers



Each individually coloured line represents one of the five volunteers in Group A3. The responses to vaccination are shown over time, with each vaccination being highlighted by a labelled arrow. The y-axis shows T cell responses to the vaccine immunogen as determined by IFN-γ ELISpot assay. These responses comprise the summed (background subtracted) responses from the six peptide pools, and are shown per million PBMC. Time is shown on the y-axis (not to scale), with each entry representing a study visit.
IFN-γ ELISpots were performed by Anthony Brown, Rachel Townsend and Leo Swadling.

Figure 6-7; IFN-γ ELISpot responses in Group A4 volunteers



T cell responses to vaccine immunogen as determined by IFN-γ ELISpot assay are shown on the y-axis, with time in weeks on the x-axis. IFN-γ ELISpot assay responses comprise the summed (background subtracted) responses from the six peptide pools, and are shown per million PBMC. All A4 volunteers were previously enrolled in Group A2 and for comparison additional time points from group A2 visits are shown to the left of the split in the x-axis. Lines with different colours and symbols represent the four different volunteers in Group A4. In the key the number of weeks shown in parentheses under each volunteer number is the time interval between enrolment in Group A2 and enrolment in Group A4 (i.e. the time interval between the first vaccination with ChAd3-NSmut and the second vaccination with ChAd3-NSmut). The mean of the responses is shown by the dashed grey line. IFN-γ ELISpots were performed by Anthony Brown, Rachel Townsend and Leo Swadling.

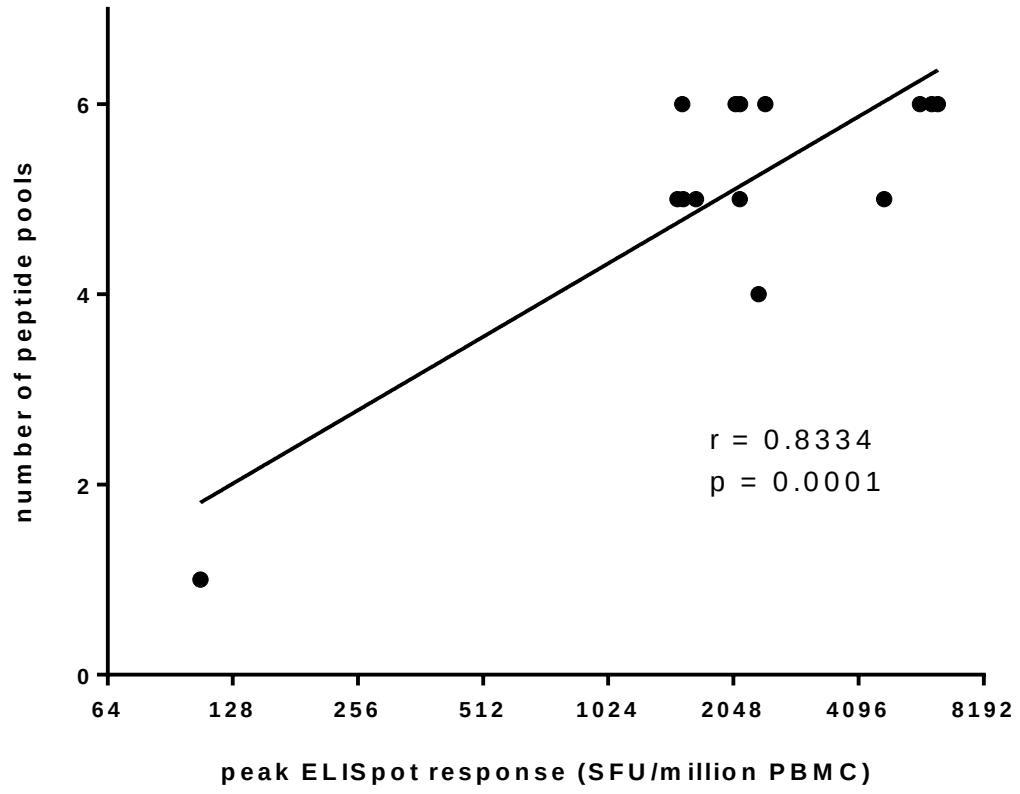
6.5.2 RE-BOOSTING WITH A SECOND DOSE OF MVA-NSmut

For Group A3 the second dose of MVA-NSmut resulted in an increase in IFN-γ ELISpot assay responses in all 5 volunteers. However the kinetics of the response was altered compared to the dynamics of response seen in Group A2. Instead of a sharp decline in responses following a peak at one week post-MVA-NSmut, 3 of the 5 volunteers saw an increase in IFN-γ ELISpot assay response between 1 week and 4 weeks post-MVA-NSmut. The responses to a second dose of MVA-NSmut in Group A4 were heterogenous: Two subjects responded similarly to Group A3, with peak responses occurring 4 weeks post- MVA-NSmut rather than 1 week post-MVA-NSmut. One volunteer responded similarly to Group A2, and one volunteer (334) had a negligible response.

6.6 BREADTH OF RESPONSE

The breadth of response is important to control of HCV. It has previously been demonstrated using a prime-boost combination of heterologous adenovirus vectored vaccines that as the magnitude of response increases so does the breadth (Barnes et al., 2012). I therefore analysed IFN-γ ELISpot data to determine whether the breadth of responses also correlated with the magnitude of response post-MVA-NSmut boost. Data were plotted from the 10 volunteers in Group A2 and the 5 volunteers in Group A3. Only data from the first MVA-NSmut boost in Group A3 were used. The initial appearance of the plotted data suggested a non-linear relationship (close to exponential) therefore data was re-expressed with a log2 scale and is shown in Figure 6-8.

Figure 6-8; Relationship between breadth and magnitude of IFN-γ ELISpot responses



Correlation between the magnitude of T cell response to NS peptides (as determined by IFN-γ ELISpot assay) and the breadth of T cell response (as determined by the number of peptide pools involved in the response). Each data point represents a single volunteer from either Group A2 or Group A3 (n=15).

6.7 SEROPOSITIVTY FOR HEPATITIS C VIRUS FOLLOWING VACCINATION

Vaccination with ChAd3-NSmut and/or MVA-NSmut will lead to the production of NS proteins in clinical trial subjects. Therefore it was theoretically possible that these NS proteins could stimulate an anti-HCV antibody response which would subsequently lead to a positive ELISA if the volunteers were subsequently screened for exposure to HCV. In order to minimise the likelihood of this, the vaccine immunogen was not optimised for extracellular expression.

Nevertheless, previous experience with the HCV001 trial was that 7% (3/41) of subjects had either a positive or equivocal HCV screening test at the end of the study (unpublished data). For HCV003 group A2 10% (1/10) of subjects had a positive HCV screening test at the end of the study. All subjects tested from Groups A1 and A3 were negative, however 50% (2/4) of subjects from Group A4 were positive. Overall, from twenty subjects who participated in Arm A of the HCV003 trial 15% (3/20) were positive for HCV antibodies as measured by a commercially available ELISA and then confirmed by the reference laboratories of Health Protection Agency (now Public Health England).

6.8 DISCUSSION

This was a first in man study for the vaccine MVA-NSmut. I have demonstrated here that this vaccine is safe at a dose of 2 x10^8 pfu, either when administered alone, or as a boosting vaccination following a ChAd3-NSmut prime.

When comparing the safety profile of MVA-NSmut to that of ChAd3-NSmut, it would appear that adverse events are more frequent and tend to be more severe following MVA-NSmut. However, the doses of viral vectored vaccines are major determinants of reactogenicity, and it is difficult to adjust for the difference in dose when comparing these vectors. For example, while adenoviral vectored vaccines are dosed according to viral particles, MVA vectored vaccines are dosed by plaque forming units (i.e. a measure of infectivity).

Comparing the safety profile between viral vectored vaccines is additionally difficult for a number of reasons: Firstly, tolerability is based on a range of symptoms reported by vaccinees, however different symptoms do not carry equal significance. As an example,

vomiting would be a far greater clinical concern than sneezing. This means that it is not prudent to simply count the total number of adverse events in one group and compare that to the number of adverse events in a second group. Secondly, how should durability of symptoms be weighted in comparison to severity? Clearly mild pain that lasts for three days is worse than mild pain that last for one day, however is mild pain that last for three days equivalent to severe pain lasting for one day? Furthermore, duration is typically recorded in units of days, and subtlety within the data is lost by rounding up to one day for symptoms that last either 5 minutes or 5 hours.

I propose that a practical approach to comparing adverse event profiles would be to dichotomise volunteers based on whether they experienced any negative impact to their daily activities following vaccination. Across our clinical vaccine trials we ascribe a severity score to each adverse event. The method for ascribing these scores is defined by the clinical trial protocol and is well harmonised between vaccine trials. In this approach, those subjects who reported either no or mild symptomatology would be combined, and volunteers who reported either moderate or severe symptomatology would be combined. When comparing the reactogenicity profile of vaccines in a given group of subjects this approach can be applied either to each symptom in turn, or can be applied to groups of symptoms (i.e. local or systemic). For example, how many volunteers experienced at least one systemic symptom that was either moderate or severe, versus how many volunteers experienced adverse reactions that were only ever mild?

I used this approach to compare ChAd3-NSmut with MVA-NSmut in the HCV003 study and summarise the analysis in Table 6-3. The proportion of volunteers with disruption to daily activities was greater following MVA-NSmut compared to ChAd3-NSmut for both local and systemic adverse events. These differences can be analysed by Fishers exact test and are

significant for local adverse events (p = 0.0006) but not significant for systemic adverse events (p = 0.2450).

Table 6-3; A proposed method for the comparison of safety profiles between vectors						
	Local adverse events			Systemic adverse events		
Vector	None/mild	Mod/severe	Total	None/mild	Mod/severe	Total
ChAd3	21	4	25	19	6	25
MVA	10	18	28	16	12	28
Total	31	22	**53**	35	18	**53**
Comparing combined frequencies of adverse events is challenging due to the diverse nature of symptoms and compounded by the additional variable of duration. The proposed method dichotomises the data based on whether (or not) a subject has experienced a negative impact on daily activities. Fisher's exact test was then applied to calculate p values for local adverse events (p = 0.0006) and systemic adverse events (p = 0.2450) respectively.						

Data from Group A1 of the HCV003 study have shown that MVA-NSmut will not effectively prime a T cell response to the NSmut insert in HCV-seronegative individuals. This lack of immunogenicity may have been predicted from similar results in a small (n=3) study of vaccination with MVA-NSmut in Rhesus macaques (unpublished data). However, in murine models, MVA vectored vaccines (including MVA-NSmut) are capable of priming naive mice (Mullarkey et al., 2013) and unpublished data.

Many studies have confirmed the observation that recombinant MVA is a highly immunogenic boosting vaccine and a less than effective priming vaccine. However, fewer studies have attempted to address why this may be the case. One obvious difference between the two vectors is the relative size of their genomes. As MVA is much larger, there will be a higher ratio of non-transgenic genes expressed compared to the desired (transgenic) immunogen than there would be for adenovirus. Additional potential contributing factors might include; i) the type of cells infected by the vector, ii) the quantity of (transgenic) antigen expressed, iii) the number of insert-derived peptides:MHC complexes that are presented on the surface of

infected cells, iv) the activation status of infected cells, and v) the milieu of inflammatory cytokines produced.

It may also be the case that the nature of the antigenic insertion can have an impact on the ability for a recombinant MVA vectored vaccine to prime immune responses. For example in contrast to MVA-NSmut, MVA85A is able to induce IFN-γ ELISpot assay responses at magnitudes of over 1,000 SFU in individuals who have never received vaccination with BCG (McShane et al., 2004). However, there is likely to be a role for prior exposure to environmental non-tuberculous mycobacteria for priming healthy volunteers prior to receiving MVA85A. As more clinical trial data with recombinant MVA vectored vaccines becomes available, more insight will be gained into the role of the insert.

There are multiple streams of evidence for T cell immunity to protect against the development of chronic HCV infection, however no single correlate of protection has been identified. Whether there is a threshold at which HCV-specific T cells can provide protection against HCV is unknown. In a chimpanzee challenge model of hepatitis C, Forlogi et al. were able to demonstrate a link between recall of vaccine induced memory responses and prevention of HCV chronicity (Folgori et al., 2006).

Vaccine trials seek to maximise the peak immune response, and with good reason. I have demonstrated here that the size of the IFN-γ ELISpot assay response correlates with the breadth and durability of immune response following vaccination with ChAd3-NSmut/MVA-NSmut. Previous studies have suggested that a broad T cell immune response is one of the factors that predict the likelihood of clearance of acute hepatitis C (Spada et al., 2004, Lechner et al., 2000).

What factors determine the magnitude of the peak IFN-γ ELISpot assay response post-boost? I calculated the correlation coefficient between post-prime IFN-γ ELISpot assay responses and post-boost IFN-γ ELISpot assay responses for the volunteers in Group A2. The results suggest that maximising the post-prime response can result in improvements to the post-boost response (Spearman r = 0.673, p = 0.039).

I then examined whether repeated administration of these viral vectored vaccines could further enhance IFN-γ ELISpot assay responses. In Group A3, the vaccines were delivered with only 8 week intervals in the sequence ChAd3-NSmut/MVA-NSmut/ChAd3-NSmut/MVA-NSmut. In these volunteers the second dose of ChAd3-NSmut was not accompanied by a subsequent peak and indeed there was no difference in responses at week 18 between Groups A2 and A3 (p = 0.62, Mann Whitney test). However, in Group A4 a second dose of ChAd3-NSmut did result in an increase in IFN-γ ELISpot assay responses in all four subjects. Two factors are likely to be responsible for this observation. Firstly, the contraction of T cells post MVA-NSmut boost is occurring relatively rapidly at 16 weeks (8 weeks post-MVA-NSmut). Secondly, anti-adenoviral immunity will be relatively strong at 16 weeks, potentially blunting the immune response to the insert. In Group A4, the improved responses to a second dose of ChAd3-NSmut may be explained by a waning of anti-vector immunity, and the fact that IFN-γ ELISpot assay responses are relatively stable and comprise mostly of a population of memory T cells.

In a related malaria study of vaccine scheduling, one consistent finding has been that IFN-γ ELISpot assay responses did not rise about the initial post-MVA-NSmut boost threshold. Interestingly in Group A4 I observed one case where after the second MVA-NSmut boost, IFN-γ ELISpot assay responses were elevated compared to the first (4,090 vs 2,355 SFU/10^6 PBMC).

Finally, the durability of vaccine induced responses are essential for vaccine efficacy to be demonstrated in field studies. For Group A2 subjects I examined whether the magnitude of IFN-γ ELISpot assay responses 26 weeks post-boost correlated with peak IFN-γ ELISpot assay responses. For the nine subjects on whom data were available, this correlation was significant (Spearman r = 0.7667, p = 0.021).

In summary, I have shown that a heterologous prime-boost vaccination regimen with ChAd3-NSmut and MVA-NSmut is safe in healthy adults. IFN-γ ELISpot assay responses to HCV peptides were significantly increased post-boost compared to post-prime, which substantially improves upon the responses observed with heterologous adenoviral vectors (Barnes et al., 2012). The presented data supports the use of this vaccine regimen in a clinical trial of efficacy in a high risk population.

While this combination of vaccines can be safely re-administered to healthy volunteers, the timing of repeat vaccinations has a major impact on immunogenicity. With a longer delay, post-MVA-NSmut responses can match those observed with the initial round of vaccination.

The way in which these two vaccines act to stimulate an immune response and the differences or similarities between these mechanisms is still largely unknown. In the following Chapter I will attempt to explore these mechanisms by examining host transcriptional response to vaccination on a genome wide level.

CHAPTER 7: COMPARING HOST TRANSCRIPTIONAL RESPONSES TO ADENOVIRUS AND MVA VECTORED VACCINES FOR HEPATITIS C VIRUS

7.1 INTRODUCTION

We would expect viral vectored vaccines to behave differently because of their different virological properties. They will infect different cell types in different tissues. They will use different proteins to take over host cellular machinery and they may have different mechanisms for subverting the host immune response. They may be detected via different pattern recognition receptors, and they may activate different combinations of host immune cells – NK cells, plasmacytoid dendritic cells, other DC subsets.

Vectors may meet pre-existing adaptive host immune responses which have been generated from prior exposure to related viruses that circulate freely (this is true for adenovirus). Or they may not encounter these pre-existing adaptive immune responses (as in most individuals receiving vaccination with a recombinant MVA vaccine).

We can see evidence of viral vectored vaccines acting in different ways from different properties that we observe from the vaccination of volunteers. For example some vectors are effective at priming whereas others are virtually incapable of generating an immune responses in immunologically naïve individuals. Secondly, the time period over which a vector stimulates a T cell response is different. For example most recombinant MVA vaccines produce a peak in T cells which occurs at 1 week post-vaccination, whereas for adenoviral vectors the peak in T cell responses occurs as late as 4 weeks in some individuals.

However in other ways they are remarkably similar – they result in a very similar pattern of adverse events, which onset over a similar period of time and last for a similar duration. While

the frequency and type of local adverse events can be influenced by the route of administration, the pattern of systemic adverse events remain similar despite vaccines being delivered either intramuscularly or intradermally. The functionality of the T cell responses produced by viral vectored vaccines can also be very similar: For example multi-functional T cell responses secreting different Th1 associated cytokines can be induced from either adenoviral vectored vaccines given to immunologically naïve individuals (Barnes et al., 2012) or following MVA vectored vaccines given to individuals already primed by natural exposure/infection (Antrobus et al., 2012) .

Despite these overall patterns of behaviour for different vectors, there is huge variation within a population of vaccinees for how different individuals respond. Examples of this include individuals receiving the malaria vaccine ChAd63 ME-TRAP (O'Hara et al., 2012) and individuals receiving the hepatitis C vaccine Ad6-NSmut (Barnes et al., 2012). In the latter example individuals vaccinated with 2.5 $x10^{10}$ vp Ad6-NSmut had a range of responses from less than 100 SFU/million PBMC to over 4,000 SFU/million PBMC (E. Barnes, personal communication). Within a population the differences in host genetics are one of the factors that will explain this diversity, even when the exposure history can be controlled for (as in the examples given).

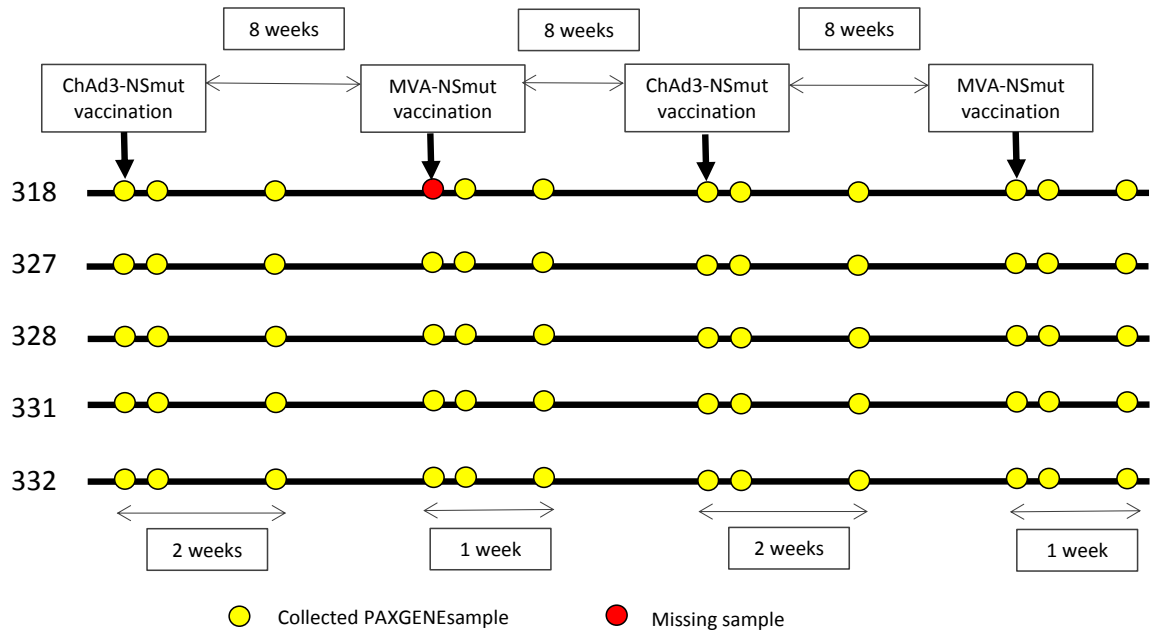
This Chapter focusses on microarray experiments performed with samples obtained during the HCV003 trial. By studying gene expression before and after vaccination with different viral vectored vaccines I aim to:

- Characterise host transcriptional responses to vaccines that use different viral vectors.
- Understand why different viral vectors may have different properties.

- Understand the host transcriptional responses that associate with individual reactogenicity and immunogenicity outcomes.

To assist the reader, a summary of the trial subjects relevant to this chapter, together with the timing of both vaccinations and blood samples is provided in Figure 7-1:

Figure 7-1; Summary of volunteers in HCV003 Group A3



In this schematic representation each subject's progress through the trial is represented by a black line. Vaccinations are shown with arrows at the top of the figure. Major time intervals are also shown in boxes with arrows. Yellow circles are the points where blood was successfully collected into PAXgene tubes. The red circle indicates the one point where this was unsuccessful.

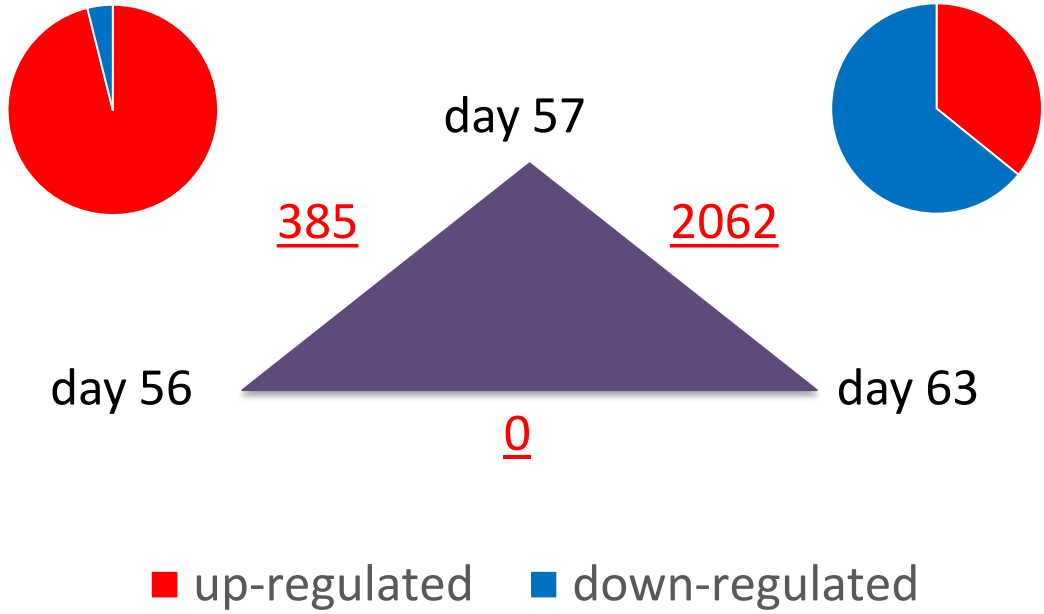
7.2 HOST TRANSCRIPTIONAL RESPONSES TO MVA

7.2.1 DYNAMICS OF GENE EXPRESSION FOLLOWING MVA-NSmut

Volunteers in Group A3 (n=5) received their first dose of MVA-NSmut eight weeks after ChAd3-NSmut prime. Peripheral blood samples collected into PAXgene tubes were taken pre-vaccination and then at 1 day and 7 days post-vaccination. Unfortunately due to difficulties with venous access the pre-vaccination sample was unavailable for one volunteer (318). For the remainder of this section the term DEGs is used to refer to differentially expressed gene transcripts, but the reader should note that this term is interchangeable with the term "probe". The distinction is important because multiple probes can relate to the same gene. Furthermore, when describing the numbers of DEGs between groups of interest, the reader should note that this does not reflect a "mean" number of DEGs. In the method of analysis used (called Limma – see Section 2.5.5) the expression values of each probe is compared between the two groups and an adjusted p value is ascribed. The number of DEGs therefore refers to the number of probes for which the adjusted p value is below the threshold for significance.

Following vaccination with MVA-NSmut, 385 probes were identified with significant differential expression (DEGs) at day 57 compared to baseline (day 56). Of these DEGs, 370 (96%) were found to be up-regulated at day 57 compared to baseline (Figure 7-2).

Figure 7-2; Schematic representation of the numbers of DEGs following the first vaccinations with MVA-NSmut



The three points of the central purple triangle represent the time points at which samples were taken. The day 56 baseline sample was taken immediately prior to vaccination. The day 57 sample was taken 24 hours after vaccination. The day 63 sample was taken one week after vaccination. The three sides of the triangle represent the individual comparisons that can be made between these time points. The numbers given in red (and underlined) represent the number of DEGs identified between the time points (q value < 0.05).Where DEGs have been identified, the pies represent whether transcripts are either increased or decreased at the second time point relative to the first time point.

Gene expression at day 57 was then compared to gene expression at day 63 (7 days post-vaccination). Here a total of 2,062 DEGs were identified (Figure 7-2). These 2,062 DEGs included 372 of the 385 DEGs (97%) that were differentially expressed between day 57 and baseline. Most of these overlapping DEGs were returning to baseline levels at day 63 – i.e. 360 of the 370 DEGs up-regulated at day 57 (1 day post-vaccination) were then significantly down-regulated at day 63, and 12 of the 15 DEGs down-regulated at day 57 were then significantly up-regulated at day 63. Of the remaining 1,690 DEGs, 963 (57%) were down-

regulated at day 63 compared to day 57, and 727 (43%) were up-regulated at day 63 compared to day 57. For the 963/1,690 down-regulated probes, the corresponding day 63 expression levels were lower than they had originally been at baseline. Similarly, for the 727/1,690 up-regulated probes, the corresponding day 63 expression levels were higher than they had originally been at baseline.

When day 63 was compared to the pre-vaccination time point, no significant DEGs were identified. Thus, following MVA-NSmut there is a strong transcriptional signature at 24 hours post-vaccination which is subsequently lost by 7 days post-vaccination. The total number of probes differentially expressed across the three time points was 2,075, of which 1,333 (64%) were up-regulated at 24 hours post-vaccination. After being up-regulated at 24 hours post-vaccination, the expression of these genes falls to levels that are lower at 7 days post-vaccination than they are at baseline. The same is true for the 36% of genes which were down-regulated at 24 hours post-vaccination, in that their final expression level at day 63 was higher than it had been at baseline. These expression changes can be appreciated visually using heatmaps (Figure 7-3, left hand panel).

7.2.2 DIFFERENCES BETWEEN FIRST AND SECOND DOSES OF MVA-NSmut

The same 5 volunteers subsequently received a second dose of MVA-NSmut at week 24 of the study. Peripheral blood samples collected into PAXgene tubes were taken at the same time points namely pre-vaccination, 1 day post-vaccination and 7 days post-vaccination. One volunteer (327) could not attend clinic 24 hours after vaccination and therefore this sample was taken 48 hours after vaccination.

The data were analysed with Limma in an identical method to that used for the first dose of MVA-NSmut. The expressions changes (in terms of the number of DEGs and the temporal dynamics) was very similar for the second dose of MVA-NSmut compared to the first (Figure 7-3). There was a strong gene expression signature at 24 hours post-vaccination which was lost by 7 days post-vaccination. Again, a larger number of DEGs were identified between day 1 and day 7 than between day 0 and day 1. This was as a result of the gene expression not just returning to baseline levels at day 7, but either increasing or decreasing further beyond the baseline level.

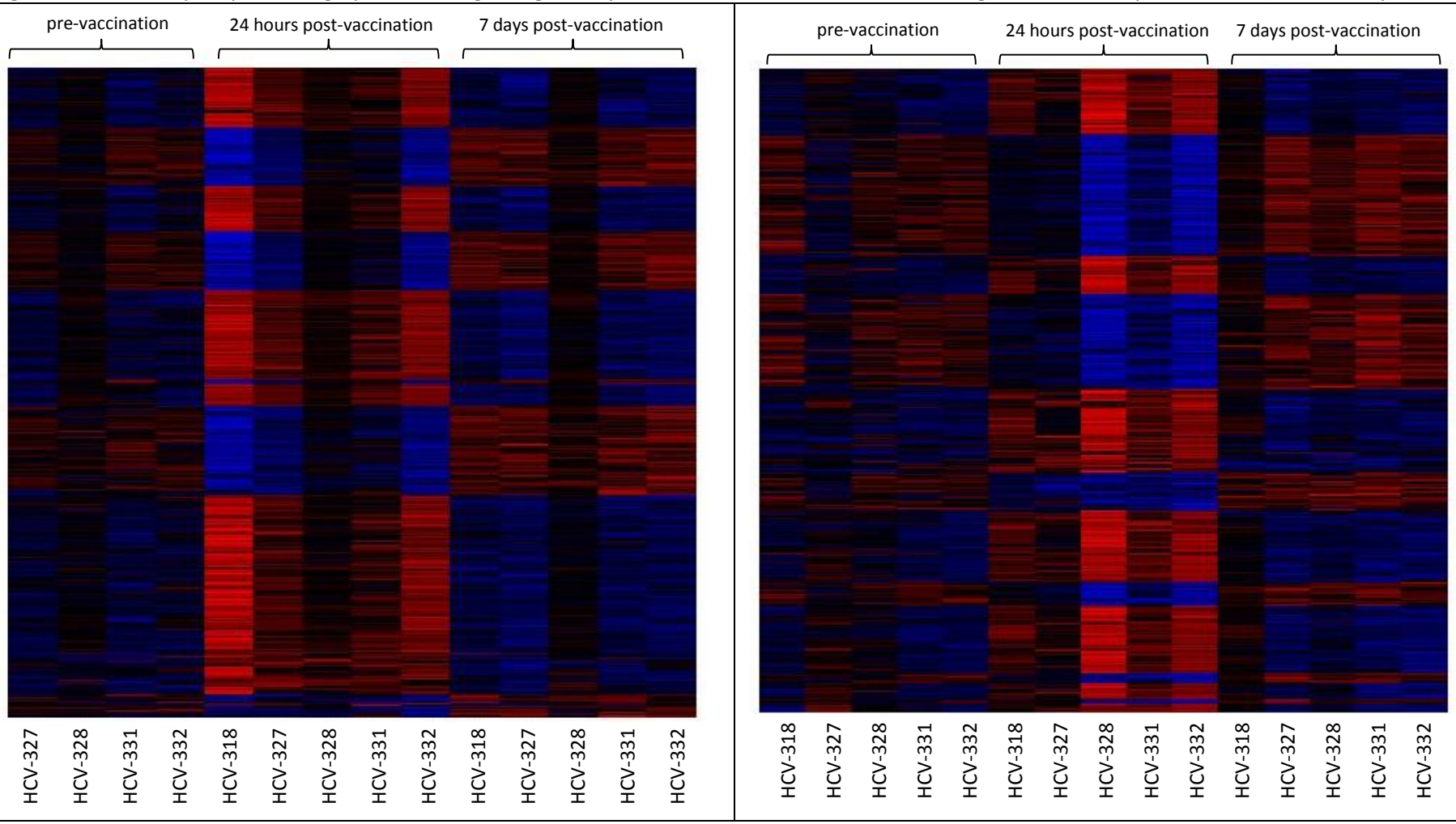
A larger number of DEGs was identified between day 0 and day 1 for the second dose of MVA-NSmut compared to the first. However, for the second dose of MVA-NSmut more day 0 samples were available (5 vs 4), which will have affected the power of the analysis. The numbers of DEGs and the overlap between these DEGs for the two doses in shown in Table 7-1.

Table 7-1; Number of, and overlap between DEGs following 1st and 2nd doses of MVA-NSmut

	1st dose MVA-NSmut	2nd dose MVA-NSmut	Overlapping DEGs
day 0 vs day 1	385	611	262
day 1 vs day 7	2,062	1,643	812
day 0 vs day 7	0	0	0
total DEGs	2,075	1,714	830

For each dose of vaccine the table shows the number of DEGs found between the two time points. The right hand column shows the number of DEGs which are common to both the first and second dose of MVA-NSmut. For these results the numbers relate to probes and unique probe IDs but not to unique genes (i.e. multiple probes can relate to the same gene).

Figure 7-3; Heat maps representing dynamic changes in gene expression for those DEGs which were significant at the q < 0.05 level (Limma analysis)



The relative expression levels for significant DEGs across the three time points can be visualised using heatmaps. In these two heatmaps the rows represent different probes and the columns represent different samples.
Colour intensity is representative of the row Z score for that probe rather than indicating absolute expression level.
Heatmaps of the left and right show data from the first and second vaccinations with MVA-NSmut respectively.

7.2.3 FUNCTIONAL ANNOTATION OF GENE LISTS

The lists of DEGs obtained from the analyses in sections 7.2.1 and 7.2.2 were uploaded for functional annotation by DAVID (Section 2.5.5). This software allows gene lists to be analysed according to whether they contain a number of genes which are related by a common biological process. In order to conduct this analysis the DAVID database links "gene ontology (GO) terms" to each of the genes in the list of interest. GO terms can be broad (for example *immune response*), or can be more specific (for example *T cell activation*). DAVID is also used to calculate a p value for each GO term, to establish whether it is enriched to statistical significance. The Benjamini–Hochberg procedure is used to correct for multiple comparisons when performing this analysis. When referring to GO terms, I am specifically referring to GO terms that relate to biological processes, however it is also possible to use DAVID to analyse gene lists to look for shared molecular functions or shared cellular components.

Analysis relating to the first dose of MVA-NSmut administered:

For the first dose of MVA-NSmut there were 25 GO terms that were significantly enriched (adjusted p value <0.05, Benjamini and Hochberg method) from the 385 DEGs between day 0 and day 1. Of these 25 terms, 21 were related to immune/inflammatory/defence responses, the NFkB pathway, responses to viruses, responses to cytokines and apoptosis/programmed cell death. Using the 2,062 list of DEGs (day 1 vs day 7), 105 GO terms were significantly enriched and 79 were related to the same terms listed above. In addition, 5 terms were related to responses to bacteria/bacterial proteins or to pathogen associated molecular pattern (PAMP) recognition pathways.

Specific groups of genes that were up-regulated at day 1 post-vaccination included all 4 member of the OAS family (12 probes), MX1 and MX2, TAP1 and TAP2, STAT1 and STAT2 (4 probes), and EIF2AK2 (encoding the enzyme PKR).

Comparisons between MVA-NSmut and influenza virus challenge data:

Thus many of the genes identified above were familiar from my previous analysis of the FLU002 challenge study described in Chapter 5. I therefore compared these DEGs (from volunteers receiving MVA-NSmut) to those from volunteers with moderate-severe laboratory confirmed influenza. This list of DEGs will now be referred to as the "FLU002 gene list".

Of the 385 DEGs following the first dose of MVA-NSmut, 321 (83%) were also found in the FLU002 gene list. The 64 DEGs that were specific to MVA-NSmut had a narrower range of log2 fold changes (-0.967 to 1.52) compared to the FLU002 gene list (-2.80 to 5.18). Amongst the genes that were specific to MVA-NSmut were members of the proteasome (identified by 4 probes), the receptor NOD1 and the kinase JAK2. Caspases are up-regulated in both sets of data however following MVA-NSmut caspases 4 and 10 are increased, whereas in the FLU002 gene list it is caspase 1 and caspase 5 that are increased.

Analysis relating to the second dose of MVA-NSmut administered:

Functional annotation (again using DAVID) was then performed for the gene lists generated following the second dose of MVA-NSmut. For the 611 DEGs between day 0 and day 1, 34 GO terms were significantly enriched, of which 23 (68%) were related to immune/inflammatory/defence responses, the NFkB pathway, responses to viruses, responses to cytokines and apoptosis/programmed cell death. For the 1,643 DEGs found

between day 1 and day 7, 35 GO terms were significantly enriched of which 27 (77%) related to the biological processes lists above.

I then compared the list of 611 DEGs to the gene list identified in volunteers with moderate-severe laboratory confirmed influenza. In this case 66% of genes from the list of 611 were also found on the FLU002 gene list.

In Section 7.2.2 I showed that when comparing lists of DEGs between the first dose of MVA-NSmut and the second dose of MVA-NSmut, approximately 40-50% of the DEGs were shared, and the remainder being unique to either the first or the second dose. In order to explore whether these overlapping and unique genes were representative of the same biological pathways I analysed them separately in DAVID. Smaller gene lists yield fewer enriched GO terms, with less specific biological processes. Given that 88-96% of the DEGs identified in the day 0 vs day 1 comparisons were also present in the day 1 vs day 7 comparisons I therefore performed this analysis from the latter gene lists.

There were 705 unique genes (using official gene symbols) that were found between the gene lists from the first and second doses of MVA-NSmut. From this list 126 GO terms were significantly enriched at $q < 0.05$ and 94 terms were significantly enriched at $q < 0.01$. Of these 94 terms, 74 (79%) related to immune responses/defence responses and their regulation, the activation and proliferation of immune cells, responses to viruses, bacteria or bacterial molecules, production of and responses to cytokines, the NFKB pathway and TLR-signalling pathways, inflammation and apoptosis.

For the first dose of MVA-NSmut there were 988 unique genes (Entrez Gene IDs) that were differentially expressed, but which were not found following the second dose of MVA-NSmut.

Analysis of this gene list with DAVID did not show any significantly enriched terms (adjusted p value <0.05, Benjamini and Hochberg method). The same analysis was then performed for the 685 unique genes that were differentially expressed following the second dose of MVA-NSmut. Again, the analysis of this gene list with DAVID did not reveal any significantly enriched GO terms.

7.2.4 COMPARING HOST TRANSCRIPTIONAL RESPONSES TO MVA-NSmut AND MVA85A

I next wanted to determine whether recombinant MVA-vectored vaccines with different antigenic insertions activated the same pattern of host transcriptional responses. Similar gene expression studies have been performed at the Jenner Institute with the vaccine MVA85A (comprising MVA expressing antigen 85A from *mycobacterium tuberculosis*). In the clinical trial TB022 (clinicaltrial.gov Identifier: NCT01181856), 24 volunteers were vaccinated with 1 x 10^8 pfu MVA85A (Meyer et al., 2013). One group of volunteers received vaccination via the intramuscular route and a second group of volunteers received vaccination via the intradermal route. Overall, there were no substantial differences in immunogenicity between the two routes of injection. Systemic adverse events were also similar between the two groups, however local adverse events were more frequent in the group who received intradermal vaccination (Meyer et al., 2013).

During this clinical trial peripheral blood was collected for subsequent RNA extraction. Three samples were collected from each volunteer: a baseline sample was collected immediately prior to vaccination, an early sample was collected 2 days post-vaccination and a final sample was collected 7 days post vaccination.

The data set generated from this trial therefore has some fundamental differences to my own data. Firstly, the RNA used in these experiments was not extracted from whole blood collected into PAXgene tubes. Instead, peripheral blood was collected and PBMC were isolated and frozen. RNA was then extracted from thawed PBMC and thus no RNA from neutrophils was present. Secondly, the early post-vaccination time point is different, and given the rapidity with which the gene expression signature from MVA-NSmut was lost, this timing of the early post-vaccination samples could have a significant impact on the nature of the DEGs found. Thirdly, half of the vaccinations were delivered by the intradermal route (although in a subgroup analysis, the nature of the DEGs was not found to be different between intramuscular and intradermal administration). Finally, as RNA was extracted from PBMC, no globin mRNA depletion step was required.

Microarray hybridization and subsequent filtering and normalisation of data was performed by Magali Matsumiya, Jenner Institute, Oxford, UK. Analysis of gene expression following vaccination with MVA85A revealed similar dynamics to that seen with MVA-NSmut. A substantial host transcriptional response was observed early post-vaccination, but this signal was subsequently lost by 7 days post vaccination.

A total of 992 probes were found to be differentially expressed between day 0 and day 2 ($q < 0.05$). These probes related to 870 unique genes with official gene symbols (all duplicates having been removed).

From the lists of DEGs obtained following MVA-NSmut, there were 830 probes which were found after both the first and second doses. This corresponded to 705 unique genes with official gene symbols (all duplicates having been removed).

These two gene lists were then compared and only 205 genes were overlapping. There was therefore exactly 500 genes which were specific to MVA-NSmut and 665 genes which were specific to MVA85A. I proceeded to analyse these gene lists using DAVID, starting with the 205 genes that were common to the two vaccines. Surprisingly, only 12 GO terms were significantly enriched, and these were very similar to the 15 GO terms that were significantly enriched when all 870 genes from the MVA85A microarrays were analysed in DAVID. Thus it would appear that the majority of the significance in functional annotation observed in the MVA85A data can be accounted for by this subset of 205 genes, and the remaining 665 genes that were specific to MVA85A did not reflect a functionally co-ordinated group of genes (Table 7-2).

I then moved on to analyse the gene lists which were specific to either of the two vaccines. For MVA85A, there were no GO terms which were significantly enriched. This would support the above suggestion that the genes in this list are representative of a number of different biological processes rather than being representative of a co-ordinated biological response. However, in contrast to the gene list which was specific MVA85A, for the gene list which was specific to MVA-NSmut there were 91 GO terms that were significantly enriched. Of these terms, 75 (82%) were related to immune responses/defence responses and their regulation, the activation and proliferation of immune cells, responses to viruses, bacteria or bacterial molecules, production of and responses to cytokines, apoptosis/programmed cell death, the NFKB pathway and protein kinase cascades.

Table 7-2; Significant GO terms identified by DAVID for DEGs relating the MVA85A

Both vaccines (205 genes)		MVA85A (992 probes, 870 unique genes)	
immune response	6.00E-20	immune response	2.20E-11
response to virus	3.90E-11	response to virus	3.90E-06
defense response	1.60E-06	defense response	4.40E-05
positive regulation of immune response	2.10E-04	positive regulation of immune response	3.30E-04
response to wounding	4.40E-04	activation of immune response	1.10E-02
positive regulation of immune system process	5.50E-04	positive regulation of immune system process	7.50E-03
innate immune response	6.00E-04	innate immune response	4.90E-04
inflammatory response	5.50E-04	inflammatory response	1.10E-02
positive regulation of response to stimulus	1.90E-03	positive regulation of response to stimulus	1.20E-02
response to molecule of bacterial origin	6.30E-03	lymphocyte mediated immunity	3.20E-02
immune response-activating signal transduction	3.30E-02	immune response-activating signal transduction	4.10E-02
immune response-regulating signal transduction	4.30E-02	leukocyte mediated immunity	4.30E-02
		regulation of cell death	4.20E-02
		I-kappaB kinase/NF-kappaB cascade	4.20E-02
		protein kinase cascade	3.50E-02

Two lists of DEGs were subjected to an identical analysis with DAVID. The right hand column shows results from all DEGs relating to MVA85A. The left hand column shows the results from the subset of DEGs which were common to both MVA85A and MVA-NSmut. The similar nature of the two lists suggests that much of the functional significance of the total gene list is accounted for by the subset of genes which are common to both vaccines.

7.2.5 ASSOCIATIONS WITH IMMUNOGENICITY

One of my objectives was to explore whether different gene expression signatures could be associated with T cell responses to the vaccine transgene. In order to explore this, it is first necessary to categorise volunteers according to their response. ELISpot data can be viewed in a number of ways to try and rank responses – peak response in SFU/million PBMC may be used, however this will be influenced by pre-vaccination levels of T cells. An area under the curve analysis may be used, but also has the same caveat. Alternatively it is possible to compare the fold change increase in T cells post-vaccination. Successful categorisation into biologically relevant groups also requires there to be sufficient variation amongst the subjects in their response to vaccination.

However, this variation was not present in Group A3 of the HCV003 study: Following the first dose of MVA-NSmut, 4 of the 5 volunteers had very similar ELISpot assay responses. Volunteers 318, 327, 331 and 332 all had pre-vaccination ELISpot assay responses of <150 SFU/million PBMC which were boosted to between 1,543 and 2,105 SFU/million PBMC. The remaining volunteer (328) achieved a much higher peak post-boost response (6,356 SFU/million PBMC) however this is also likely to reflect the higher baseline in T cell response (2,273 SFU/million PBMC at week 8).

Following the second vaccination with MVA-NSmut all the volunteers had unique changes in their ELISpot assay responses. Volunteer 318 showed a peak in response at 7 days post-vaccination (day 175), volunteer 327 showed a peak response at 28 days post-vaccination (day 196), volunteer 328 had a very high pre-vaccination ELISpot assay response which continued to rise even at the final study time point (day 336), volunteer 331 had a modest boost in their ELISpot assay response 7 days post-vaccination which increased slightly to day 224 and volunteer 332 had a modest boost in their IFN-γ ELISpot assay response which decreased slightly to day 336.

With only 5 subjects, and 10 vaccination episodes, it would have been necessary to categorise volunteers into “high-responders” and “low-responders” before attempting to find significant associations with gene expression. Without these clear phenotypic distinctions, this kind of analysis was not justified.

7.3 HOST TRANSCRITPIONAL RESPONSES TO CHAD3-NSMUT

I next wanted to establish the host transcriptional response to vaccination with the simian adenoviral vectored vaccine ChAd3-NSmut. This vaccine was administered twice, firstly on the day of enrolment and then again at week 16. Samples were collected immediately prior to vaccination, with two subsequent samples taken at the safety visit (days 1-5 post-vaccination) and at 14 days post-vaccination. In addition, 4 of the 5 volunteers had RNA processed from samples taken at day 28 of the study.

Ideally, the time interval between vaccination and collection of the first post-vaccination samples would be harmonious between the volunteers. Samples would also ideally have been taken on day 1 post-vaccination, as not only would this be consistent with the subsequent MVA-NSmut data, but would also be more likely to detect the full range of the host transcriptional response.

The clinical trial protocol allowed for volunteers to be seen for safety review at day 3 (+/- 2 days) and therefore unfortunately at the beginning of the clinical trial, all five volunteers had a ≥4 day interval between vaccination and the collection of the first post-vaccination blood sample. This was subsequently corrected for the second round of vaccinations with ChAd3-NSmut, where all five volunteers had post-vaccination samples taken on the first day post-vaccination.

7.3.1 THE TRANSCRIPTIONAL RESPONSE FOLLOWING VACCINATION WITH CHAD3-NSMUT IS LESS DISTINCT COMPARED TO MVA-NSMUT

Initially I performed an identical Limma analysis for the 15 samples relating to the first dose of ChAd3-NSmut (5 subjects, 3 samples per subject). Three comparisons were made; day 0 vs day 4, day 4 vs day 14 and day 0 vs day 14. For each comparison there were no significantly differentially expressed genes. Having already characterised the extent of differential gene expression at 7 days post-vaccination with MVA-NP+M1 and MVA-NSmut, the fact that no differentially expressed genes were found between these time points was not surprising. The most parsimonious explanation is that the post-vaccination changes in gene expression would have occurred over the first 48 hours and then normalised by day 4 post-vaccination.

I therefore went on to analyse the equivalent data relating to the second round of vaccinations with ChAd3-NSmut. Again, three comparisons were made. When the pre-vaccination samples (day 112) were compared to the two week post-vaccination samples (day 126), no differentially expressed genes were found. This negative finding was entirely consistent with the findings previously described for MVA-NSmut. When the pre-vaccination samples (day 112) were compared to the early post-vaccination samples (day 113), no differentially expressed genes were found. This was a much more surprising finding, given that the equivalent analysis for the MVA-NSmut samples yielded over 350 probes which showed differential expression. Finally, the early post-vaccination samples (day 113) were compared to the two week post-vaccination samples (day 126). For this analysis, 102 probes were found to be significantly differentially expressed between these time points.

Therefore, far fewer DEGs were found following either the first or the second dose of ChAd3-NSmut than for MVA-NSmut. The possible explanations for why this might be the case are debated in Section 7.5, however I next wanted to evaluate the dynamics of the expression

changes and the nature of these DEGs. Signal data from the probes in this list is shown as a heatmap in Figure 7-4. From this heatmap it can be seen that, unlike for MVA-NSmut, almost all the DEGs were up-regulated at 1 day post-vaccination, returning to levels that were less than baseline by 14 days post vaccination.

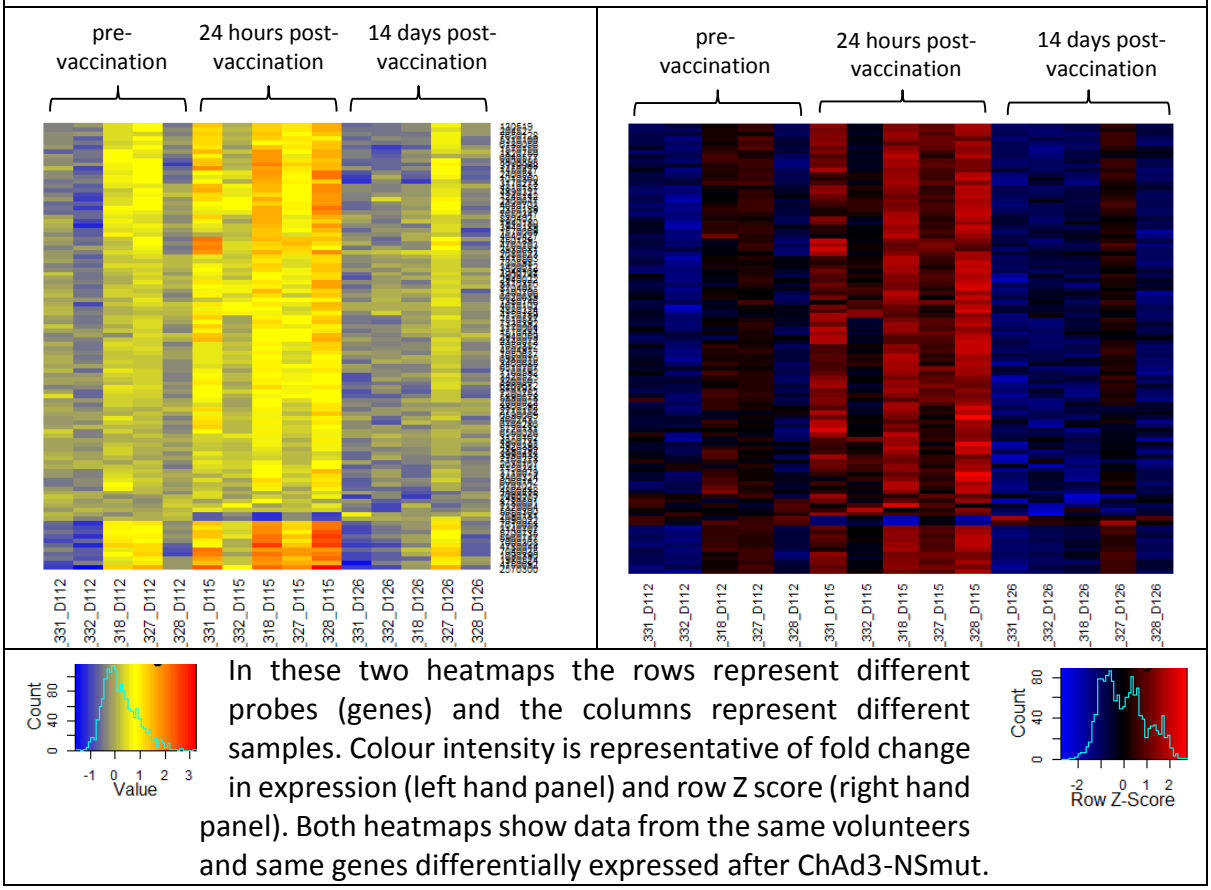
The heatmap also reveals that there is considerable variation between the 5 volunteers. The gene expression signature is strongest for volunteer 328 (with 318 and 331 also being strong) and weakest for volunteers 327 and 332. Previous studies have suggested that the innate immune response stimulated following vaccination with viral vectored vaccines is attenuated by high levels of pre-existing antibodies to the viral vector (Zak et al., 2012). This may therefore have accounted for the variation between individuals that I observed. In order to explore this I utilised neutralising antibody data (provided by Dr S Colloca, Okairos) for the five volunteers in Arm A3 of the HCV003 study (Table 7-3).

Table 7-3; Anti-vector neutralising antibodies in HCV003 (A3) volunteers

	Screening	4 weeks	16 weeks	20 weeks
327	31	642	263	866
328	235	1,474	1,339	1,095
331	791	2,645	3,821	3,804
318	170	2,059	1,099	3,004
332	43	457	809	574

Neutralising antibody data is shown for the 5 volunteers in Arm A3 of the HCV003 trial. Serum samples were taken at screening (up to 90 days prior to enrolment) and then:
4 weeks into the trial (which relates to 4 week post the first dose of ChAd3-NSmut)
16 weeks into the trial (which is immediately prior to the second dose of ChAd3-NSmut)
20 weeks into the trial (which relates to 4 week post the second dose of ChAd3-NSmut)
Antibody titre data was provided by Dr S Colloca (Okairos)

Figure 7-4; Heatmaps representing gene expression following vaccination with ChAd3-NSmut



In these two heatmaps the rows represent different probes (genes) and the columns represent different samples. Colour intensity is representative of fold change in expression (left hand panel) and row Z score (right hand panel). Both heatmaps show data from the same volunteers and same genes differentially expressed after ChAd3-NSmut.

The explanation that pre-existing antibody levels reduce innate immune signals post-vaccination does not match my observations for the data presented. The strongest transcriptional signals were produced by volunteers 318, 328 and 331. These three volunteers had the highest pre-vaccination levels of neutralising antibodies (>1,000), and conversely the weakest transcriptional responses were found in the two volunteers with the lowest levels of neutralising antibodies (<1,000). The mechanism by which pre-existing neutralising antibodies reduced innate immune responses was not discussed by Zak et al. however, it could be assumed that neutralisation leads to a reduction in the number of potential infective particles and results in an effective dose reduction. However, an alternative hypothesis could account for the opposite effects that are observed here. An increase in anti-vector antibodies could result in an increase in opsonised viral particles (the majority of which will not be

infective in a recombinant vaccine). Increased opsonisation then leads to greater activation of innate immune cells via Fc receptors and greater uptake of viral particles by antigen presenting cells.

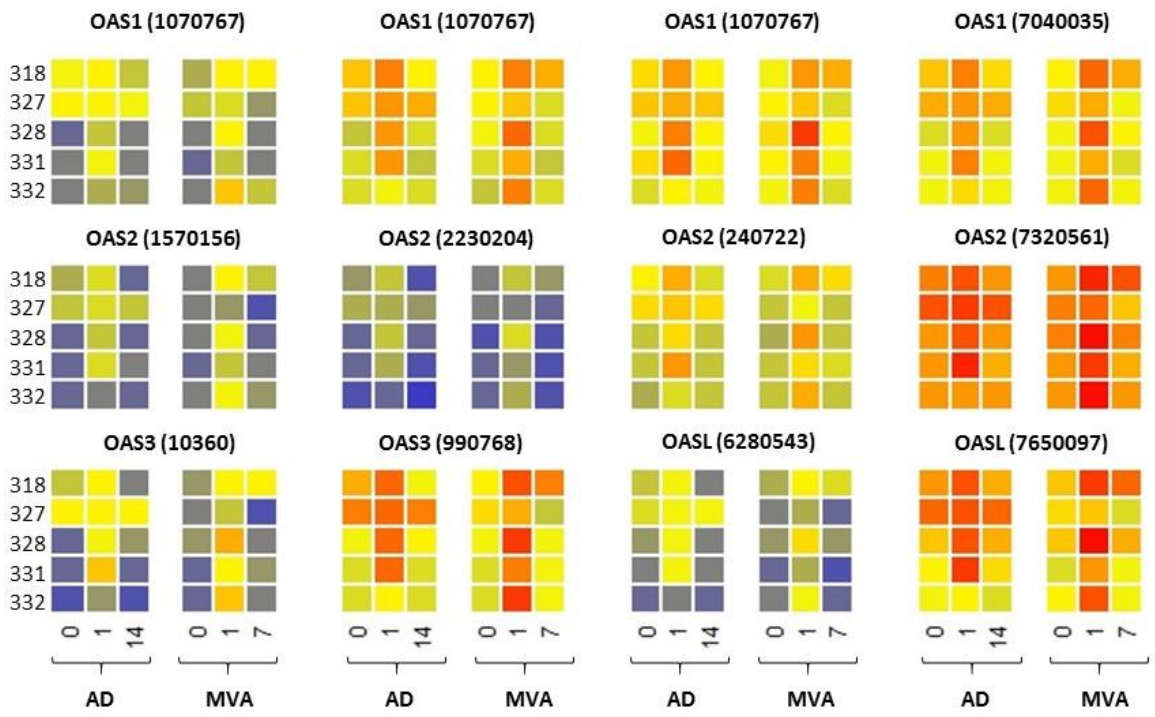
7.3.2 COMPARISON OF GENE EXPRESSION FOLLOWING CHAD3-NSMUT AND MVA-NSMUT

As mentioned in Section 7.3.1, following vaccination with the second dose of ChAd3-NSmut there were 102 differentially expressed probes, which after further analysis corresponded to 92 unique genes. This list of genes was then compared to the 705 genes that were differentially expressed following both first and second doses of MVA-NSmut and 76% of the DEGs identified following ChAd3-NSmut were also differentially expressed following MVA-NSmut.

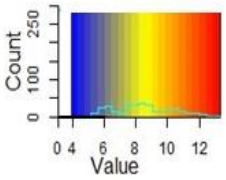
While similar pathways of genes were being activated by the two different viral vectors, it appeared that the fold changes in the expression of these genes was different. Specifically, the expression changes amongst the significant DEGs were apparently lower for the same volunteers following ChAd3-NSmut compared to MVA-NSmut.

I explored this further by examining expression in the OAS family of genes, which were significantly up-regulated early post-vaccination following both vectors (Figure 7-5). For MVA-NSmut all four members of the OAS family (detected by 12 different probes) were significantly over-expressed, whereas for ChAd3-NSmut only two OAS members (OAS1 and OAS2, detected by 3 probes) were significantly over-expressed. I therefore went on to examine the expression of these genes across the different volunteers to understand how much variation there was within the groups as well between the groups.

Figure 7-5; Gene expression amongst OAS gene family member



- Volunteer 318: Fold changes in OAS expression were generally greater following MVA-NSmut than following ChAd3-NSmut (9 versus 3 probes)
- Volunteer 327: Fold changes in OAS expression were greater following MVA-NSmut than following ChAd3-NSmut (11 versus 1 probe)
- Volunteer 331: Fold changes in OAS expression were greater following ChAd3-NSmut than following MVA-NSmut (for all 12 probes)
- Volunteers 328 and 332: Fold changes in OAS expression were greater following MVA-NSmut than following ChAd3-NSmut (for all 12 probes).



In this figure, colour blocks represent absolute gene expression from an individual sample. All colour blocks are on the same scale. The blocks are arranged into 12 panels representing the 12 probes that relate to members of the OAS gene family. Each panel comprises two halves – the left hand side representing ChAd3-NSmut and the right hand side representing MVA-NSmut. Each half consists of 15 blocks with volunteers arranged by row and time point arranged by columns.

The first observation was that, within a single volunteer (and for a given vaccine), there was close agreement between different probes aimed at detecting expression from the same gene. Secondly the different OAS transcripts were closely regulated, so that if a volunteer had a strong increase in OAS1 expression, they also demonstrated large increases to OAS2, OAS3

and OASL. Indeed, all the transcripts for all the volunteers showed increases in expression, despite only a minority achieving significance following vaccination with ChAd3-NSmut. Thirdly, for a given vaccine and a given probe, there was substantial inter-individual variation. For example at 1 day post vaccination with MVA-NSmut volunteer 328 had a 3.44 change in log2 expression, whereas volunteer 327 only had a 0.66 change in expression. Fourthly, for a given probe, the maximum changes in log2 expression were always seen in an individual who had received MVA-NSmut. However, one individual (331) had consistently greater changes in log2 expression following ChAd3-NSmut compared to MVA-NSmut. Overall this indicates that at the doses of the viral vectors used, while MVA-NSmut induces stronger type-I interferon responses in most individuals, ChAd3-NSmut is capable of inducing stronger responses in some individuals.

Volunteer 331, who had a stronger transcriptional response for OAS genes to adenovirus than following MVA-NSmut interestingly had no symptoms following vaccination with ChAd3-NSmut. This volunteer also had high titres of neutralising antibodies.

Overall, it is difficult to conclude from these data that ChAd3-NSmut induces gene expression at either lower levels, or amongst fewer genes than MVA-NSmut. Instead, what is clear is that there is considerable inter-individual variation in the transcriptional responses following vaccination. This degree of variation is perhaps not surprising in light of the inter-individual variation seen in immunogenicity following any given vaccine, both in terms of antibody responses and T cell responses to either the transgene or the vector.

7.3.3 COMPARISION OF ChAd3-NSmut WITH OTHER ADENOVIRUS VECTORS

In a study of innate immune responses by Zak (Zak et al., 2012), the transcriptional response to vaccination with MRKAd5/HIV was studied in 10 volunteers. The MRKAd5/HIV vaccine is a mixture of three E1-deleted recombinant Ad5 viruses (McElrath et al., 2008), each containing a different insert (derived from HIV-1 gag, pol and nef proteins). For these microarray experiments PBMC were isolated from peripheral blood samples taken pre-vaccination and then at 4-6 hours, 24 hours, 72 hours and 168 hours post-vaccination. RNA was then extracted from unstimulated PBMC and analysed using the Human Exon ST 1.0 microarrays from Affymetrix.

Amongst the 10 volunteers studied, 7 were seronegative for Ad5, as defined by neutralising antibody titres ≤200. For these 7 volunteers, transcriptional responses were maximal at 24 hours post-vaccination with 1,026 genes demonstrating enhanced expression and 1,048 genes demonstrating repressed expression levels compared with baseline (Zak et al., 2012). The dynamics and extent of these transcriptional signatures are therefore more similar to my observations following vaccination with MVA-NSmut than following ChAd3-NSmut.

Seventy-three percent of the genes differentially expressed following ChAd3-NSmut were differentially expressed following MRKAd5/HIV. This compared to 76% of genes which were shared between ChAd3-NSmut and MVA-NSmut, and only 61% of genes which were shared between MVA-NSmut and MRKAd5/HIV. Thus, greater differences in DEGs are observed when unrelated vectors are compared, however even larger differences are found when gene lists are compared between microarray experiments that use different cell populations for the basis of RNA extraction.

7.4 ASSOCIATIONS WITH REACTOGENICITY

One of the objectives of this study was to explore whether distinct transcriptional signatures were associated with, and could potentially predict, reactogenicity outcomes. In Group A3 of HCV003 there were 20 vaccine episodes in which to look for these associations. However, for a combination of reasons it was difficult to test for these associations.

Firstly, for vaccinations with ChAd3-NSmut, no differential gene expression was found following the first dose. As I have described in Section 7.3, this is likely to have occurred because blood sampling did not occur early enough post-vaccination for differential gene expression to be detected. Thus the number of vaccine episodes in which to examine reactogenicity outcomes was reduced to 15.

Secondly, for the remaining vaccinations with ChAd3-NSmut although there were some significantly differentially expressed genes, the numbers of DEGs were low. In addition there was very little reactogenicity observed in this group. Only one volunteer developed any systemic adverse events, compared to 80% of volunteers who developed systemic adverse events following the first dose of ChAd3-NSmut. Hence these next five vaccines episodes did not provide sufficient phenotypic diversity for reactogenicity associations to be explored. Overall, this combination of factors meant that gene expression signatures that may associate with reactogenicity could only be examined for the ten MVA-NSmut vaccine episodes.

Prior to examining the data for individual subjects, I adopted the following hypothesis: Individuals with the most severe spectrum of adverse events would have the highest degree of signal changes. This hypothesis was based on the fact that signal change was largely driven by genes involved in type I interferon pathways (Chapter 7.2.3).

Following the first dose of MVA-NSmut, subject 318 had the most severe profile of adverse events and appeared to have one of the strongest signal changes consistent with the above

hypothesis. A complicating factor here however was that there was no pre-vaccination sample (day 56) available for this volunteer. However for the remaining volunteers, there was no consistent pattern between the degree of signal change and the severity of adverse events: Volunteers 328 and 332 had similar reactogenicity profiles with moderate local adverse events and mild systemic adverse events. However the degree of signal change observed in the microarray experiment was substantially different between these two individuals – volunteer 328 had a very weak signature following MVA-NSmut whereas volunteer 332 had a very strong signature similar to that observed for volunteer 318.

The associations between the strength of signal change and the extent of adverse events was equally inconsistent following the second dose of MVA-NSmut. Volunteers 318 and 327 both had similarly weak patterns of gene expression change following vaccination, however while volunteer 318 was one of the most symptomatic volunteers following vaccination (developing multiple moderate systemic adverse events), volunteer 327 was the least symptomatic (developing only mild discomfort at the vaccine site).

Finally, in support of the above hypothesis, following the second vaccination with MVA-NSmut, volunteer 332 had one of the strongest signal changes and was also one of the most symptomatic volunteers (developing headache, myalgia and a fever of 37.8 C).

One avenue for analysing this data further would have been to use the pamr method that I described in Chapter 5. This would have required access to a “training set” of data for another MVA-vectored vaccine. The data I obtained following MVA-NSmut could then have been used as a “test set” of data to evaluate if gene expression signatures could be associated with reactogenicity across viral vectored vaccines that utilised the same vector but differed in the transgene they expressed. However, I could not use the microarray data I collected following

MVA-NP+M1 (described in Chapter 4) for this purpose because there was no significant differences in gene expression between day 0 and day 7 in the group that received vaccine co-administration. Finally, for the alternative option of using MVA85A as a comparator, the relevant microarray data could not be accessed.

7.5 DISCUSSION

Firstly, I have shown that vaccination with MVA-NSmut results in a significant host transcriptional response which is readily detectable in peripheral blood mononuclear cells. However this response is short-lived, with differences in gene expression being undetectable between a pre-vaccination baseline and 7 days post-vaccination. More subtle differences in gene expression may be detectable at 7 days or beyond if a larger sample size had been available or if individual cell subsets were examined. Indeed, significant changes in gene expression have been found 14 days after vaccination with TIV (Bucasas et al., 2011).

When comparing the DEGs found following the first and second doses of MVA-NSmut, 40-50% of genes were common between the two lists. One possibility for this observation is that the nature of the host transcriptional response changes according to whether the subject's immune system is naïve to the MVA vector, or whether memory responses are being activated. However, further evaluation showed that the gene list unique to the first dose of MVA-NSmut was not enriched for functionally related gene ontology terms. The same was true for the gene list unique to the second dose of MVA-NSmut. This suggests that instead of there being specific biological pathways that are only activated when MVA-NSmut is given on the first or second occasion, there may in fact be a significant amount of "noise" in the data.

I went on to compare lists of DEGs between two different MVA vectored vaccines, namely MVA-NSmut and MVA85A. I found that there was an overlapping group of genes with significantly enriched GO terms, indicating that expression of these genes was consistently affected by the MVA vector, and that these genes represent regulation of functionally

relevant biological pathways. In making comparisons between MVA-NSmut and MVA85A specific gene lists, it became apparent that the genes specific to MVA85A were not significantly associated with distinct biological pathways. In contrast, the genes specific to MVA-NSmut were significantly associated with distinct biological pathways. Although it is possible that these differences are the result of vaccine-specific properties, an alternative hypothesis is that the differences reflect changes in different populations of leukocytes. For the MVA-NSmut data there could be a group of DEGs in the PBMC fraction and then a separate group of DEGs in the granulocyte fraction. The data from MVA85A will only share the former, and hence when common DEGs are excluded from the analysis the MVA-NSmut data could reveal DEGs that are specifically related to changes in granulocytes.

It may also be the case that these differences are due to contrasting methodologies for RNA collection and extraction. For the MVA-NSmut data, collection of peripheral blood into PAXgene tubes allowed for RNA to be immediately stabilised. For the MVA85A data, RNA submitted for microarray had been extracted from PBMCs which had first been isolated from peripheral blood by density centrifugation and then exposed to DMSO and a freeze thaw cycle. This *ex vivo* manipulation may have resulted in gene expression changes which were not specific to vaccination, and this may have added "noise" to those particular microarray experiments. If this latter hypothesis were correct then future microarray experiments would benefit from being conducted from PAXgene tube derived RNA rather than RNA derived from frozen PBMC. It may however be worthwhile investigating whether RNA extracted from fresh PBMC could result in improvements to either of these two options.

In both of these analyses I found that the functional annotation tool DAVID was of greater assistance in comparing two gene lists rather than in describing the content of a single gene

list. The terms describing the biological pathways enriched in a single list of genes are frequently too vague for the investigator to appreciate whether they are of relevance. In contrast, by performing the same DAVID analysis on two separate gene lists it allows for an unbiased comparison to be made between gene lists and informs the investigator of which gene list is more representative of a cohesive, biologically relevant process.

One of the most striking findings was the contrast in gene expression between the two viral vectors. There are several factors that may explain why the transcriptional signatures following vaccination were found to be so different between ChAd3-NSmut and MVA-NSmut:

1. Clearly for the first dose of ChAd3-NSmut, the timing of the blood sampling was suboptimal. Data from this Chapter, from earlier Chapters in this thesis, from previous experience at the Jenner Institute, and from the literature all suggest that the strongest gene expression changes occur early post-vaccination. However, while this can explain the lack of DEGs found following the first vaccination, it does not explain the reduced number of DEGs following the second dose of vaccine.
2. It may be that when the second dose of ChAd3-NSmut is given, pre-existing anti-hexon antibodies effectively reduce the dose of vaccine. Although anti-hexon antibodies were detectable in volunteers prior to the first dose of ChAd3-NSmut, these titres substantially increased following vaccination and these levels were sustained to week 16 (date of second vaccination).
3. The differences observed in gene expression between the vaccines may be a reflection of the fact that ChAd3-NSmut infects a different cell type to MVA-NSmut. Different target cells would potentially activate downstream immune effector cells to different extents. For example, a viral vector which infects antigen presenting cells such as

dendritic cells (as is the case for MVA) would be expected to activate type I interferon pathways more readily.

4. Vaccination with ChAd3-NSmut may not have resulted in strong transcriptional responses because of a batch effect rather than the intrinsic properties of the simian adenovirus. It is difficult to prove this hypothesis, however there is some (circumstantial) supporting evidence. Firstly, it was unusual that only 2 of the 5 volunteers mounted detectable ELISpot assay responses to vaccination at priming (Figure 6-6), and secondly there was no evidence of immunopotency (based on ELISpot assays) following the second dose of vaccine because all the responses were still declining following the first dose of MVA-NSmut.
5. Finally, it was evident from both the MVA-NSmut and ChAd3-NSmut data that there was significant inter-individual variation in gene expression following vaccination. The fact that there is such large transcriptional variation is consistent with the observed variation in reactogenicity and immunogenicity. Because the numbers in this study were small, it is possible that the observed differences in gene expression between the vectors reflects chance variation in transcriptional response. Should similar studies be conducted on future vaccine trials, it would be advisable to increase the sample size.

In this chapter (and in previous chapters) I used a whole blood gene expression profiling approach rather than examining gene expression in leukocyte subpopulations (including potentially examining populations of antigen-specific lymphocytes). Each approach has its advantages and disadvantages: Using whole blood has the advantage that samples can be collected into specialised tubes which are then frozen without the need for further

processing. Samples can subsequently be thawed and processed to extract RNA in batches, which is a cost efficient and time efficient strategy. It also has the advantage that gene expression can be halted immediately at the point when the sample was collected. This is important because gene expression can be greatly affected by the *ex-vivo* manipulation of cells, including the density centrifugation methods I used to separate PBMC.

Unfortunately this approach also has disadvantages: Firstly, with whole blood being a mixed population of cells it is difficult to detect transcripts that are present at low levels or that are expressed by less abundant cell types. This is a reflection of the high degree of signal noise that is created by cells which do not express the transcript of interest. Secondly, the gene expression signature obtained from whole blood may simply reflect the relative frequency of the different cell subtypes rather than reflecting a change to their gene expression activity.

However, there are methods that can be employed to mitigate against some of the disadvantages of a whole blood transcriptome approach: Firstly, in order to compensate for the differences in cellular composition between samples, full blood count differentials can be measured at the same time points that the RNA samples are collected. During the analysis these differentials can be introduced as co-variables. Unfortunately for the data presented here, the full blood counts differentials were not available. However, it is possible to perform a similar compensation using information from within the gene expression microarray data set. For each different cell type there are genes which are expressed constitutively. These genes can be arranged into modules, and from the relative expression of these modules an estimate can be made of the relative frequencies of cells within a mixed population. Therefore even without full blood count differentials, the frequencies of different cell subsets can be introduced as co-variables.

It was not possible to perform the necessary analyses to establish what host transcriptional responses were associated with either vaccine immunogenicity or reactogenicity. However there are important lessons that can be learnt from these data to help inform future study designs. Firstly, it is important to use larger sample sizes: I would want to use ten volunteers per group and would ideally like to have a replication set of data to validate potential results (i.e. 20 volunteers per vaccination episode). The FLU005 study is currently in progress and aims to recruit volunteer numbers of this order, so it would be anticipated that the transcriptomic data for the ChAdOx1 vector would be able to test these associations. The HCV003 trial is still ongoing, now with the ChAd3-NSmut1 vaccine replacing ChAd3-NSmut. If this study also wants to test these associations then similarly large group sizes will be required.

8. CONCLUDING REMARKS

This thesis explores the use of viral vectored vaccines for two important viral diseases, namely influenza and hepatitis C. While the two pathogens cause extremely diverse patterns of disease, I have described how they are linked immunologically by their evasion of the innate immune system, their subversion of protective antibody responses and the phenomena of immunodominance in T cell responses to their conserved epitopes. These themes are crucial to rational vaccine design. In addition to evaluating vaccine response using well established immunoassays, I have used RNA microarray technologies to further evaluate the host immune response to vaccination and infection at the transcriptional level.

The goal of protective immunity against pathogens through the generation of specific T cell responses has been pursued for many years. A range of technologies have been employed in addition to viral vectored vaccines but even the most advanced vaccine candidates remain in phase III clinical trials. For many infectious diseases it is children who are at most risk, hence these vaccine candidates must be immunogenic and efficacious in the younger population.

For influenza however, the elderly are the more relevant population and in Chapter 3 I show that influenza virus-specific memory T cells can be safely boosted in elderly individuals using the viral vectored vaccine MVA-NP+M1. I go on to show that these T cells are polyfunctional in respect of their ability to secrete cytokines. For traditional influenza vaccines it appears that response rates decline with age, perhaps from as early as 40 years old. This robust finding is one of the primary streams of evidence to support the notion that the functionality of the immune system declines with ageing (termed immunosenescence). My data shows that individuals aged 50-70 have equally good responses to vaccination with MVA-NP+M1 as

young volunteers. My study had no upper age limit for vaccination and I recruited individuals up to the age of 86. Although cancer vaccines have been given in the extremes of age, there have not to date been any reports of viral vectored vaccines against infectious diseases being given to such aged individuals.

Interestingly, I found that volunteers over 70 years old had lower immune responses to vaccination with MVA-NP+M1, despite having equivalent baseline levels of memory T cells. This observation may still be explained by immunosenescence. If the mechanisms which underlie impaired responsiveness to seasonal influenza vaccine are different to those that affect responsiveness to MVA-NP+M1 then it follows that the onset of these phenomena could occur at different decades of life. This explanation is plausible given the immunological differences between seasonal influenza vaccination and viral vectored vaccines such as MVA-NP+M1. The former involves the activation of naive B cells via the helper functions of T cells. The latter involves antigen recognition by memory T cells followed by lymphocyte proliferation. The immunological danger signals provided by a viral vectored vaccine (therefore activating the innate immune system) may be part of the reason that MVA-NP+M1 remains equally immune-potent up to the 7th decade of life.

In order to pursue this hypothesis, I next wanted to examine the innate immune signatures that accompany vaccination. These experiments, which were performed at a transcriptome wide expression level, formed part of my second investigative chapter (Chapter 4). However, I also wanted to establish whether by virtue of this or alternative mechanisms, the immuno-activatory properties of MVA-NP+M1 could be utilised to enhance immune responses to seasonal influenza vaccines. These experiments involved the co-administration of both

vaccines at the same injection site, and were the first time that such experiments had been performed in human subjects.

In Chapter 4 I demonstrated that the TIV:MVA-NP+M1 vaccine co-administration regimen was safe and well tolerated. I established that, unlike with other vaccine combination regimens, there was little suggestion of immune interference, with peak T-cell responses being similar to controls. I demonstrated that while MVA-NP+M1 is capable of enhancing immune responses to seasonal influenza vaccine, these increases appeared to be strain specific. The reasons for such strain specificity remain to be established but other studies have reported this phenomenon with other immunological adjuvants.

The experiments to explore innate immune signatures at the genome wide level did not show any significant differential expression when the full groups of data were compared. One explanation for this is that gene expression changes are greatest (and therefore easiest to detect in the setting of small sample sizes) during the first 24 hours following vaccination. This explanation for the lack of positive findings is supported by data from subsequent experiments that I performed in my final investigative chapter.

In Chapter 5, I used similar microarray experiments to explore the host response to infection with influenza virus - as opposed to vaccination with either live viral vectored or inactivated influenza vaccines. There was no convincing evidence that vaccination with MVA-NP+M1 modulated the host immune response to influenza virus. The most striking finding was that individuals who shed virus and who were moderately or severely symptomatic, represented a distinct biological phenotype. I was able to validate this finding by conducting a parallel

analysis of data obtained from a separate influenza virus challenge study. This information may prove helpful in performing future influenza virus challenge studies as it provides an unbiased and biologically meaningful outcome measurement. By identifying a panel of 6 genes which can account for the transcriptional variability between the two groups of interest, I have provided a method for this outcome to be measured in a low-cost and time efficient manner.

In Chapter 6 I explore the use of viral vectored vaccines against HCV using a heterologous prime-boost regimen in healthy adults. I show that MVA-NSmut cannot prime immune responses, but that it is extremely potent at boosting immune responses following prior vaccination with ChAd3-NSmut. Immune responses in these individuals were highly variable, and I was able to correlate the magnitude of immune responses with the breadth of the responses against different epitopes. Strong and broad immune responses are desirable outcomes as they are likely to correlate with protection. I therefore wanted to explore both the reasons for the variability of responses between individuals, and the reasons for the profound differences between different viral vectors expressing the same transgenic immunogen. I therefore went on to perform additional experiments which looked at whole-transcriptome gene expression following vaccination with both MVA-NSmut and ChAd3-NSmut.

In Chapter 7 I performed RNA microarray experiments on a cohort of healthy volunteers whom I vaccinated twice with ChAd3NSmut and twice with MVA-NSmut. The transcriptional signature was extremely strong following MVA-NSmut, but more subtle following ChAd3-NSmut. These signatures were short-lived, explaining the previous lack of positive findings in

Chapter 4. While there was 76% overlap in the identity of differentially expressed genes following administration of the two viral vectored vaccines, expression levels were generally lower following ChAd3-NSmut. However, as with immunogenicity, expression levels were also extremely heterogenous between individuals within vaccine groups.

Repeated vaccination with MVA-NSmut identified a core group of genes that were consistently differentially expressed. Using functional annotation software I have argued that these "common genes" more accurately describe the host transcriptional response to vaccination. Such findings highlight the value of including biological replicates in microarray experiments instead of solely performing technical replicates.

Variability within the group of vaccinees led to some analytical difficulties. With reactogenicity, the heterogeneity in the frequency and severity of adverse events meant that it was difficult to categorise the phenotype of the volunteers' responses. For example, how does one weight the relative importance of localised versus systemic side effects? With immunogenicity there was co-incidentally a number of volunteers who had numerically similar responses to vaccination. How then does one legitimately dichotomise the volunteers by response? One important lesson is that larger sample sizes are needed if such sub-group analyses are deemed to be important analytical outcomes.

Overall, this body of work has led to some important strands of evidence which have helped to progress vaccine development both for influenza viruses and for HCV: Plans are in place for the adjuvanting properties of MVA-NP+M1 (with regard to TIV) to be explored further. This Phase IIb study of 1,000 subjects (aged ≥65 years) will potentially start in 2015. Meanwhile

the potent ChAd3-NSmut prime followed by MVA-NSmut boost vaccine regimen is being evaluated in a phase II efficacy study being conducted in the United States (ClinicalTrials.gov Identifier: NCT01436357).

On a personal level this project has allowed me to develop skills that are not common amongst Clinical Immunologists in the United Kingdom, but which are highly relevant. Patients at the extreme end of variability in response to vaccination are being defined as having immunodeficiency. We need better tools to establish how these patients differ from 'normal'. If these patients do have genuine immunological defects, we need to establish whether these exist at the level of a single gene, a protein, cell or beyond. I hope that I will be able to apply my learning in the area of transcriptomics to clinical immunology and vaccinology.

APPENDICES

APPENDIX 1: List of differentially expressed genes for volunteers with moderate-severe laboratory confirmed influenza at 48 hours post-challenge. PubMed symbols are used and only the most significant genes are listed (n = 994). Genes described in this thesis are highlighted in bold and underlined.

ABCC5	ATF3	C6orf190	CD3D	CXorf21
ABCE1	ATF5	C6orf204	CD40LG	CXXC5
ABHD14A	ATF6	C6orf48	CD52	CYBB
ABI2	ATG3	C7orf23	CD68	CYBRD1
ABTB2	ATOX1	C9orf109	CD74	D4S234E
ACACB	ATPIF1	C9orf123	CD8B	DAPP1
ACAD11	AXUD1	C9orf127	CDC42EP2	DCUN1D3
ACOT9	B4GALT5	C9orf91	CDKN1A	DCUN1D4
ACPL2	BACH2	CA5B	CDKN1C	DDO
ACSL3	BATF2	CACNA1E	CEACAM1	**DDX58**
ACTA2	BATF3	CACNA1I	CEP78	DDX60
ADAP2	BAZ1A	CADM4	CERK	DDX60L
ADAR	BCKDHB	CALCOCO2	CHMP5	DENND1A
ADHFE1	BEND5	CAMK2D	CLC	DFFA
ADM	BIVM	CAPN12	CLDN23	DHRS9
AFF1	BLVRA	CARD14	CLEC2D	DHX58
AGL	BOLA1	CARD16	CLIC3	DIP2A
AHCTF1	BRSK1	CARD17	CLK1	DISC1
AIM2	BST2	CASP1	CLYBL	DKFZp761E198
AKR1C3	C10orf21	CASP5	CMPK2	DNAJC25
ANKFY1	C11orf1	CAST	CNDP2	DPEP2
ANKRD22	C11orf67	CBX7	CNOT6	DPEP3
ANKRD36	C11orf75	CBY1	CNPY4	DPH5
APEX1	C12orf29	CCDC102A	CPEB3	DRAP1
APOBEC3A	C12orf57	CCDC14	CREBZF	DSC1
APOBEC3F	C13orf27	CCDC66	CRIP2	DTX3L
APOBEC3G	C16orf42	CCDC72	CROCCL2	DUSP3
APOL1	C16orf7	CCDC76	CROP	DUSP5
APOL2	C16orf79	**CCL2**	CRTAP	DUSP6
APOL6	C17orf87	CCL8	CS	DYNLT1
AQP12A	C19orf66	CCR1	CSGALNACT1	DYSF
ARHGAP17	C1GALT1	CCRL2	CTLA4	EBI2
ARHGAP23	C1orf138	CD160	CTNNA1	ECGF1
ARSB	C1orf41	CD1C	CTSK	EEF1A1
ASCL2	C1orf59	CD2	CTSL1	EEF1AL7
ASGR2	C1QB	CD244	CTSZ	EEF1B2
ASNSD1	C3AR1	CD274	CUL1	EEF1D
ASPHD2	C5	CD2AP	CXCL10	EFHC2
ASPRV1	C6orf108	CD38	CXCL16	EFNA1

EIF2AK2	**GBP2**	HIF1A	**IRF1**	LGALS9
EIF2AK3	**GBP3**	HINT1	**IRF2**	LGALS9B
EIF3L	**GBP4**	HINT3	IRF7	LHFPL2
EIF4B	**GBP5**	HIST1H2BD	**IRF9**	LILRA5
EIF4G3	**GBP6**	HIST1H3D	IRS2	LILRB1
ELF1	GCH1	HIST1H4H	**ISG15**	LILRB4
ELF4	GIMAP4	HIST2H2AA3	ISG20	LIMK2
EMILIN2	GIMAP8	HIST2H2AA4	ITK	LMNB1
ENO2	GK	HIST2H2AB	ITM2A	LMO2
ENOSF1	GK5	HIST2H2AC	ITPK1	LOC100128060
EPAS1	GLOD4	HIST2H2BE	ITPRIP	LOC100128196
EPB41L3	GLS	HLTF	ITPRIPL2	LOC100128274
EPHB1	GM2A	HNRNPA1L2	JUP	LOC100128410
EPSTI1	GMCL1	HNRPA1L-2	KBTBD11	LOC100128771
ERN1	GNB4	HNRPA1P4	KCND1	LOC100128936
ETV6	GNG2	HNRPLL	KCNJ15	LOC100129067
ETV7	GNLY	HOPX	KIAA0040	LOC100129237
FAM101B	GNPTAB	HPSE	KIAA0082	LOC100129334
FAM111A	GNS	HSH2D	KIAA0114	LOC100129553
FAM134B	GOLGA6B	HSPA1B	KIAA0226	LOC100129657
FAM190B	GOLGA8A	ID3	KIAA0319L	LOC100129668
FAM26F	GOLGA8B	IDO1	KIAA0895L	LOC100129681
FAM46A	GORASP1	IER2	KIAA1370	LOC100129742
FAM8A1	GPAM	IFI16	KIAA1618	LOC100130070
FAR2	GPBAR1	IFI30	KIAA1712	LOC100130553
FAS	GPD2	IFI35	KIAA1958	LOC100130624
FBXO6	GPIHBP1	IFI44	KIF27	LOC100130775
FCER1A	GPR141	IFI44L	KLF12	LOC100130828
FCGBP	GPR183	**IFI6**	KLF5	LOC100130892
FCGR1A	GPR56	**IFIH1**	KLHDC2	LOC100131196
FCGR1B	GPR84	**IFIT1**	KLHL3	LOC100131526
FCGR1C	GPX7	**IFIT2**	KLRB1	LOC100131572
FCGR2B	GRAMD1B	**IFIT3**	KLRC2	LOC100131662
FER1L3	GRAMD1C	IFIT5	KLRD1	LOC100131787
FFAR2	GRIN3A	IFITM1	KLRF1	LOC100131905
FFAR3	GRN	**IFITM3**	KPNB1	LOC100132291
FGD2	GSDMD	IFITM4P	KREMEN2	LOC100132488
FGFBP2	GSTM2	IGF2BP3	KRT72	LOC100132499
FLJ43681	GTPBP2	IL15	LACTB	LOC100132516
FRMD3	GZMA	IL1B	**LAMP3**	LOC100132547
FTSJD2	H2AFJ	IL1RN	LAP3	LOC100132673
FYB	HDGF2	IL27	LARP1	LOC100132742
GADD45B	**HERC5**	IL7R	LBA1	LOC100132804
GALM	HERC6	IL8	LDHB	LOC100133045
GAS6	HES4	IL8RBP	LDLR	LOC100133185
GBA	HESX1	INDO	LGALS3BP	LOC100133329
GBP1	HHEX	INPP5A	LGALS8	LOC100133812

LOC100133923	LOC401206	LOC646527	LOC727984	MAFB
LOC100133931	LOC401537	LOC646672	LOC728031	MARCO
LOC100134504	LOC401676	LOC646688	LOC728060	MASTL
LOC130773	LOC401717	LOC646766	LOC728093	MATK
LOC144383	LOC401817	LOC646784	LOC728126	MBNL2
LOC153561	LOC402057	LOC646785	LOC728207	MDK
LOC158345	LOC402251	LOC646791	LOC728216	MEF2D
LOC202134	LOC402677	LOC646793	LOC728484	MEFV
LOC255783	LOC440093	LOC646909	LOC728553	MEGF6
LOC284230	LOC440396	LOC646949	LOC728590	MGC4677
LOC284672	LOC440595	LOC646966	LOC728643	MICB
LOC284837	LOC440731	LOC647030	LOC728658	MIR21
LOC285296	LOC441013	LOC647135	LOC728672	MLKL
LOC285741	LOC441019	LOC647276	LOC728744	MLLT11
LOC285900	LOC441073	LOC647436	LOC728772	MOAP1
LOC286444	LOC441246	LOC647673	LOC728843	MOBKL2C
LOC339192	LOC441506	LOC647856	LOC728877	MOV10
LOC346950	LOC442232	LOC648000	LOC728973	MR1
LOC387825	LOC641814	LOC648099	LOC729102	MRFAP1L1
LOC387867	LOC641848	LOC648249	LOC729196	MRPL44
LOC387882	LOC641849	LOC648343	LOC729301	MRPS21
LOC387930	LOC641922	LOC648366	LOC729340	MSRB2
LOC388122	LOC642250	LOC648622	LOC729342	MT1A
LOC388339	LOC642741	LOC648659	LOC729409	MT1E
LOC388654	LOC642808	LOC649049	LOC729423	**MT1G**
LOC388707	LOC642989	LOC649076	LOC729646	MT2A
LOC388720	LOC643007	LOC649150	LOC729686	MTE
LOC389141	LOC643308	LOC649214	LOC729798	MTF1
LOC389223	LOC643358	LOC649839	LOC729839	MTHFD2
LOC389342	LOC643384	LOC650276	LOC730004	MUC1
LOC389386	LOC643779	LOC651149	LOC730029	**MX1**
LOC389404	LOC643863	LOC651436	LOC730167	**MX2**
LOC389672	LOC643870	LOC651655	LOC730187	MXD4
LOC390183	LOC643873	LOC651816	LOC730255	MYB
LOC390578	LOC644315	LOC652287	LOC730704	MYBL1
LOC391126	LOC644464	LOC652616	LOC730754	MYD88
LOC391655	LOC644511	LOC652726	LOC731049	MYL5
LOC391769	LOC644907	LOC653162	LOC732371	MYOF
LOC391825	LOC644937	LOC653610	LOC91561	NADK
LOC391833	LOC645231	LOC653658	LOC93622	NAE1
LOC399804	LOC645296	LOC653702	LPIN2	NAGK
LOC400027	LOC645387	LOC653773	LRRN3	NAP1L1
LOC400304	LOC645436	LOC653881	LY6E	NAPA
LOC400455	LOC645693	LOC654194	LYRM1	NARG1L
LOC400657	LOC645968	LOC654350	LYSMD2	NAT6
LOC400759	LOC646200	LOC727865	LZTFL1	NBN
LOC400986	LOC646483	LOC727970	MACF1	NCALD

NCF1	PDCL3	PTPN4	RPS20	SKAP1
NCF1B	PDE3B	PYHIN1	RPS21	SLAMF8
NCF1C	PDZD4	RAB24	RPS24	SLC12A2
NCOA7	PEBP1	RAB8A	RPS25	SLC16A3
NCRNA00219	PECR	RABEP1	RPS27	SLC22A4
NELL2	PGM2L1	RANGAP1	RPS27A	SLC25A28
NEXN	PHACTR2	RARRES3	RPS3A	SLC26A11
NFIL3	PHF11	RASGRP3	RPS4X	SLC26A8
NFKBIE	PI4K2B	RASSF1	RPS5	SLC27A3
NLRC5	PIK3AP1	RASSF5	RPS6	SLC37A1
NME3	PIK3IP1	RBCK1	RPS6P1	SLC4A7
NOV	PITPNC1	RBM43	RPS8	SLC6A12
NPC2	PKIA	RBMS2	RPSA	SLC6A13
NR3C2	PLAC8	RCN2	RRBP1	SMAD5
NSMCE2	PLAUR	RDH10	RSAD2	SMCHD1
NSUN5B	PLEKHA1	RGL1	RSL24D1	SMYD4
NT5C2	PLEKHO2	RHBDF2	RSPH9	SNHG5
NT5C3	PLSCR1	RHOBTB3	**RTP4**	SNHG6
NT5E	PLXDC2	RIN2	RUNX2	SNHG7
NTNG2	**PML**	RIOK2	S1PR5	SNORA43
NUB1	PNPT1	RIPK2	SACS	SNTB1
NUCB1	POL3S	RNASE6	SAMD3	**SOCS1**
OAS1	POLR1E	RNF213	SAMD4A	SORT1
OAS2	PPAT	RORA	SAMD9	SP100
OAS3	PPIL3	RPAIN	SAMD9L	SP110
OASL	PPM1H	RPGR	SAT1	SP140
OCIAD2	PPM1K	RPL10A	SCARB2	SPAG7
ODF3B	PPP1R2	RPL13A	SCO2	SPATS2L
OGT	PPP2R2B	RPL15	SEC24D	SPTLC2
OR52K2	PRIC285	RPL17	SECTM1	SQRDL
ORM1	PRKAG2	RPL22	SELL	SRBD1
OSM	PRKCH	RPL23	**SEPT4**	SRC
OTOF	PRKD2	RPL23AP13	SERF1B	SRGAP2
P2RX7	PRKRIR	RPL24	SERPINB1	SRGAP2L
P2RY10	PRR11	RPL26	SERPING1	ST3GAL5
P2RY14	PRRG4	RPL27	SESN1	STARD8
P2RY6	PSG3	RPL3	SFI1	**STAT1**
PANK2	PSIP1	RPL31	SFXN5	**STAT2**
PARP10	PSMB10	RPL35	SGK	STAT3
PARP11	PSMB8	RPL4	SGK1	STAT4
PARP12	PSMB9	RPL5	SH2D1A	STOM
PARP14	PSME1	RPL6	SH3YL1	STX11
PARP9	PSME2	RPL7	SHISA5	STX2
PASK	PSTPIP2	RPL9	SIDT2	STXBP5
PATL1	PTGDR	RPLP0	SIGLEC1	SYTL1
PBX4	PTP4A1	RPS17	SIGLEC14	**TAP1**
PCMTD2	PTPN22	RPS18	SIGLEC16	**TAP2**

TARP	TRAT1	WARS
TBC1D26	TRIM14	WDFY1
TBC1D4	TRIM21	WSB1
TBC1D8	TRIM22	WSB2
TC2N	TRIM26	XAF1
TCEA3	TRIM38	XRN1
TCEAL8	TRIM5	ZBED5
TCN2	TRIM56	ZBP1
TDRD7	TRIM6	ZC3HAV1
TFEC	TRIM78P	ZCCHC17
TIGA1	TSEN54	ZCCHC2
TIMM10	TSPAN3	ZFP82
TINP1	TSTD1	ZFYVE26
TLR5	TTC13	ZMYND11
TLR7	TTC21A	ZNF200
TMEM110	TTC26	ZNF211
TMEM123	TUBE1	ZNF232
TMEM131	TXK	ZNF256
TMEM140	TXNDC12	ZNF30
TMEM181	TYMP	ZNF337
TMEM194A	TYW3	ZNF438
TMEM229B	**UBA7**	ZNF439
TNFAIP6	UBE1DC1	ZNF529
TNFRSF25	**UBE2L6**	ZNF540
TNFSF10	UBQLNL	ZNF589
TNFSF13B	**UNC93B1**	ZNF684
TOMM7	**USP18**	ZNF83
TOP2B	USP25	ZNF831
TOR1B	USP41	ZNF84
TP53INP1	VAMP1	ZNFX1
TPT1	VAMP5	ZNHIT6
TRAFD1		

APPENDIX 2: List of 92 unique genes differentially expressed following vaccination with ChAd3-NSmut. Those genes which were also differentially expressed following vaccination with MVA-NSmut are shown in bold and underlined.

ACOT9	FUT4	**MUTYH**	**SLAMF8**
ADAP2	**GBP2**	**NECAB2**	SLC36A3
AFF1	GBP3	**NUB1**	**SP140**
ATF5	**GBP4**	**OAS1**	**SPPL2A**
BLVRA	**GCH1**	**OAS2**	**STAT1**
C11orf75	HIATL1	**ODF3B**	**STAT2**
C17orf87	**HLA-DMB**	PARP11	**TAP1**
C18orf25	**IFI44**	**PARP12**	TBK1
C9orf91	**IFIT5**	**PARP14**	**TDRD7**
CASP1	**IL15**	**PARP9**	TFEC
CHMP5	IQCA1	PRNP	**TICAM2**
CTSL1	**IRF9**	PSMA3	**TIMM10**
DDX58	**JAK2**	**PSMB9**	**TLR7**
DDX60	**KPTN**	**PSME2**	TMEM123
DHX58	**KYNU**	**RAB24**	TMX1
DMXL2	**LACTB**	**RHBDF2**	**TOR1B**
ECGF1	**LOC100129697**	RPS6KC1	**TRIM22**
ETNK1	**LOC389386**	**RTP4**	**TYMP**
FBXO6	LOC642373	**SAMD4A**	**UBE2L6**
FER1L3	LOC728877	**SAMD9**	**UNC93B1**
FLJ23152	MED28	**SCO2**	**WARS**
FLJ35816	**MICB**	**SESTD1**	**WDFY1**
FTSJD2	**MTHFD2**	SIGLEC16	**XAF1**

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