OPTIMISING T CELL VACCINATION STRATEGIES FOR THE PREVENTION OF HCV INFECTION

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CONTRIBUTIONS BY OTHERS

Chapter 4: HCV003 trial

I helped with the blood processing and to run some of the HCV ELISpot assays. The majority of the blood process and ELISpot work of the HCV samples was performed by Anthony Brown (Peter Medawar Lab).

The chapter refers to work previously done in the trial (groups A1-A2) – this work was completed by Leo Swadling and Anthony Brown.

Chapter 5: PEACHI-04 trial

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Chapter 7: HCV CD4 phenotyping

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ABSTRACT

Optimising T cell vaccination strategies for the prevention of HCV infection

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There remains an unmet global need to develop a vaccine for the prevention of HCV infection. Adenoviral and MVA vectors have been established as promising tools in the development of a prophylactic HCV vaccine strategy. A Phase I trial in humans has demonstrated the induction of broad, robust and polyfunctional HCV specific T cells following prime-boost vaccination with heterologous AdCh3/MVA-NS encoding the HCV non-structural (NS) region. An important next step therefore is to optimise strategies in which viral vectored HCV vaccines are administered. Furthermore, with the emergence of highly efficacious yet prohibitively costly directly acting antiviral therapy to treat HCV infection, an imperative question is whether individuals cured of chronic HCV infection may be amenable to vaccination to prevent re-infection.

The first section of this thesis assesses re-boosting strategies of AdCh3/MVA-NS following initial prime-boost in healthy adult volunteers. The primary aim was to establish whether either the peak magnitude and/or the memory 'set-point' of the HCV specific T cell response could be improved. Re-vaccination with AdCh3/MVA-NS could be demonstrated to re-boost the antigen specific T cell response, however this was more effective following a longer interval between primary and secondary vaccination rounds.

Next, in a separate phase I study, I sought to establish whether the co-administration of distinct adenoviral vectors encoding both HIV-1 and HCV immunogens could be safely administered without compromising immunogenicity of either vector. Co-administration was shown to be safe and did not compromise the immunogenicity of either immunogen.

Thirdly, I addressed whether the reversal of T cell exhaustion is possible following DAA mediated HCV cure. A functional analysis of HCV specific T cells prior to commencing and following treatment was performed. Little recovery of proliferation or cytokine production was observed, furthermore T cell responses were significantly attenuated in comparison to spontaneous viral resolution and vaccine induced T cell responses.

Finally, I performed a detailed *ex vivo* phenotypic analysis of HCV specific CD4+ T cells induced by vaccination and compared them with spontaneous viral resolution. Phenotypic markers suggestive of highly functional effector and memory CD4+ T cells were observed.

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ABBREVIATIONS

Ad	Adenovirus
AE	Adverse reaction
ALT	Alanine aminotransferase
APC	Antigen presenting cell
CBF	Clinical BioManufacturing Facility
CMV	Human cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTL	Cytotoxic T lymphocyte
DAA	Directly acting antiviral
DMSO	Dimethyl sulfoxide
DSMC	Data safety monitoring committee
DSUR	development safety update report
EBV	Epstein Barr Virus
ELISpot	Enzyme-linked immunospot assay
Eomes	Eomesodermin
EOT	End of trial
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FMO	Fluorescence minus one
Foxp3	Forkhead box protein 3
GCP	Good clinical practice
GGT	Gamma-glutamyl transpeptidate
GMP	Good manufacturing practice
GWAS	Genome wide association tudies
HAART	Highly active antiretroviral therapy
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus
HLA	Human leucocyte antigen
HVR	Hypervariable region
i.m.	Intramuscular
ICS	Intracellular cytokine staining
IDT	Impfstoffwerk Dessau-Tornau
IFN	Interferon
Ii	Human invariant chain
IL-2	Interleukin 2
ISG	Interferon-stimulated genes
IVDU	Intravenous drug use
LTNP	long-term non-progressor (HIV)
MIP	macrophage inflammatory protein-1beta

MFI	geometric mean fluorescence intensity
MHC	Major Histocompatibility Complex
MHRA	Medicines and Healthcare products Regulation Agency
MMR	Measles mumps rubella vaccine
MSM	Men who have sex with men
MVA	Modified vaccinia Ankara
NHP	Non-human primate
NK	Natural killer (cells)
NS	Non structural
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Peg-IFN	Peglyated interferon
PMA	Phorbol 12-myristate 13-acetate
Riba	Oral ribavirin therapy
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSV	Respiratory syncytial virus
SAE	Serious adverse event
SD	Standard deviation
SFU	Spot forming unit
SNP	Single nucleotide polymorphism
SUSAR	Suspected unexpected serious adverse reaction
SVR	Sustained virological responses
Tbet	T-box transcription factor TBX21
TF	Transcription factor
Th_1	T helper 1 cells
TNF	Tumour necrosis factor
Treg	T regulatory cells
TW	Trial week / treatment week

CHAPTER 1: INTRODUCTION

1.1 HEPATITIS C VIRAL INFECTION

1.1.1 GLOBAL BURDEN OF HCV INFECTION

Hepatitis C virus (HCV) infects approximately 180 million people worldwide, with an estimated 3-4 million new cases occurring annually (Muhlberger et al. 2009). In many patients, infection results in chronicity and may ultimately result in decompensated liver cirrhosis and hepatocellular carcinoma (HCC), causing HCV and HCV related complications to be a primary indication for liver transplantation in the western world (Davis et al. 2003). HCV prevalence has a geographic and economic distribution. Rates are reported to be the highest in less economically developed countries, with the highest rates reported in North Africa/Middle East (rate >15 million) and East Asia (>50 million), with a population prevalence of >3.5% (Mohd Hanafiah et al. 2013). On the other hand, HCV prevalence is lower in economically developed countries, estimated between 0.5-1% in Western Europe and Australia (Shepard, Finelli, and Alter 2005). However, despite these estimates the true global prevalence is challenging to accurately determine, and most statistics are based on HCV seroprevalence studies in select populations, for example blood donors and patients with disease complications (Shepard, Finelli, and Alter 2005).

HCV exists as 7 distinct genotypes and 67 subtypes (Smith et al. 2014). The genotypes vary in terms of their global prevalence and distribution. Genotype 1 has by far the highest global prevalence (46.2%), and is most common in Latin America, Australasia, Central

Europe and the Caribbean (Messina et al. 2015). Genotype 3 is the next most common genotype (30.1% of global infections), and is most prevalent in South Asia. Genotype 2, 4 and 6 account for the majority of the remaining (Messina et al. 2015).

1.1.2 RISK FACTORS AND TRANSMISSION

Transmission of HCV occurs through exposure to contaminated blood and blood products. A major source of infection during the 1970's and 1980's was via blood and blood product transfusions. Originally described as 'non A non B transfusion associated hepatitis' (Feinstone et al. 1975), transmission of the virus was reported to be up to 39% in a case series of 66 transfusion recipients undergoing heart transplant in the 1060's (Engle et al. 2014). With the development of a successful screening tool in 1990, transmission through this route dropped to virtually zero in the developed world (Alter and Houghton 2000). However, transmission with blood products still remains a significant source of transmission in the developing world, particularly Africa and parts of Asia, where universal blood donor screening has not been as successfully implemented. The other major source of transmission in the developing world is from reusing contaminated injecting equipment without proper sterilisation, resulting in an estimated 2.3 to 4.7 million cases of HCV yearly (Kane et al. 1999). In the developed world the major source of transmission is through the sharing of contaminated needles and drug preparation equipment in the intravenous injecting drug using (IVDU) population, which serves as a reservoir for ongoing re-exposure and re-infection (Cox and Thomas 2013). Additional risk-factors in this group include incarceration, male gender and increased duration of injecting drug use (Lamden et al. 1998). Other possible sources of infection in both the developed and developing worlds include unsafe injecting practices such as tattooing, acupuncture and body piercing,

however there is insufficient data to quantify the overall contribution of these modes of transmission (Shepard, Finelli, and Alter 2005). Sexual transmission has traditionally been thought to not be a significant mode of transmission, accounting for approximately 0.6% of cases (Terrault et al. 2013). In recent years however, there has been a significant increase in transmission between HIV-positive non IVDU men who have sex with men (MSM's) as a result of higher risk sexual practices (Schmidt et al. 2011). Vertical transmission remains a rare mode of transmission (Rehermann and Nascimbeni 2005).

1.1.3 CLINICAL COURSE OF HCV INFECTION

Acute HCV infection is largely asymptomatic and therefore usually goes undetected (Lauer and Walker 2001). Symptoms when present are usually non-specific, including malaise, jaundice and anorexia. Fulminant hepatitis is extremely rare, although has been reported (Farci et al. 1996). Following acute infection, a minority of individuals will spontaneously clear the virus, however approximately 80% will develop chronic infection, from which spontaneous resolution is rare (Lauer and Walker 2001). The main complications of chronic HCV infection include chronic hepatic inflammation, fibrosis, cirrhosis and ultimately liver failure (Lauer and Walker 2001). The duration in which these complications occur is highly variable, and some patients have no progression of liver disease at all (Poynard, Bedossa, and Opolon 1997). However, once cirrhosis is established and the compensatory mechanisms of the liver are overwhelmed, manifestations of decompensated liver disease become evident, including portal hypertension, ascites, variceal bleeding, hepatic encephalopathy and splenomegaly (Gomez et al. 2014). The progression to liver failure is influenced by additional factors such as heavy alcohol consumption, male gender, increasing age and co-infection with HBV or HIV-1 (Poynard,

Bedossa, and Opolon 1997; Zarski et al. 1998). Once cirrhosis is established, the annual risk of developing HCC is approximately 1-4% (Tsukuma et al. 1993). Complications arising from chronic HCV infection are the most common indications for liver transplantation in the Western world (Wise et al. 2008).

In those individuals that spontaneously clear the virus and are then re-infected, both a lower peak viral load and higher re-clearance rates have been observed (Sacks-Davis et al. 2015), an observation that has also been demonstrated in chimpanzee studies (Bassett et al. 2001). Factors associated with reclearance after reinfection include female sex and IFNλ4 C/C genotype (Sacks-Davis et al. 2015) (discussed below). Additionally some cohorts of patients have been able to spontaneously clear subsequent HCV infection of a different genotype compared with initial infection (Osburn et al. 2010), suggesting the development of cross-reactive immunity. These studies indicate individuals able to spontaneously clear the virus may develop protective but not necessarily sterilising immunity to the virus.

Finally, there is a small minority of patients who have apparent resistance to HCV. These individuals, largely studied in the injecting drug user cohort, remain aviremic and seronegative despite repeated exposure to HCV through sharing of injecting equipment. Possible mechanisms for this and distinction from early spontaneous resolution will be discussed later in the chapter.

1.1.4 DIAGNOSTIC AND LABORATORY MARKERS

Diagnosis of HCV infection can be made by both serological testing and detection of HCV RNA. HCV antibody testing is used as a screening tool. Diagnosis is made on detection of anti-HCV antibodies against both structural and non-structural proteins, and current

methods carry a sensitivity and specificity of over 99% as early as 4-6 weeks post exposure (Lee et al. 1995). A positive HCV antibody result requires further molecular testing to be performed to detect active HCV infection. HCV antibodies are thought to gradually decrease over time, but may remain in the circulation for up to 18-20 years post infection (Takaki et al. 2000).

Active infection is determined by the presence of HCV RNA viral particles in quantitative PCR testing (to determine viral load), which appear 1-3 weeks after exposure (Chevaliez and Pawlotsky 2008). Additionally sequencing is performed to determine genotype and possible resistance mutations, which can be used to guide initial treatment and subsequent treatment after failure respectively. HCV RNA testing is also useful for immunocompromised patients or infants born to infected mothers, where false negative antibody testing may occur (Firdaus et al. 2015).

Other non-specific markers of liver inflammation such as alanine aminotransferase (ALT), gamma-glutamyl transpeptidate (GGT) and bilirubin can also be elevated in both acute and chronic infection – often in the absence of symptoms – and can be used to aid diagnosis, monitor disease activity and evaluate response to treatment. However, liver enzymes are variable, can often fluctuate and are non-specific, and therefore should not be relied solely upon (Fabris et al. 2008).

1.1.5 HCV: THE VIRUS

HCV is a hepacivirus; one of three genera of the *Flavivirdiae* family. The other members of this family include yellow fever, dengue fever virus, Japanese encephalitis and Tick-borne encephalitis (Chevaliez and Pawlotsky 2006), which all share a number of structural and

virological characteristics. HCV is a positive-strand RNA virus composed of 9.6kb nucleotides. The virus is contained in a lipid bilayer where two envelope proteins are anchored, and contained within this envelope is the nucleocapsid, housing multiple copies of the core protein and the RNA genome. HCV has an open reading frame (ORF) encoding a polyprotein 3011 amino acids long (Reed and Rice 2000). Following viral replication, the polyprotein is translated and cleaved into 3 structural (core, envelope 1 (E1) and envelope 2 (E2) and 7 non-structural (NS) proteins (p7, NS2, NS3, NS4A/B, NS5A/B) (**Figure 1-1)**.

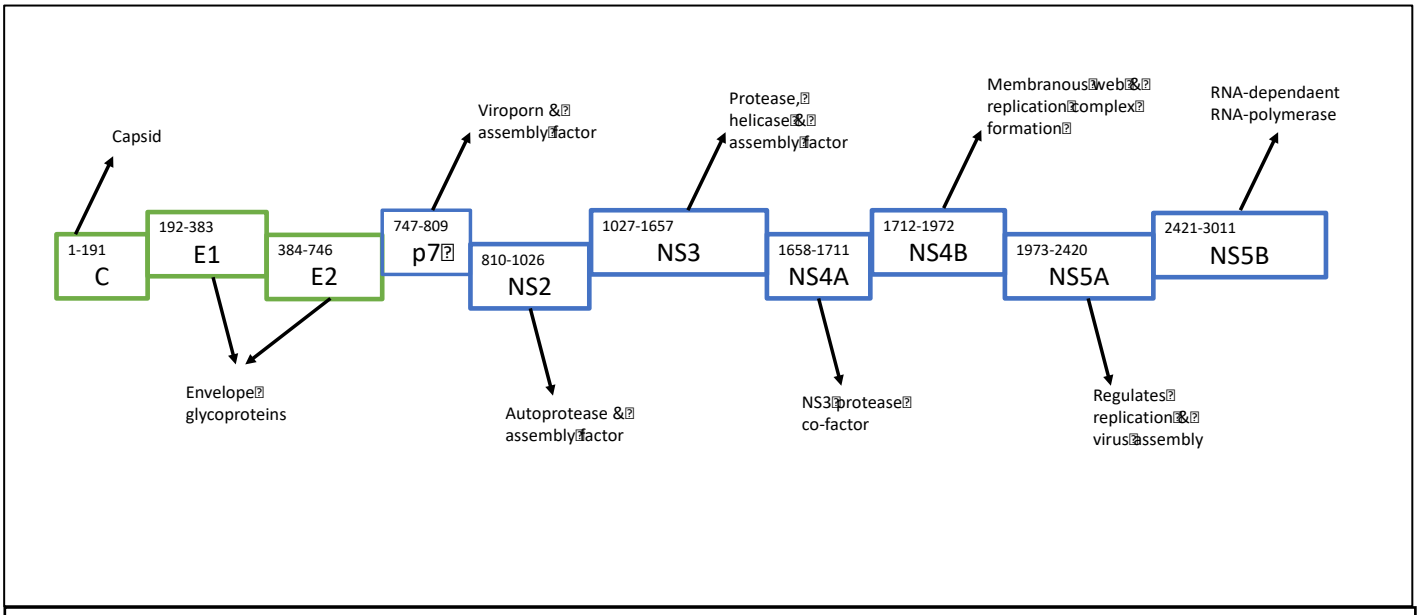


Figure 2-1: HCV polyprotein. The HCV genome is translated into a single polyprotein containing 3 structural (core, E1 and E2) [green boxes] and 7 non-structural (NS2, NS3, NS4A/B, NS5A/B) proteins [blue boxes]. E2 contains the binding site for cell entry, and also has the highest genetic variability. NS2, NS3 and NS4A function as viral proteases and assembly factors. NS5B functions as RNA polymerase. The lack of proof-reading function of NS5B results in the high mutation rate and genetic diversity of the virus**.**

The core protein forms the viral capsid (Yasui et al. 1998) and may interact with certain cellular proteins during the HCV lifecycle (McLauchlan 2000). The envelope proteins E1 and E2 are important for facilitating viral fusion and entry into the hepatocyte (Bartosch,

Dubuisson, and Cosset 2003). The non-structural proteins are made up of p7, NS2, NS3, NS4A/B and NS5A/B. p7 is thought to play a role in viral particle assembly, although its full function has not been elucidated (Saint et al. 2009). Nonetheless it is believed to be essential, as deletions or mutations result in suppression of infectivity in chimpanzee studies (Sakai et al. 2003). NS2-NS4A proteins function as viral proteases and assembly factors. NS5A is thought to play a role in regulating replication and viral assembly, and NS5B acts as an RNA-dependant RNA polymerase required for viral replication. This RNA polymerase lacks a proof-reading function, contributing to the huge mutation rate and genetic diversity of the virus (Steinhauer, Domingo, and Holland 1992).

The virus initially attaches to the hepatocyte via interactions between apolipoprotein E and cell surface heparin sulfate proteoglycans (Jiang et al. 2012) or the Scavenger Receptor class B type I (SR-B1) (Dao Thi et al. 2012). This attachment allows the E1 and E2 envelope glycoproteins to gain hepatocyte entry in which a complex interaction between a number of cell surface proteins are involved. The predominant entry receptors include SR-B1 (Scarselli et al. 2002), CD81 (Pileri et al. 1998; Evans et al. 2007), the tight junction proteins claudin-1 (CLDN1) (25) (Evans et al. 2007) and Occludin (OCLN) (Ploss et al. 2009).

Following viral attachment to the hepatocyte via a number of identified receptors including LDL receptor, CD81, SRb1 and Claudin (Flint, Quinn, and Levy 2001), the virion envelope fuses with the cell membrane and the viral nucleocapsid is released into the cytoplasm. Translation from the viral genome into the polyprotein occurs, which is then cleaved into structural and non-structural proteins. Replication (via the viral replication complex) and formation into virions also occur in the cytoplasm, and finally mature virions are exocytosed into the extracellular space (Chevaliez and Pawlotsky 2006).

As discussed above, HCV has a high mutation rate, with an estimated *in vivo* rate of about 2.5×10^{-5} per nucleotide per genome replication (Ribeiro et al. 2012). The regions required for essential functions, such viral translation and replication are the best conserved regions (Bukh, Purcell, and Miller 1992). E1 and E2 are the most variable regions of the virus. E2 contains the hypervariable regions (HVR 1 and 2), the sequence of which may differ by more than 50% between individuals (Smith 1999). This variability is thought to be a consequence of the HVR region being the main target of neutralising antibodies and subsequent development of escape variants. As well as the array of genotypes and subtypes discussed previously, the high mutation rate of HCV results in a number of similar but genetically distinct viral sequences existing within the same host, known as 'quasi-species' (Enomoto et al. 1994). The genetic diversity both between genotypes and within the same host represents one of the challenges to the development of a prophylactic vaccination for the prevention of HCV infection.

HCV and HIV-1 coinfection

There is a significant global burden of HCV and human immunodeficiency virus (HIV-1) co-infection, carrying a number of clinical and economic consequences. There are an estimated 2.3 million cases of HCV co-infection in HIV-1 positive individuals globally, with the highest rates of co-infection occurring in Eastern Europe and Central Asia, accounting for nearly 30% of the global burden of coinfected individuals (Platt et al. 2016). The largest risk factor for coinfection is IVDU, followed by MSMs. The increased risk of transmission

in the MSM population is a growing trend as a result of changing social practices such as increased sexual and drug-injecting risk behaviours, use of psychoactive substances and serosorting within these groups (van der Helm et al. 2011; Wandeler et al. 2012).

Coinfection has a significant impact particularly on the course and outcome of HCV infection. HCV is transmitted more efficiently in persons co-infected with HIV-1, and the vertical transmission rate is suggested to double with mothers infected with HIV-1 (Sulkowski 2008). There are lower rates of spontaneous clearance of HCV in the setting of HIV treated with antiretroviral therapy (Frias et al. 2017). Furthermore, HCV/HIV-1 coinfection leads to faster rates of (Benhamou et al. 1999) and more extensive (Soto et al. 1997) liver fibrosis, decompensated liver disease and death compared with either infection alone (Hernando et al. 2012; Macias et al. 2009). The mechanisms for this are likely multifactorial. Expression of pro-fibrotic cytokine TGF-β1 is upregulated in the presence of both HCV proteins (core and NS) (Presser, Haskett, and Waris 2011) and HIV (envelope protein gp120) (Lin et al. 2008), resulting in activation of hepatic stellate cells and production of inflammatory cytokines with subsequent liver fibrosis (Hernandez-Gea and Friedman 2011). HIV and HCV also increase the production of reactive oxygen species in hepatic stellate cells, further accelerating liver fibrosis (Lin et al. 2011). Increased rates of hepatocyte apoptosis in the setting of coinfection has been proposed to increase liver fibrosis. Furthermore, increased microbial translocation from lymphoid depletion in the gut (in the setting of HIV-1) causing increased levels of lipopolysaccharide – a component of bacterial cell wall – and subsequent liver fibrosis has been thought to play a role (Brenchley et al. 2006; Sandler et al. 2011). A final factor resulting in hepatotoxicity in the setting of coinfection is the development of metabolic disease and insulin resistance

causing non-alcoholic fatty liver disease (NAFLD) as a consequence of some antiretrovirals (Lemoine, Serfaty, and Capeau 2012).

No licensed vaccine is currently available for either HCV or HIV-1, largely due to the significant challenges they present in inducing a protective immune response. They share a number of similarities immunologically, both stimulating a predominantly cell mediated immune response. There is a significant economic burden on healthcare systems treating co-infected patients, particularly with newly available directly acting antiviral drugs (DAA's).

For these reasons, a vaccination strategy aimed at prevention of both viruses is an attractive goal. This will be addressed in greater detail in Chapter 5.

1.2 THE IMMUNE RESPONSE TO HCV INFECTION

1.2.1 THE INNATE RESPONSE

The innate immune response to any invading pathogen involves the recruitment of non-specific immune cells including neutrophils, macrophages, dendritic cells and natural killer (NK) cells during the early stages of infection, and aims to control the infection prior to induction of the antigen specific adaptive immune response.

NK cells, defined by the presence of cell surface marker CD56 and absence of CD3, are enriched in the liver (first described as 'pit cells') (Wisse et al. 1976), and have been observed to be increased in acute HCV infection, with a progressive decline after 1-3 months in spontaneous viral resolution (Amadei et al. 2010). Another study found NK activity to be increased during acute HCV infection, and that this was correlated with T cell activity, implying potential coordination between these two subsets (Pelletier et al. 2010).

Interestingly CXCL10, a chemokine produced by liver homing lymphocytes and hepatocytes during HCV infection to attract T cells and NK cells to the site of infection, is associated with the development of chronic HCV infection (Grebely et al. 2013). Additionally, higher levels of this chemokine is a negative predictor of response to pegylated interferon (peg-IFN) (Romero et al. 2006). The proposed mechanism for this is that higher levels of a truncated form of CXCL10 are observed in those that develop viral persistence, and this truncated form binds CXCR3 on T cells and NK cells but does not induce cell signalling, resulting in a negatively antagonistic effect (Riva et al. 2014)

The innate response to HCV is largely mediated by the interferon (IFN) family of cytokines, consisting of interferons type I-III. Type I IFNs (IFNα and IFNβ) and Type III IFNs (IFNλ1 [IL29], IFNλ2 [IL28A] and IFNλ3 [IL28B]) are produced by virally infected cells, macrophages and dendritic cells. IFNα is also given exogenously to treat chronic HCV infection. Type II IFN (IFNγ) is produced by NK cells in the innate response, and by antigen specific CD4+ and CD8+ T cells in the adaptive response (Heim and Thimme 2014).

Following receptor binding, all IFNs induce a number of signals from the cell surface to the nucleus via the JAK/STAT pathway, ultimately resulting in the upregulation of a large number of genes – termed interferon-stimulated genes (ISGs) – to promote an antiviral state. Chimpanzee experiments have demonstrated hepatic ISG's to be upregulated very early following infection, with a positive correlation observed between viral load and the extent and duration of ISG induction (Su et al. 2002). However, it is suggested the induction of ISG's alone is insufficient in clearing the virus, as both a high viral load and high ISG expression are observed in infected cells (Heim and Thimme 2014). It has also

been hypothesised that chronic ISG induction, as occurring in chronic HCV infection, may result in lower efficacy of exogenous IFNα therapy (Sarasin-Filipowicz et al. 2008).

There is evidence that host genetics play an important role in the innate response to HCV infection. A number of genome wide association studies (GWAS) (Duggal et al. 2013; Rauch et al. 2010; Tanaka et al. 2009; Ge et al. 2009; Thomas et al. 2009) observed a strong association between single-nucleotide polymorphisms (SNPs) in the IL-28B locus (encoding IFN-λ3) and HCV treatment response and disease outcomes, with certain haplotypes correlated with higher spontaneous viral clearance and better responses to IFNα therapy. The study by Thomas et al observed the strongest association between IL28B genetic variation and spontaneous viral resolution. In a cohort of 388 spontaneous resolvers and 620 chronically infected individuals, IL28B C/C genotype was strongly associated with spontaneous viral resolution ($p = <10^{-12}$) compared with the C/T or T/T genotype (Thomas et al. 2009)

A more recent GWAS in 2013 identified a new gene that overlaps with IFNλ3 (named IFNλ4) that is more strongly correlated with a positive treatment response and spontaneous HCV clearance in African-Americans compared with IFN-λ3 (Prokunina-Olsson et al. 2013).

As well as host genetics determining the success or failure of the innate antiviral response, it is likely that the virus itself has adapted mechanisms over time to subvert the innate response, including the prevention of IFN production, disruption of IFN mediated signalling or inhibition of effector mechanisms of ISG's (Randall and Goodbourn 2008).

Finally, the killer cell immunoglobin-like receptors 2D3 (KIR2DL3) that recognises HLA-C1 is significantly higher in the exposed but uninfected population compared with both

spontaneous resolvers and chronically infected individuals (Knapp et al. 2011) suggesting a possible protective role for this innate receptor.

1.2.2 THE HUMORAL RESPONSE TO HCV INFECTION

The role of HCV specific neutralising antibodies in the immune response to HCV infection is incompletely defined, and there is much conflicting evidence regarding their significance. Neutralising antibodies work by binding to the envelope glycoproteins (E1 and E2) to prevent binding and entry into hepatocytes. The most immunogenic region of the envelope proteins is the hypervariable region 1 (HVR-1) (Sabo et al. 2011), and perhaps because of this evolutionary selection pressure on HVR-1, the epitopes in this region have amongst the highest mutation rate in the viral genome (Smith 1999).

Most patients have detectable HCV antibodies between 1 and 3 months following infection, regardless of disease outcome (Lavillette et al. 2005). Evidence supporting a role of neutralising antibodies in viral clearance comes from a well-documented study of women infected with the same HCV strain following contaminated anti-D, which found a correlation between the early development of neutralising antibodies to spontaneous viral clearance (Pestka et al. 2007). Furthermore, a cohort study of IVDU's that were re-infected following previous viral clearance found an association between viral clearance upon reinfection and the induction of broadly neutralising antibodies (Osburn et al. 2014). On the other hand, various studies have reported the absence of neutralising antibodies in the majority of patients with acute HCV that subsequently clear the virus (Kaplan et al. 2007; Logvinoff et al. 2004), and spontaneous viral clearance has even been reported in patients with agammaglobulinaemia (Adams et al. 1997).

1.2.3 THE ROLE OF T CELLS IN HCV INFECTION

There is a significant body of evidence supporting a critical role for both CD4+ and CD8+ T cells in the control of HCV infection.

Early evidence for a predominant T cell mediated response comes from viral challenge studies in chimpanzees, the only other species susceptible to HCV infection. A natural history study of 6 infected chimpanzees observed viral clearance to be associated with a strong multi-specific CD8+ T cell response (Cooper et al. 1999). Further evidence for the role of CD8+ T cells comes from a study by Shoukry et al. (Shoukry et al. 2003), in which 2 chimpanzees, following two successful viral challenges, were re-infected a third time in the context of antibody-mediated depletion of their CD8+ T cells. Following the first and second rounds of exposure to the virus, the animals cleared the infection within 4 months and 14 days respectively. Following CD8+ T cell depletion however, a third viral challenge resulted in prolonged viraemia – despite the presence of HCV specific memory CD4+ T cells – and ultimate viral clearance was only achieved with recovery of intrahepatic CD8+ T cells. Evidence supporting the necessary for CD4+ T cell help comes from a similar study by Grakoui et al. (Grakoui et al. 2003). Here two previously immune chimpanzees were re-infected with the same viral strain following antibody mediated CD4+ T cell depletion. Despite the presence of circulating virus specific memory CD8+ T cells, viraemia persisted for >300 days, and resulted in the emergence of mutations in CD8+ specific epitopes.

Additionally, several patient cohort studies have found associations between viral clearance and the induction of HCV specific CD4+ and CD8+ T cell responses. An analysis of MHC Class II restricted epitopes in acutely infected patients observed more vigorous proliferative responses in viral clearance compared with chronic infection (Missale et al. 1996). Another CD4+ T cell analysis of a cohort of acutely infected patients found a strong

association of a robust CD4+ T helper 1 (Th_1) response with early viral control and clearance (Gerlach et al. 1999), findings which were also observed in a prospective study of 8 individuals with acute HCV infection (Guobuzaite et al. 2008) Interestingly, in a small number of patients, subsequent loss of the antiviral CD4+ T cell response was associated with rebound viraemia (Gerlach et al. 1999). With regards to the role of CD8+ T cells in viral control, a strong correlation was observed between IFNγ secreting cytotoxic T lymphocytes (CTLs) and self-limiting disease compared with chronic infection in 18 acutely infected patients (Gruner et al. 2000). Furthermore, a temporal relationship between a reduction in viral load and the emergence of IFNγ producing CD8+ T cells has been observed (Lechner, Gruener, et al. 2000). In contrast, weak, narrowly focused or even absent HCV specific CD4+ and CD8+ T cell responses have been widely reported in chronic infection (Gerlach et al. 1999; Lauer et al. 2004; Urbani et al. 2006). These studies suggest important roles of both CD4+ and CD8+ T cells in the early stages of infection. However, the exact roles of both T cell subsets in control of the virus are still incompletely defined. Hypothesised mechanisms of success and failure of the cell-mediated response are discussed in further detail below.

Finally, protective HLA alleles specific for both MHC Class I and II have been reported. In a cohort of Irish women infected with the same HCV strain, a strong protective association was observed between HLA A*3, B*27 and Cw*01 (McKiernan et al. 2004). Other studies have reported a correlation between HLA B57 and viral clearance (Kim et al., 2011; Kuniholm et al., 2010). Similar reports have been published about HLA Class II alleles and disease outcome. A GWAS study performed in 2401 individuals (919 spontaneously resolved; 1482 chronically infected) observed a number of significant allelic differences near HLA Class II, including a DQB1*03:01 SNP, indicating a protective role of this allele

(Duggal et al. 2013). Furthermore, the same Irish cohort described above reported protective associations with DRB1*01, DRB1*0401 and DRB1*15 (McKiernan et al. 2004).

1.3 ELEMENTS OF A SUCCESSFUL IMMUNE RESPONSE

The evidence presented above suggests a predominantly cell-mediated immune response in determining HCV viral control, with a likely role of an early robust innate response and a possible role of broadly neutralising antibodies. What then are the correlates of a successful immune response, and the mechanisms of the HCV specific T cells in controlling the virus? Determining this will lead to better understanding of the host-virus interactions, and will aid in the development of a T cell mediated vaccine against HCV. Spontaneous viral resolution offers a model of a successful immune response to HCV infection. The study of spontaneously resolved infection (and how it compares to chronic infection), as well as primate and other animal studies, has greatly contributed to the understanding of what a protective response to HCV infection may be.

1.3.1 BREADTH

There are a number of studies that suggest the breadth of HCV epitopes targeted by CD4+ and CD8+ T cells determine infection outcome. A broadly directed CD4+ and CD8+ T cell response targeting a range of epitopes has been associated with viral clearance (Diepolder et al. 1997; Missale et al. 1996; Schulze zur Wiesch et al. 2005). One study in particular reporting an average of 10 CD4+ T cell epitope specificities in resolved infection compared to one or none in chronic infection (Day et al. 2002). In particular, both CD4+ and CD8+ T cell responses targeting the non-structural proteins have been correlated with HCV control

(Diepolder et al. 1995; Pape et al. 1999) with a number of immunodominant epitopes reported particularly within NS3. Interestingly, one study reported the most immunodominant CD4+ epitopes within the NS region to be highly promiscuous, binding a range of different HLA DR molecules (Schulze zur Wiesch et al. 2005).

1.3.2 MAGNITUDE

An important determinant of any adaptive immune response is the magnitude, or size of population, of the antigen specific T cells. Although no defined cut-off has been correlated with a protective response, a robust anti-HCV T cell response is observed in most who clear the virus, and remains detectable for many years (Takaki et al. 2000). However, there is conflicting evidence regarding the importance of the response magnitude in determining viral clearance, as well as the T cell response kinetics.

Challenge studies in chimpanzees have reported rapid viral control upon secondary exposure to be associated with a vigorous expansion of antigen specific T cells (Grakoui et al. 2003; Shoukry et al. 2003), however this may have been more dependent on the rapid induction of effector T cells by the memory T cell population. Robust, early proliferative CD8+ and CD4+ T cell responses have been associated with favourable disease outcome in humans, whereas chronicity is correlated with weak or even absent HCV T cells (Diepolder et al. 1995; Gruner et al. 2000; Lauer et al. 2004; Lechner, Gruener, et al. 2000).

The kinetics of the HCV specific T cell response have been observed to affect HCV control. Following initial viral control, a contraction of the CD4+ T cell response preceded a rebound viraemia resulting in chronic infection (Gerlach et al. 1999). In another study, an effective

antiviral CD8+ T cell response was observed at a later time point and after recovery from infection, presumably following CD4+ mediated priming (Urbani et al. 2006).

However these observations are associated with other variables, notably the breadth and effector functions of the antiviral response. Conversely, a number of other studies have observed no correlation of the magnitude of the CD8+ (Kaplan et al. 2007) or CD4+ (Schulze Zur Wiesch et al. 2012; Thimme et al. 2001) T cell response in acute infection with rate of viral clearance. The lack of a clear correlation of response magnitude independent from other factors suggests that size alone is not the most important determinant of a successful immune response. Nonetheless, T cell magnitude is a parameter that is easy to measure in vaccine induced responses, and in association with other T cell functions will continue to be assessed in the development of T cell vaccines.

1.3.3 MEMORY AND FUNCTIONAL PROPERTIES

Finally, the ability of HCV specific CD4+ and CD8+ T cells to produce effector cytokines and rapidly expand on exposure to antigen are likely to be important features of a successful immune response. In particular the production of tumour necrosis factor-α (TNFα) (Locksley, Killeen, and Lenardo 2001), IFNγ (Schroder et al. 2004), interleukin 2 (IL-2) (Malek 2008) and macrophage inflammatory protein-1beta (MIP-1β) (Florholmen et al. 2011) are regarded to be important effector cytokines. Evidence of this comes from an SIV vaccine challenge study, where it was demonstrated the production of TNFα and IFNγ were associated with robust antiviral CD4+ and CD8+ T cell responses (Hansen et al. 2011). Furthermore a successful immune response against HCV also requires a population of long lived antigen specific memory cells to be maintained in the absence of antigen, through

homeostatic self-renewal mechanisms (Wherry and Ahmed 2004). However, the exact phenotypic features, particularly of CD4+ T cells, have been difficult to elucidate due to the challenges of identifying and analysing *ex vivo* antigen specific CD4+ T cells. CD4 and CD8 T cells exhibiting memory cell surface markers have been observed to be enriched in the liver in chronic HCV infection, however with attenuated ability to produce IFNγ, postulated to be a result of the tolerogenic liver environment (Wang et al. 2006). Due to the difficulty in sampling liver tissue in otherwise healthy individuals with spontaneously resolved infection, the population size, role and phenotype of intrahepatic HCV specific T cells is difficult to assess. It could be hypothesised that they would play an important role in the prevention of reinfection as they are at the site of infection, however they may also have an attenuated response to reinfection as a consequence of the tolerogenic liver environment.

Finally, in addition to the production of specific antiviral cytokines by both CD4+ and CD8+ T cells, the specific cytotoxic capacity of CD8+ T cells is likely to be an important mechanism of viral clearance, in keeping to that which occurs in acute viral infections (Lechner, Wong, et al. 2000). Cytotoxic mediated killing of virally infected cells by antigen specific CD8+ T cells occurs through the secretion perforin and granzymes, which induce apoptosis of the target cell on delivery (Bots and Medema 2006). Although this has only been demonstrated *in vitro* (Jo et al. 2009), the importance of perforin can be inferred from studies demonstrating deficient or absent perforin expression in CD8+ T cells in chronic HCV infection (Jo et al. 2012).

1.4 MECHANISMS OF FAILURE OF THE CELL MEDIATED RESPONSE

Various hypotheses have been suggested as to the underlying mechanisms causing the failure of the host response in chronic HCV infection. The complex nature of the virus, host response and interplay between the two suggest that there are likely a number of factors involved which will be discussed below. It is important to understand the nature of these mechanisms to aid in T cell vaccine development, and more importantly determine if the dysfunctional phenotype associated with failure may be reversed following cure of HCV infection with DAA therapy.

1.4.1 INEFFICIENT T CELL PRIMING

It is hypothesised that the early adaptive immune response induced in the acute phase of infection largely determines the quality of the subsequent cell mediated response. HCV specific CD8+ T cell responses in early infection have been described as having a 'stunned' phenotype, as evidenced by reduced production of IFNγ, diminished proliferative potential, and impaired cytotoxicity (Lechner, Gruener, et al. 2000; Thimme et al. 2001; Urbani et al. 2006). This initial weak and narrowly focused CD8+ T cell response is universally detected regardless of disease outcome (Urbani et al. 2006). It is hypothesised that a robust, broad and highly functional early Th1 CD4+ response is needed to prime the CD8+ T cell to acquire effector functions capable of viral killing, as well as differentiate into long lived memory CD8+ T cells (Park and Rehermann 2014). This differentiation is also accompanied by the loss of PD-1 – which is expressed early in infection (Kasprowicz et al. 2008) – and the acquisition of anti-apoptotic markers such as Bcl-2 and CD127 (Shin et al. 2013; Thimme et al. 2001; Urbani et al. 2006). In the absence of early CD4+ T cell help,

CD8+ T cells develop into an exhausted phenotype, continuing to express PD-1 (Penna et al. 2007) and incapable of eliciting effector functions.

The interplay between the CD4+ and CD8+ T cells in acute infection is incompletely understood. A study of acutely infected patients reported a robust and broadly targeting CD4+ T cell response in acute infection regardless of outcome, and it was only after chronicity was established that the CD4+ T cell population diminished (Lucas et al. 2007; Schulze Zur Wiesch et al. 2012). It could be speculated that it is the functional characteristics of the CD4+ T cell response that determines the fate of the CD8+ T cells, a hypothesis that is supported by the observation of an increase in immunosuppressive T regulatory cells (Tregs) in acute infection resulting in chronic viraemia (Kared et al. 2013). However, more research is needed to elucidate the interactions between the CD4+ and CD8+ T cells early in HCV infection.

1.4.2 VIRAL ESCAPE

Viral persistence may also be caused by the acquisition of escape mutations in epitopes targeted by the host cell mediated response. The development of escape variants is plausible considering the vast replication rate of the virus (10^{12} virions/day) (Neumann et al. 1998) and the lack of proofreading by the RNA polymerase (Bukh, Miller, and Purcell 1995). Evidence for this comes from analysis of acutely infected chimpanzees (Erickson et al. 2001), which demonstrated T cell escape variants developing within the first 16 weeks following infection. This has been hypothesised to be influenced by the absence of CD4+ T cell help (Grakoui 2004), although the mechanisms are not well understood. The escape of viral variants occurring early in infection is supported by analysis of viral sequences in

both acutely infected (Timm et al. 2004) and chronically infected (Chang et al. 1997) patients, the latter demonstrating fixed viral sequences over a four year follow up period. It should be noted that CD4+ epitope escape mutations are rarely reported, and are not thought to be a predominant factor underlying CD4+ T cell failure (Fuller et al. 2010).

1.4.3 T CELL EXHAUSTION

T cell exhaustion is a collective term for a number of changes that occur in the dysfunctional cell mediated response to many chronic viral, bacterial and parasitic infections, as well as cancers (Wherry 2011). Accordingly, it is also believed to be one of the key mechanisms behind the failure of the adaptive response in chronic HCV infection. T cell exhaustion was first described in mouse models of chronic LCMV infection (Zajac et al. 1998). Here it was observed that persistent antigen exposure in a chronically infected host resulted in epitope specific CD8+ T cells either being deleted or persisting indefinitely without effector functions. Furthermore, these defects were more prominent in the absence of CD4+ T cell help. Additionally, it was found that deletion of antigen specific CD8+ T cell populations in chronic LCMV infection was dependant on the antigen load, where excessive antigen stimulation led to apoptosis (Gallimore et al. 1998).

Over the past decade T cell dysfunction and exhaustion in chronic viral infection has been widely studied, and in particular in chronic HCV, HBV and HIV infection (Betts et al. 2006; Lechner, Gruener, et al. 2000; Xu et al. 2006). It is believed similar mechanisms of exhaustion exist between mouse and human models of chronic infection, albeit the dominant mechanisms driving exhaustion may differ between different pathogens (Virgin, Wherry, and Ahmed 2009). It is accepted that a hierarchical loss of function occurs in

antigen specific CD8+ T cells with *ex vivo* killing ability, proliferative capacity and production of IL-2 amongst the first functions lost (Wherry et al. 2003). Thereafter the ability to secrete TNF is lost, followed by the loss of production of IFNγ and beta-chemokines. CD8+ T cells become unable to degranulate and finally in the severe stages of exhaustion may become deleted entirely (Moskophidis et al. 1993). Much less is known about the role of antigen specific CD4+ T cells in chronic viral infection, likely due in part to the challenge in studying the functional diversity and range of T helper subsets. However, several key observations have been noted. Firstly – and particularly the case in chronic HCV infection – deletion of antigen specific CD4+ T cell populations may occur to a greater extent than CD8+ T cells, with the CD4+ T cell populations unable to be detected as early as 3 months following infection (Schulze Zur Wiesch et al. 2012). Secondly, and perhaps intuitively, the loss of CD4+ T cell help leads to more severe CD8+ T cell exhaustion (Wherry et al. 2003).

Regulation of exhaustion

Negative immunoregulatory pathways are thought to be key in mediating T cell exhaustion, and can be broadly classified into three groups: 1) regulatory T cell subsets, 2) cell surface markers, and 3) cytokines and other secretory factors. Although these pathways and mechanisms are critical in normal functional immune responses in order to prevent autoimmunity, regulate normal immune responses and avert excessive immune responses to non-cytopathic organisms, in chronic infections and cancer these mechanisms may dominate the immune response and result in dysfunctional cells.

Regulatory T cells (Tregs), characterised by CD4+CD25+FoxP3+ expression and immunosuppressive cytokine release (Belkaid and Rouse 2005), have been observed in increased numbers in chronic HCV infection (Cabrera et al. 2004; Sugimoto et al. 2003), and are associated with suppression of the HCV specific CD8+ T cell response (Boettler et al. 2005; Ebinuma et al. 2008; Rushbrook et al. 2005). In normal immune responses they act to prevent autoimmunity and regulate immune responses through a number of mechanisms such as inhibition of activation of antigen presenting cells (APCs) and CD8+ T cells (Shevach 2009), therefore it would seem likely they would employ similar mechanisms to induce a tolerogenic environment in chronic HCV infection. However, it is incompletely understood exactly how Tregs contribute to T cell exhaustion, and the mechanisms involved in doing so (Wherry 2011).

Inhibitory cell surface markers are often presented transiently during a normal immune response. However, in a dysfunctional response elicited by exhausted T cells they are expressed disproportionately (Virgin, Wherry, and Ahmed 2009). A range of inhibitory markers have been described, but PD-1 is arguably the most widely studied. Expression of PD-1 and the effect of PD-1-ligand interactions limit antigen specific T cell efficacy during responses to infections and cancers (Freeman et al. 2006), affect the proliferative potential and survival of exhausted CD8+ T cells (Petrovas et al. 2006), cause blunted intracellular signalling responses (Chemnitz et al. 2004) and can even induce transcriptional factor changes to promote molecules that actively inhibit T cell function (Quigley et al. 2010). Furthermore, the pattern and level of co-expression of PD-1 with other inhibitory cell surface markers, such as CTLA-4, TIM-3 or LAG-3 can have a range of inhibitory functions (Blackburn et al. 2009; Crawford and Wherry 2009).

Immunosuppressive cytokines are also thought to play a role in the development and function of exhausted T cells. IL-10 and TGF-β in particular have been observed to have higher rates of secretion in chronic viral infection in humans (Alatrakchi et al. 2007), and blockade of these receptors in mouse LCMV models resulted in improved CD8+ T cell functionality and better viral control (Blackburn and Wherry 2007).

Finally, transcriptional changes are believed to occur during the transition of CD8+ T cells to an exhausted phenotype, and it is possible, although incompletely defined, that exhausted T cells have a distinct transcriptional signature (Wherry et al. 2007). For instance, Blimp-1 has been found to be highly expressed in exhausted CD8+ T cells, which is associated with high expression of a range of inhibitory receptors (Shin et al. 2009). Furthermore, the transcriptional factors Tbet and eomes are thought to play key roles in exhausted CD8+ T cell differentiation. A recent study using the LCMV model showed both of these transcription factors to be upregulated on exhausted T cells, but persistent antigen stimulation caused the T-bet^{hi} population to proliferate and give rise to the eomes^{hi} subset. This was a significant observation as the T-bet^{hi} population may be reinvigorated by PD-1 blockade, whereas the eomes^{hi} population were terminally differentiated (Paley et al. 2012).

Potential of reversal of exhaustion

A critical question, and one that remains largely unanswered, is the fate of exhausted T cells, and importantly whether or not reversal of exhaustion is possible. Restoration of T cell function has been observed following blockade of the PD-1 pathway alone (Barber et al. 2006) and in combination with other inhibitory receptors and immunosuppressive

cytokines (Brooks et al. 2008; Nakamoto et al. 2009), providing proof of principle that at least partial reversal of exhaustion is possible in some settings. Reversal of T cell exhaustion and its relevance in chronic HCV infection will be discussed in more detail in chapter 6.

1.5 TREATMENT OF HCV

1.5.1 INTERFERON BASED THERAPY

Since its approval in 1991 until 2015, interferon-α based regimes have been the mainstay of treatment for HCV. Initial treatment responses based on a 48 week regimes was 15-20% sustained virological response (SVR) (Hoofnagle and di Bisceglie 1997). Efficacy rose after the addition of oral ribavirin (riba) therapy in 1998 (McHutchison et al. 1998). The exact mechanism of ribavirin is unclear, however a number of hypotheses have been proposed, including a direct antiviral effect (on RNA dependant RNA polymerase) (Hofmann et al. 2007), upregulation of IFN dependant genes (Chung et al. 2008), and a drive from a Th2 to Th1 mediated immune response (Peavy, Powers, and Knight 1981; Tam et al. 1999). HCV clearance rates then improved again in 2002 after the introduction of peg-IFN. Pegylation involved linking the interferon molecule with polyethylene glycol to produce peg-IFNα, which extended the serum half-life and caused constant viral suppression. This allowed for once-weekly dosing, better clinical efficacy and slightly higher tolerability (Heathcote et al. 2000). Nonetheless all interferon regimes have unwanted and often intolerable adverse effects, including flu-like symptoms, myelosuppression and neuropsychiatric symptoms, such as depression and irritability.

1.5.2 DIRECTLY ACTING ANTIVIRALS

Over the past few years however, the treatment landscape of chronic HCV infection has undergone revolutionary changes with the development of directly acting antiviral drugs (DAA's). DAA's work by inhibition of a particular non-structural HCV protein and are broadly grouped into three categories: NS3/4 inhibitors (also known as protease inhibitors), NS5a inhibitors and NS5b inhibitors (targeting NS5b polymerase). NS3/4 protease inhibitors telaprevir and boceprevir were the first to be approved in 2011, and were used in combination with peg-IFN and ribavirin. They remarkably improved SVR rates, particularly for genotype 1 patients, up to this time the most difficult genotype to control. SVR rates for this genotype were 75% and 66% for treatment naïve and treatment experienced patients respectively (Sarrazin et al. 2012), compared with <10% SVR following the introduction of IFN regimens (Poynard et al. 1996). The introduction of the NS5b polymerase inhibitor sofosbuvir in 2013, and second generation DAA's approved thereafter allowed for the first time all-oral IFN-free regimes to treat HCV. DAA's are now used in different combinations depending on various antiviral and clinical attributes such as genotype, prior treatment failure, adverse effects and drug interactions (Gotte and Feld 2016). Of note the most recent combination of sofosbuvir and velpatasvir is pangenotypic and induces very high rates of SVR, which may reduce or even eliminate the need to do genotype testing in the future (Everson et al. 2015; Feld et al. 2015). In general, DAA therapy is highly efficacious and well tolerated.

However there are two important distinctions between DAA mediated cure and cure with spontaneous viral resolution. Firstly, DAA cure does not induce a successful host HCV specific cell mediated response potentially capable of clearing the virus with re-exposure, and therefore the host has no protection against re-exposure. Secondly, it is likely that the

HCV specific immune response remains attenuated following HCV cure – this observation will be further explored in chapter 6 of this thesis.

Finally, an area of recent controversy has been the risk of developing HCC following DAA treatment. Early studies assessing the correlation reported a higher incidence of HCC with DAA's (Conti et al. 2016; Ravi et al. 2017). One theory for this mechanism was a change in pro-tumour and anti-tumour immunity with rapid reduction in viral load (Farazi and DePinho 2006). However more recently there have been a number of studies (Kanwal et al. 2017; Calvaruso et al. 2018), including an American Gastroenterology Association clinical practice update (Singal, Lim, and Kanwal 2019) reporting no specific increased risk with DAA's, and the highest predictor of developing HCC is the degree of fibrosis.

1.6 HCV VACCINATION

1.6.1 RATIONALE FOR A HCV VACCINE

Despite significant changes in recent years in the treatment of HCV following the introduction of DAA therapy, there is still a strong rationale for the development of a prophylactic HCV vaccine. Firstly, the treatment cost associated with DAA regimes is unaffordable to most countries, and will continue to be so for many years (Hagan, Wolpe, and Schinazi 2013). This is of particular relevance to developing economies, where resources may be limited and infection rates high (Ford et al. 2012). Secondly, following viral cure, many individuals will continue to be at risk of reinfection through either social practices or exposure to contaminated medical equipment (Sacks-Davis et al. 2013). Thirdly, as discussed previously, HCV is often clinically silent until irreversible

complications are established, and therefore without stringent screening measures, many infections will go untreated.

1.6.2 CHALLENGES TO HCV VACCINE DEVELOPMENT

There are a number of challenges facing the development of a successful preventative HCV vaccine.

Firstly, a great impediment to studying the HCV immune response has been the paucity of animal and other *in-vitro* models. Due to the fastidious nature of the virus, chimpanzees are the other only species susceptible to HCV infection. The study of chimpanzees in HCV research has greatly aided the understanding of the immune response to the virus (Lanford et al. 2001), and has allowed for the assessment of multiple vaccines. However, the use of these animals has certain logistic and ethical challenges, the latter of which led to a moratorium of their use in research in 2013 in most countries. Other *in vitro* models, such as the JFH-1 strain isolated from a Japanese patient with fulminant hepatitis (Lindenbach et al. 2005) and *in vivo* models, such as the humanised mouse model (Washburn et al. 2011) have been developed over the past several years which have contributed to HCV research and in particular to the development of DAA therapy.

Additionally, the HCV virus itself poses significant challenges to the development of a preventative vaccine for two reasons. Firstly, there is significant genetic variability between the genotypes and subtypes. Immunodominant epitopes that are targeted by T cells in the immune response to HCV are often highly variable with distinct T cell specificity (Yewdell 2006; Giugliano et al. 2009) and it has previously been observed that vaccine induced T cell have attenuated cross-reactivity against both other genotypes (Barnes et al.

2012) and quasispecies in the same individual (Kelly et al. 2015). A recent study assessing HCV sequence diversity between genotypes 1 and 3, as well as genotypes 1 to 6 calculated conserved viral regions of 94.7% and 90.2% respectively (von Delft et al. 2018), although it should be noted that this was across the entire genome, whereas our vaccine design, and most T cell dominant epitopes are confined to the non-structural region. Nonetheless there are several studies reporting diversity within a number of immunodominant T cell epitopes within the NS region across different genotypes (Giugliano et al. 2009; Lopez-Labrador et al. 2004; von Delft et al. 2016). Secondly, the high mutation rate of many HCV epitopes – in particular the hypervariable regions of E1 and E2 – results in a significant number of escape mutations. The envelope proteins would be the main target of a traditional antibody mediated vaccine, but given the diversity of epitopes within E1 and E2, a vaccine aimed at inducing neutralising antibodies is particularly challenging. Finally, considering the demographic of people infected with HCV in the developed world – being largely confined to the IVDU population – proceeding past a Phase I trial to assess vaccine efficacy presents logistical difficulties.

1.6.3 COMPONENTS OF A SUCCESSFUL HCV VACCINE

Correlates of a successful vaccine for HCV are not precisely defined, as the correlates of "a successful" immune response to the virus are not fully known. However, based on the present knowledge, the essential elements of a protective HCV vaccine are likely to be **a)** safe and well tolerated **b)** induction of both a CD4+ and CD8+ T cells that are of high magnitude, broadly targeting, polyfunctional and durable **c)** cross-reactive between HCV

genotypes **d)** contain epitopes specific to a variety of HLA alleles and **e)** allow for global distribution, defined predominantly by affordability and ease of storage.

1.6.4 PREVIOUS HCV VACCINE ATTEMPTS

The majority of currently licensed vaccines work by inducing an antibody response and include live attenuated vaccines, inactivated microorganisms, subunits and toxoids (Rollier et al. 2011). However, as discussed previously, an antibody mediated approach alone is unlikely to be effective as a prophylactic HCV vaccine. Several other vaccine approaches have been trialled with little progression in human trials. Recombinant protein vaccines have been trialled, which involve cloning of bacteria, yeast or mammalian cell lines expressing HCV protein. Although pre-clinical studies were initially promising (Choo et al. 1994; Houghton and Abrignani 2005; Verstrepen et al. 2011), no human trial has progressed past Phase I (Drane et al. 2009; Frey et al. 2010; Habersetzer, Baumert, and Stoll-Keller 2009; Leroux-Roels et al. 2004). Efforts have been made at developing a synthetic peptide vaccine (Firbas et al. 2006; Huang et al. 2013) involving coupling HCV peptides with adjuvants to induce both a humoral and cell mediated response. Initial immunogenicity results demonstrated in IFNy secreting enzyme-linked immunospot (ELISpot) assays were promising (Firbas et al. 2006), but when trialled as a therapeutic vaccine in HCV infected patients no change in viral load was observed (Klade et al. 2012). Furthermore, this approach requires immunogenic epitopes associated with specific HLA types. Finally DNA vaccines, involving transfer of recombinant plasmids expressing viral DNA into the host is a potential evolving technology (Sallberg, Frelin, and Weiland 2009),

however a single study in chronically infected patients demonstrated only a modest effect, in combination with IFN/riba therapy (Weiland et al. 2013).

1.6.5 DEVELOPMENT OF VECTOR BASED VACCINES

Over the past decade adenoviral and modified vaccinia Ankara viral vectors have become established as a powerful tool to generate a robust T cell response to many pathogens including respiratory syncytial virus (RSV) (Green et al. 2015), malaria (Afolabi et al. 2016), Ebola (Ewer et al. 2016), tuberculosis (Sheehan et al. 2015) and HCV (Swadling et al. 2014). Adenoviral vectors in particular have been found to be potent vectors for the induction of antigen specific T cell responses to encoded antigens. As well as their immunogenic potential, they are attractive vectors for several reasons. They are relatively cheap to manufacture, can be manufactured to high titres in cell culture, and have an excellent safety profile (Rollier et al. 2011). They have also been made replication defective and non-pathogenic by deletion of specific loci (Brave et al. 2007).

Extensive research has been done using human adenovirus serotype 5 (Ad5) vector, which has demonstrated high immunogenic potential (Harro et al. 2009; Ledgerwood et al. 2010). However, due to the almost universal exposure to the virus in the first few years of life, it has a high seroprevalence of cross neutralising antibodies, resulting in vector clearance and reduced efficacy (Catanzaro et al. 2006; McElrath et al. 2008; Priddy et al. 2008).

The use of adenoviruses for the induction of HCV specific T cells was initially demonstrated with rare human adenovirus serotypes 6 and 24 (Ad6 and Ad24), encoding the non-structural region of HCV genotype 1b delivered into five chimpanzees followed by

electroporated DNA boosting (Folgori et al. 2006). The vaccines generated broad and high magnitude HCV-specific CD8+ and CD4+ T cell responses which were effective against heterologous viral challenge, resulting in a shortened and blunted viraemia when compared to controls. A similar study was performed in macaques using the NS immunogen (comprising genotype 1b NS3-NS5b) in adenoviral vectors Ad6 and Ad24 in a heterologous prime-boost strategy, and a broad and durable T cell response was observed, notably much higher than observed following homologous boosting (Fattori et al. 2006). The reason for the attenuated responses seen with homologous boosting is thought to be due to the induction of neutralising anti-vector antibodies with priming vaccination, causing rapid clearance of the vector upon re-administration.

Chimpanzee adenoviruses were then considered as vectors. They are not known to cause disease in humans and have a low seroprevalence (Tatsis and Ertl 2004). A study of over 1000 chimpanzee adenoviral vectors was performed to determine suitable vectors with the highest immunogenic potential (Colloca et al. 2012). Chimpanzee adenovirus serotype 3 (AdCh3) was selected for progression to human trials of a HCV candidate vaccine based on its replication incompetency in humans and high immunologic potential (Colloca et al. 2012).

The first human trial of HCV adenoviral vectored vaccines involved both homologous and heterologous prime-boost regimens with either AdCh3 or human Ad6 encoding genotype 1b NS3 to NS5b in healthy adults. Priming with both Ad6 and ChAd3 induced high magnitude and broadly targeting peak T cell responses that were polyfunctional and proliferative, and persisted for up to a year post vaccination (Barnes et al. 2012). However, neither adenoviral vector successfully boosted the response. Furthermore, the HCV

specific CD4+ response, although induced, was much less robust than the CD8+ T cell response.

To address the limitations of using solely adenoviral vectors as a prime/boost regime, modified vaccinia Ankara virus was considered as an alternative boosting vector. After the discovery of the vaccinia cowpox virus to protect against variola and smallpox in 1798 by Edward Jenner, the vaccinia vector was successfully used to achieve global eradication of smallpox. A modified form of the virus was achieved in the 1970's by more than 570 passages through chick embryoblasts (Mayr and Munz 1964), leading to the loss of approximately 15% of its genome (Meyer, Sutter, and Mayr 1991) and resulting in an attenuated, non-replicative form named modified vaccinia Ankara (MVA). Despite the genomic deletions, the vector maintained its immunogenic potential (Blanchard et al. 1998; Drillien, Spehner, and Hanau 2004). Furthermore, it is not compromised by pre-existing immunity (Cottingham and Carroll 2013), and can allow for large immunogen inserts (Kremer et al. 2012). Since then it has been extensively researched for its potential application as a T cell vaccine. Pre-clinical studies in rhesus macaques and human trials using malaria and tuberculosis immunogens (Capone et al. 2010; Reyes-Sandoval et al. 2011; Sander et al. 2009) demonstrated MVA to be an excellent boosting vector following adenoviral priming, as well as inducing high magnitude and polyfunctional antigen specific CD4+ T cell responses.

For these reasons MVA was chosen as alternative boosting vaccine for the HCV immunogen. Vaccination with AdCh3 priming following by heterologous MVA boost encoding the HCV non-structural region of genotype 1b (HCV-NS) was given to 10 healthy

volunteers in a Phase I study in Oxford (Swadling et al. 2014). The vaccines were safe and well tolerated, and MVA boosting generated an HCV specific T cell population 3-4 times higher than induced with AdCh3 or Ad6 boosting. Both CD4+ and CD8+ virus specific T cells were induced, and targeted a broad range of epitopes, were polyfunctional, and developed sustained effector and memory cell phenotypes. Despite this though, the HCV specific memory response declined over time. The next part of this trial addresses whether giving additional vaccinations may either enhance the peak response or propagate the memory response of the antigen specific T cells. This will be addressed in chapter 4**.**

1.6.6 VECTOR VACCINES IN HCV PATIENTS

Despite promising results in Phase I trials in healthy adults, critical questions remain regarding the use of vector vaccines in a therapeutic setting in chronically infected HCV patients. There have been two vaccine trials to date conducted in Oxford to addressing the efficacy of HCV vector vaccination in HCV patients.

The first therapeutic HCV vaccine study in HCV patients assessed AdCh3 followed by Ad6 boost in genotype 1 patients either alone or in combination with peg-IFN + ribavirin therapy (Kelly et al. 2016). Vaccine induced HCV specific T cell responses were observed in 11/20 patients receiving concurrent therapy and 2/4 patients receiving vaccination alone. Notably this was despite 9 patients having undetectable viral loads at time of vaccination in the concurrent treatment group. Responses were significantly attenuated in comparison to healthy volunteers (Barnes et al. 2012), and in many cases the T cells induced by vaccination did not target the patient's endogenous virus, suggesting a poor cross-reactive potential of the vaccines to target escape variants. A significant

confounding factor in this trial though was the possible global immunosuppressive effects of concurrent IFN therapy.

The second trial in 13 chronically infected HCV genotype 1 patients was conducted similarly to Kelly et al, however here the patients received AdCh3 priming followed by MVA boosting at week 8 (again both with and without concurrent peg-IFN + ribavirin therapy) (Swadling et al. 2016). In contrast to the robust T cell responses induced by MVA boosting in healthy volunteers, HCV specific T cell responses were weak, partially functional and rarely induced CD4+ T cell responses.

Both of these trials highlight the challenges and limited success employing viral vectors as a therapeutic tool, and potentially the limitations of inducing viral specific responses with concurrent suppressive effects of IFN based therapy.

A yet to be explored however important question is whether a prophylactic HCV vaccine can be successfully employed to prevent reinfection. It is unknown whether primed T cells will respond to vaccine in the same way as naïve T cells, and additionally if there is any reversal of T cell exhaustion observed in chronic infection to enable an effective cell mediated response to vaccination. It is critical to answer these questions however, as many individuals cured of HCV with DAA's remain at risk of reinfection, and will be an important target group for preventative HCV vaccination. An analysis of potential restoration of the function of HCV specific T cells is addressed in chapter 6.

1.6.7 CO-ADMINISTRATION OF VIRAL VECTORS

The prospect of co-administering adenoviral vectors is highly relevant. The extensive research done over the past two decades into their potential use in therapeutic and prophylactic settings in various infections and cancers suggests there may be many applications for them in the future, and it is therefore important to establish whether they may be safely and effectively co-administered. In particular, co-vaccination with viral vectors to prevent multiple infections is an attractive goal. Vaccine combinations – such as diphtheria, tetanus and pertussis, or measles, mumps and rubella (MMR) – are routinely given as part of normal vaccine schedules, and do not compromise the safety or immunogenicity of any vaccine given alone. MMR in particular, being live attenuated viruses, may infer proof of concept that distinct viruses can be successfully co-administered.

A vaccine strategy aimed at the prevention of HCV and HIV-1 infection through co-administration of vector vaccines is ideal. There is a large global burden of both diseases, they share similar modes of transmission and as a result have similar target group (Platt et al. 2016). Furthermore, they are likely to be amenable to a T cell based vaccination strategy due to the predominant cell-mediated immune response in both viruses. A major potential limitation of this is antigenic competition, which may abrogate the immune response to either immunogen compared with single administration (Yashima et al. 2010). A more detailed discussion of this, and the analysis of serologically distinct adenoviral vectors encoding immunogens for HCV and HIV-1 in healthy volunteers, will be discussed in chapter 5.

1.7 AIMS OF THIS WORK

This thesis aims to explore mechanisms by which optimisation of T cell vaccines in the prevention of HCV infection can be achieved.

1. To determine if additional re-boosting of AdCh3/MVA-NS vaccines, or administration of low dose MVA-NS is safe and effective. I hypothesise that:
    a. Additional reboosting is safe and well tolerated
    b. Reboosting after a long interval will be more effective than a short interval
    c. Boosting with low dose MVA-NS can successfully induce HCV specific T cells
2. To determine if co-administration of distinct adenoviral vectors encoding an HCV and HIV-1 immunogen followed by heterologous MVA boosting is safe and immunogenic. I hypothesise that:
    a. Co-administration is safe and well tolerated
    b. Co-administration of distinct vectors does not impair immunogenicity to either vector
    c. Co-administration of distinct immunogens does not impair immunogenicity to either immunogen
3. To assess immune recovery following DAA mediated HCV cure. I hypothesise that:
    a. Proliferative and functional capacity of HCV specific T cells is partially restored following DAA mediated cure
    b. Reversal of phenotypic features of T cell exhaustion are observed in CD4+ and CD8+ T cells following viral cure
    c. Antigen specific T cell function in patients is attenuated in comparison to spontaneous viral resolution and HCV vaccine induced responses

4. To perform *ex vivo* phenotypic analysis of HCV tetramer specific CD4+ T cells induced by viral vectored vaccination and spontaneous viral resolution. I hypothesise that:
    a. Ex vivo HCV specific CD4+ T cells can be identified using MHC class II tetramers following both vaccination and spontaneous viral resolution
    b. The predominant T cell memory subsets induced will be Tem's and Tcm's following vaccination and spontaneous viral resolution
    c. Tetramer positive CD4+ T cells will express high levels of co-stimulatory marker CD28 and memory marker CD127
    d. Transcription factors T-bet and eomes will decrease and increase respectively following vaccination, and in spontaneous viral resolution will be upregulated and downregulated respectively

CHAPTER 2: MATERIALS AND METHODS

2.1 VACCINE TRIALS

2.1.1 OVERVIEW: HCV003 AND PEACHI-04

Two vaccine trials are included in this thesis. The first is titled 'A Phase I study to assess the safety and immunogenicity of a vaccine for Hepatitis C Virus' and will be referred to as 'HCV003'.

The second trial is titled 'A Phase I Study to Evaluate the Safety and Immunogenicity of Simultaneous Prime-Boost Immunisations with Candidate HCV and HIV-1 Vaccines, AdCh3NSmut1 / ChAdV63.HIVconsv and MVA-NSmut / MVA.HIVconsv, in Healthy Volunteers' and will referred to as 'PEACHI-04'. Both are registered in the EU clinical trials registrar (HCV003 EudraCT Number: 2009-018260-10, PEACHI-04 EudraCT Number: 2014-000730-30).

2.1.2 RECRUITMENT

Recruitment for both HCV003 and PEACHI-04 took place at the CCVTM (Centre for Clinical Vaccinology and Tropical Medicine), Churchill Hospital, Oxford. Volunteers gave their written informed consent before being enrolled, and were recruited in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice (GCP). Patients were recruited sequentially from A3 – A7 (HCV003) and from Group 1 to 3 (PEACHI-04).

2.1.3 STUDY VISITS

An outline of the study visits, including number of volunteers in each arm, vaccinations received and doses can be found in **Table 2-1** (HCV003) and **Table 2-2** (PEACHI-04).

Group	N vol	N visits	Vaccination and dose	Final visit
A1*	4	**14**	**TW0**: MVA-NS	Trial week 22
A2*	9	**14****	**TW0**: AdCh3NS; TW8: MVA-NS	Trial week 34-78**
A3	5	**20**	**TW0**: AdCh3NS; **TW8**: MVA-NS; **TW16**: AdCh3NS; **TW24**: MVA-NSmut	Trial week 48
A4	4	11	(recruited from A2 post vacc): TW0: AdCh3NS; TW8: MVA-NS	Trial week 105-151
A5	5	**23**	**TW0**: AdCh3NS; **TW8**: MVA-NS; **TW40** MVA-NS	Trial week 52
A6	5	**14**	**TW0**: AdCh3NS; **TW8** MVA-NS (2 x 10^7 pfu)	Trial week 34
A7	5	**14**	**TW0**: AdCh3NS; **TW8**: MVA-NS (2 x 10^6 pfu)	Trial week 34

Table 2-1: Study design for HCV003. N= number, vol= volunteers. All AdCh3NS vaccination doses: 2.5 x 10^{10} vp All MVA-NS vaccination doses: 2 x 10^8 pfu unless otherwise stated.
*groups completed trial and not included in this thesis
**4 volunteers from this group formed A4 - volunteers did not attend visit 14 and their final visit was week 34

Group	N vol	N visits	Week zero	Week eight	Final visit
1	8	12	AdCh3NSmut1 (2.5 x 10^5 vp)	MVA-NSmut (2 x 10^8 pfu)	TW 34
2	8	12	ChAdV63.HIVconsv (5 x 10^{10} vp)	MVA.HIVconsv (2 x 10^8 pfu)	TW 34
3	16	12	AdCh3NSmut1 (2.5 x 10^5 vp) and ChAdV63.HIVconsv (5 x 10^{10} vp)	MVA-NSmut (1 x 10^8 pfu) and MVA.HIVconsv (1 x 10^8)	TW 34

Table 2-2: Study design for PEACHI-04. *N = number. Vol = volunteers.*

2.1.4 VACCINATION ROUTE AND DOSES

All vaccinations were given intramuscularly (i.m.). This route was chosen as it has been previously demonstrated for both vectors to be safe, well tolerated, have no viral shedding and be highly immunogenic (Harro et al. 2009; O'Hara et al. 2012).

The optimal dose of adenoviral vectors used in both trials (AdCh3 and ChAdV63) were determined based on the balance between tolerability and immunogenicity observed in pre-clinical and clinical studies. Trials using Ad6 in rhesus macaques were shown to be safe and immunogenic when administered at between 10^{10} to 10^{11}vp (Capone, Meola, et al. 2006) Adenoviral vectors given to humans have demonstrated safety and immunogenicity up to 10^{11}vp, however more reactogenicity was observed at this dose (Catanzaro et al. 2006; Priddy et al. 2008). Therefore, a dose of $2.5x10^{10}$ and $5x10^{10}$ for AdCh3 and ChAdV63 respectively were chosen.

Likewise, the dose of MVA used in the HCV003 and PEACHI-04 trials were based on results in non-human primate (NHP) and clinical trials. Multiple studies in macaques and chimpanzees have demonstrated it to be safe and immunogenic at doses of up to $5x10^{8}$pfu (Draper et al. 2010; Schneider et al. 2001), and doses of between 1 and $2x10^{8}$pfu have been trialled in a number of human phase I studies (Capone et al. 2013). Doses of between 1 and $2x10^{8}$pfu have been chosen for MVA delivery in HCV003 and PEACHI-04, with the exception of 2 groups in HCV003, where volunteers will receive $2x10^{7}$ and $2x10^{6}$pfu.

2.1.5 SAFETY

As both vaccine trials were Phase I studies, the primary aim was to determine safety of vaccine administration. Patients were carefully screened before enrolment (including taking a full medical history, performing a medical examination and doing screening blood tests), and a medical summary was requested from their general practitioner. Volunteers were excluded if they had any significant medical or psychiatric co-morbidities, were HIV-1 positive, HCV positive (current or previous), were pregnant, or had ever received vector vaccination. Pregnancy tests were performed at screening and prior to each vaccination. All volunteers were observed for 30-60 mins post vaccination. For the first 4-7 days following vaccine administration, volunteers were given a diary card and asked to record local and systemic adverse events. During this period volunteers were also given a thermometer and advised to record their temperature daily. Safety bloods were done at regular intervals throughout both trials, including full blood count, liver function tests, renal function and electrolytes. Finally, any adverse events as well as changes to health and medication were reviewed at every visit. Safety data was reviewed at regular time points throughout both trials by a local data safety monitoring committee (DSMC). In addition to the DSMC, a development safety update report (DSUR) was submitted yearly for each vaccine to the Medicines and Healthcare products Regulation Agency (MHRA), local research ethics committee and trial sponsor.

There was a uniform safety scaling for assessing the severity of adverse events (**Table 2-3)**. A serious adverse event (SAE) was regarded as any event that resulted in death, was life-threatening, required hospitalisation, resulted in persistent or significant disability or capacitation, or may result in a congenital anomaly. A suspected unexpected serious

adverse reaction (SUSAR) is any serious adverse event thought likely due to vaccination. An occurrence of a SUSAR would trigger trial suspension pending safety review. A causality assessment in relation to vaccine administration was done for each adverse event according to **Table 2-4.** Re-vaccination exclusion criteria for an individual included anaphylaxis following vaccination, pregnancy or acute illness time of vaccination.

Adverse event	Grade	Definition
Pain	I	Pain on touch, easily tolerated
	II	Painful when limb moved, interferes with daily activity
	III	Severe pain at rest, prevents daily activity
Erythema (diameter)	I	30-50 mm
	II	51-100 mm
	III	>100 mm
Swelling (diameter)	I	1-20 mm
	II	21-50 mm
	III	>50 mm
Fever (oral)	I	37.6 – 38°C
	II	38.1 – 39°C
	III	>39°C
Other AE's	I	Awareness of symptom but tolerated; transient or mild discomfort
	II	Discomfort enough to cause limitation of usual activity; some medical intervention or therapy required
	III	Incapacitating, absent from work, or bed rest required

Table 2-3: Scale for severity grading of adverse events for HCV003 and PEACHI-04

No Relationship	No temporal relationship to study product *and* Alternate aetiology (clinical state, environmental or other interventions); *and* Does not follow known pattern of response to study product
Unlikely	Unlikely temporal relationship to study product; ***and*** Alternative aetiology likely (clinical state, environmental or other interventions); ***and*** Does not follow known typical or plausible pattern of response to the vaccine
Possible	Reasonable temporal relationship to study product; ***or*** Event not readily produced by clinical state, environmental or other interventions; ***or*** Similar pattern of response to that seen with other vaccines
Probably	Reasonable temporal relationship to study product; ***and*** Event not readily produced by clinical state, environment, or other interventions ***or*** Known pattern of response seen with other vaccines
Definite	Reasonable temporal relationship to study product; ***and*** Event not readily produced by clinical state, environment, or other interventions; ***and*** Known pattern of response seen with other vaccines

Table 2-4: Guidelines for assessing the relationship of vaccine administration to a given adverse event (for HCV003 and PEACHI-04)

2.2 LOCAL COHORT RECRUITMENT

2.2.1 HCV PATIENTS RECEIVING DIRECTLY ACTING ANTIVIRAL THERAPY

Chronic HCV patients undergoing treatment with directly acting antiviral (DAA) therapy were identified from hepatology clinics at the John Radcliffe Hospital, Headington. After an informed consent process, they were recruited to the Oxford local HCV cohort: 'OxBRC Prospecitve Cohort Study in Hepatitis C virus infection; The phenotypic and genotypic characterisation of a cohort of patients with current and resolved HCV infection'. This study has been approved by the local ethics committee (Ethics reference 09/H0604/20), and is open to recruit all patients with current or previous HCV infection. All volunteers are de-identified following recruitment into the cohort.

Inclusion criteria for this cohort were negative HCV viral loads by the end of treatment and the availability of both pre- and post- treatment samples. Additional patient information was collected, including current DAA treatment regime, previous HCV treatment, HCV genotype, viral load at various time points during treatment and HLA type.

Peripheral blood mononuclear cells (PBMCs) were collected prior to commencing treatment and at the end of treatment (at a minimum), and in some patients at other time points during treatment. Plasma was collected for HLA typing, which was performed at the Immunology and Tissue Typing Laboratory, Churchill Hospital, Headington. Plasma was also collected for HCV RNA PCR at every visit (as part of routine clinical practice).

2.2.2 INDIVIDUALS WITH SPONTANEOUS RESOLUTION OF HCV INFECTION

Spontaneous resolvers were identified from monthly microbiology reports at the John Radcliffe Hospital, Headington. Any reports that had HCV antibody positive but HCV RNA PCR negative results were highlighted, and those individuals were invited into the local cohort study (described above). Due to ethical restraints, the individuals were not contacted directly, but through either their primary care physician or hospital treating doctor. Following an informed consent process, confirmation was sought with the volunteer that they had never received treatment for HCV infection, and they were then de-identified and recruited to the cohort. A single 50mL blood draw was taken for PBMC isolation, as well as 5-10mL of plasma for both HLA typing and to confirm a negative viral load by PCR. Where possible information was gathered about the date of HCV transmission and clearance.

2.3 VIRAL VECTORS AND IMMUNOGENS

2.3.1 VIRAL VECTORS

In HCV003 chimpanzee adenovirus serotype 3 (AdCh3) and modified vaccinia Ankara virus (MVA) encoding HCV-NS (described below) were used, to give AdCh3NSmut1 and MVA-NSmut respectively. In PEACHI-04, the above-mentioned vaccines were used as well as chimpanzee adenovirus serotype 63 (ChAd63) and MVA encoding HIV.consv (described below), to give ChAdV63.HIVconsv and MVA.HIVconsv.

AdCh3NSmut1 (lot B.0016) was manufactured by Advent s.r.l. (Rome, Italy) under Good Manufacturing Practice (GMP) conditions, before being shipped on dry ice to the Clinical BioManufacturing Facility (CBF) in the Churchill Hospital, Oxford, UK. ChAdV63.HIVconsv (batch 01S10-02, fill 10-01) was manufactured under Good Manufacturing Practice (GMP) conditions and released to trial by the CBF. MVA-NSmut (lot 002 08 13) and MVA.HIVconsv (lot 0021212) were manufactured by Impfstoffwerk Dessau-Tornau (IDT) Biologika GmbH, Germany under GMP conditions prior to being shipped on dry ice to the CBF.

2.3.2 HIV IMMUNOGEN

To overcome the challenge posed by the rapidly mutating and extremely variable nature of HIV-1, a 'universal' T cell immunogen (HIV.consv) was developed at Oxford University (Letourneau et al. 2007). This immunogen is comprised of the 14 most highly conserved regions (each 28 – 128aa long) of the HIV-1 proteome, which are assembled into a single

chimaeric protein. The immunogen contains approximately 25% of the known HIV-1 CD8+ T cell epitopes, of which 70% are identical between the major clades. HIV.consv has been previously demonstrated to induce HIV-specific T cell responses in human trials (Borthwick et al. 2014).

2.3.3 HCV IMMUNOGEN

Designing an HCV immunogen faces the same challenges as for HIV-1, with even more extensive genetic variability encountered. To tackle this, the relatively conserved non-structural (NS) region of genotype 1b (BK strain isolate) was used. This strain has up to 80% homology with several different subtypes belonging to the six major genotypes. The NS region is 1995 amino acids in length and encodes 5 proteins: NS3, NS4A, NS4B, NS5A and NS5B (**Figure 2-1**). The NS region is known to be dense in both CD4+ and CD8+ T cell epitopes, and is considerably more conserved than the envelope region (E1 and E2) (Ward et al. 2002).

Furthermore, a mutation was introduced within the NS5b polymerase protein (AAG replaced GAA at amino acid position 1711-1713), to inhibit any potential replicative capacity of the immunogen. This replacement did not reduce NS5b gene expression and polyprotein processing in the encoded immunogen (Capone, Zampaglione, et al. 2006).

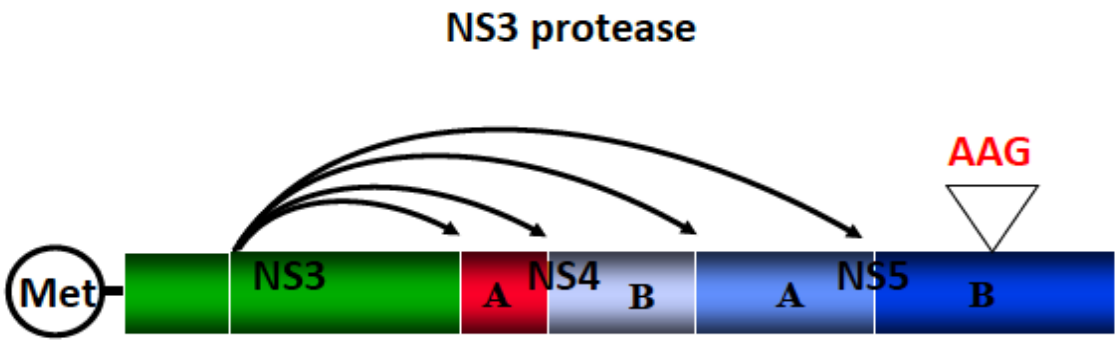


Figure 2-1: The HCV-NS immunogen. The sequence is comprised of the entire non-structural region, encoding five different proteins (NS3, NS4A, NS4B, NS5A and NS5B). A mutation has been introduced, which inhibits NS5b polymerase activity (AAG, between aa 1711-1713)

2.4 PBMC ISOLATION, FREEZING AND THAWING

Whole blood was collected in sodium heparin 10mL tubes (BD 364480). Samples were diluted in RPMI and layered onto Lymphoprep separation medium (Axis Shield Diagnostics) and spun at 900g for 24 mins at room temperature (acceleration speed 1 / deceleration speed 4). The layer of PBMCs was aspirated, diluted in RPMI and spun at 450g for 10mins. Following this the cells were incubated in 5mL of ACK lysis buffer (Gibco #A10492-01) at room temperature for 5 mins, before being washed in R10 at 450g for 7 mins and counted using a Guava Personal Cell Analysis system (Merck Millipore 0100-1430), which was calibrated daily with Guava check beads (4200-0070).

PBMCs were divided into aliquots of 8-15x10^6 PBMC and mixed in a solution of 0.5mL freezing medium (80% RPMI / 20% dimethyl sulfoxide (DMSO) and 0.5mL FBS and initially

placed in a 'Mr Frosties' freezing container at -80^{0}C. Following this the cells were placed in liquid nitrogen (-160^{0}C) for long term storage.

Thawing of PBMCs involved placing the frozen vials in a 37^{0}C water bath until partially thawed, before being transferred dropwise into 9mL R10, counted and washed.

2.5 PEPTIDES AND ANTIGENS

HCV peptides for use in the clinical trials (HCV003 and PEACHI-04) were obtained from BEI resources (genotype 1b strain BK, 1985aa – refer to **Appendix 2-1** for full list). There were 494 peptides of 15mer length in the peptide set, which overlapped by 11aa – found to be optimal for processing and presentation to T cells (Kiecker et al. 2004)**.** Peptides were dissolved in DMSO and kept in small aliquots at -80^{0}C. Peptides were divided into 6 pools with approximately 80 peptides in each pool (range 73-112), which roughly corresponded to the various non-structural proteins (see **Figure 2-2**).

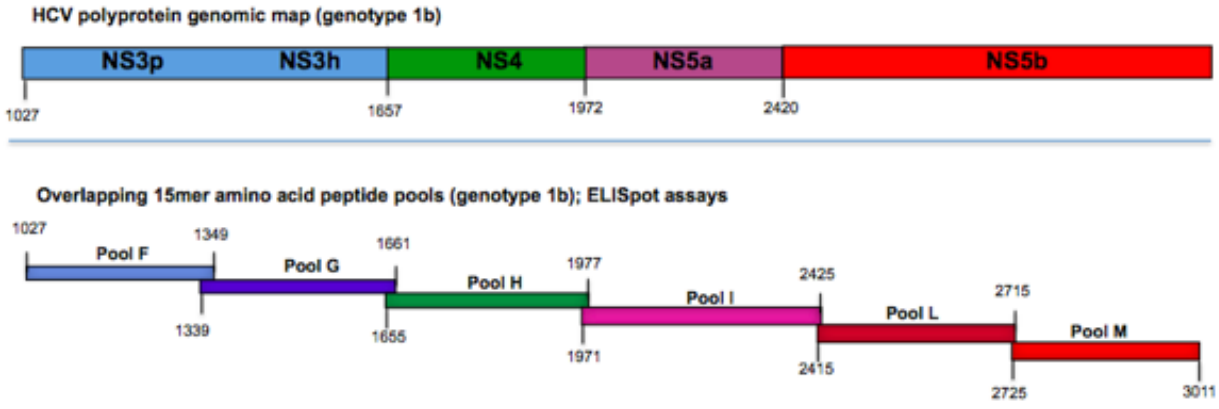


Figure 2-2: Peptide pools corresponding to the HCV immunogen. The HCV-NS immunogen spanned the entire non-structural (NS) region from HCV genotype 1b (BK isolate). For immunoassays, the peptides were divided into pools F-M, with each pool containing approximately 80 peptides of 15aa length. Pools roughly corresponded to the different NS proteins (NS3, NS4A/B, NS5A/B).

The HIV peptides used in the PEACHI-04 clinical trial corresponded to the HIV immunogen HIV.consv, and were obtained from Genscript. The peptide set contained 166 peptides of 15mer length, which also overlapped by 11aa. Peptides were divided into 6 pools of approximately 30 peptides each (refer to **Appendix 2-2** for the full list that matches the immunogen). As with the HCV peptides, small aliquots were dissolved in DMSO and stored at -80^{0}C.

Finally, peptides were obtained that matched the HCV Major Histocompatibility Complex (MHC) Class II tetramer sequences, to be used for both peptide specific cell line expansion assays and intracellular cytokine staining (ICS) assays. These peptides were obtained from Mimitopes, and dissolved and stored in aliquots of 5mg/mL in DMSO (for cell line assays) and 300ug/mL in RH10 (for ICS assays).

2.6 MHC CLASS II TETRAMERS

The MHC Class II tetramers were kindly donated from two sources, ProImmune (UK) and the NIH Core Tetramer Facility (USA). The selection and synthesis of the HCV MHC Class II tetramers are discussed in detail in Chapter 3.

2.7 ENZYME-LINKED IMMUNOSPOT ASSAY

IFN-γ secreting ELISpot assays were performed *ex-vivo* on freshly isolated PBMCs in triplicate wells. Prior to stimulation, the plates (Millipore AIP, MSIP54510) were coated with 100uL/well of anti-human IFN-γ antibody for either 3 hours in 37 degrees or overnight at 4 degrees. Following this the plates were washed five times with ELISpot wash buffer

(PBS + 0.05% tween) and then blocked by incubating with 200uL R10 for 2 hours at 37 degrees.

PBMCs were then plated out at 2 x 10^5 cells per well, and stimulated with either peptide pools F-M (for HCV-NS) or 1-6 (for HIV.consv), control antigen or negative control for 16 hours at 37 degrees. There were three positive controls and one negative control used. The negative control was DMSO (same concentration as peptide: 1uL/100uL). The positive controls used were Concavalin A (1:200 dilution; Sigma C5275) and FEC (3ug/mL; mixed HLA class I restricted peptides for influenza virus, Epstein Barr Virus (EBV) and human cytomegalovirus (CMV) lysate (0.5mg/mL, diluted 1:100). Finally, a well containing the hexon sequence from Ad5 (15mer peptides with 11 amino acid overlap) assessed induction of adenoviral specific T cells (Miltenyi 130-093-495).

Following 16 hours of incubation, the plates were washed six times with ELISpot wash buffer and incubated for 3 hours at room temperature with IFN-γ monoclonal antibody (0.5mg/mL diluted in assay diluent (0.5% BSA/PBS); 100uL/well; MAbTech clone 7-B6-1, cat. 3420-6). Following this the plate was washed five times with ELISpot wash buffer and then incubated with alkaline phosphatase-conjugated goat anti-biotin antibody diluted in assay diluent (1:750 dilution) (Vector Laboratories, cat. SP-3020). After two hours of incubation at room temperature the plate was again washed four times with ELISpot wash buffer, and finally incubated with filtered BCIP/NCT solution (50uL/well for 7 mins; Pierce cat 34042) and washed with distilled H_20. The plates were air-dried upside down overnight prior to being reading on an AID ELISpot plate reader.

The positive cut-off for the HCV ELISpot assay was determined by screening 74 healthy seronegative volunteers with the peptide pools F-M, and using the mean plus 3 standard deviations. This cut-off was determined to be 48 spot-forming units (SFU) per 10^6 PBMCs. Regarding the HIV ELISpot results, a minimum positive cut-off value has not yet been established (this will be determined in collaboration with ReiThera laboratories at the end of the trial). Therefore, all positive HIV ELISpot results are included (i.e. including 1-48 SFU's).

Any positive response must also exceed 3x the background, and the assay was determined as a failure if the DMSO wells exceeded 11 SFU per well (>55 SFU/10^6 PBMCs). Final SFU per 10^6 PBMCs were determined by subtracting the average SFU in the DMSO wells from each positive pool (following background correction), then adding the positive pools together and multiplying by 5.

2.8 SHORT TERM CELL LINES

In vitro cell lines were created for tetramer and ICS analysis. Between 2-3 x 10^6 PBMCs were added to 1mL RH10 in a 24 well flat-bottomed plate with 1ug/mL of co-stimulatory antibody CD28**.** Peptide of interest was added at 5mg/mL and the plates were left to incubate at 37 degrees. Media was changed and recombinant IL-2 (50 U/mL; Roche 11011456001) was added at days 3, 7 and 10. The cells were harvested at day 13-14, and left to rest overnight at 37 degrees in RH10 prior to tetramer analysis or re-stimulation for ICS.

2.9 FACS BASED PROTOCOLS

2.9.1 EX-VIVO PHENOTYPIC ANALYSIS

Thawed PBMCs were rested for 2 hours, followed by a cell count and viability using Guava Cell Count. They were then plated out in a 96-well U-bottom plate at 2-5 x 10^5 cells per well (cultured cells) or 10^6-10^8 cells per well (ex vivo tetramer phenotyping). Cells were stained with fixable live/dead dye in dPBS (conc 1:100; Thermo fisher L34955 APC-Cy7, ex/em 416/451) and incubated in the dark at room temperature for five minutes. They were then quenched with foetal calf serum (FCS) (50uL/well) for 5 mins in the dark at room temperature, before being washed in dPBS and spun at 450g for 5 mins. Where relevant, the cells were then stained with the tetramer of interest (attached to PE) for 60 minutes at 37 degrees Celsius (1µg/100µL) in 50 µl of RH10. Before staining, tetramers were centrifuged for 5 minutes at 16000 g at 4°C (refer to chapter 3). After this the cells were stained with the surface panel antibodies (refer to **Tables 2-5 to 2-8** for antibody panel, fluorochromes and concentrations) in 50uL dPBS at room temperature for 30 minutes in the dark. Following this (for those experiments assessing intranuclear markers) the cells were resuspended in 200uL Foxp3 Fixation/Permeabilisation working solution (eBiosciences 005123-43) and left to incubate in the dark at room temperature for 60 minutes. The cells were then spun at 450g for 5 minutes and resuspended in 200uL of permeablisation buffer (10X) (eBiosciences 00-8333-56), then spun again at 450g for 5 mins. The cells were again resuspended in 50uL permeabilsation buffer, this time containing the transcription factor antibodies of interest (**Tables 2.6 and 2.8)**, and incubated at room temperature for 45-60 mins in the dark. Following this the cells were

washed with permeabilisation buffer, spun at 450g for 5 mins, washed in dPBS and spun as previous, and finally resuspended in 200uL dPBS before being run on the LSR II.

Table 2-5: Treg staining panel (bulk PBMCs)

Fluorochrome	Antibody	Concentration	Purpose
BV570	CD3	1:100	T cell marker
AF700	CD4	1:100	CD4 + T cell subsets
BV650	CD8	1:100	CD8 + T cell subsets
APC	CD25	1:50	Treg marker
BV421	CD127	1:50	Treg marker
PE	CD161	1:50	Liver homing marker
PE-Cy7	PD-1	1:50	Exhaustion marker
FITC	FoxP3	1:50	Treg marker
Ex/em 416/451 L34955 APC-Cy7	L/D	1:1000	Live/dead marker

Table 2-6: Transcription factor panel (bulk PBMCs)

Fluorochrome	Antibody	Concentration	Purpose
BV570	CD3	1:100	T cell marker
AF700	CD4	1:100	CD4 + T cell subsets
BV650	CD8	1:100	CD8 + T cell subsets
FITC	CD57	1:50	Cytotoxic CD4+ marker
PerCP-Cy5.5	Tbet	1:50	Transcription factor
PE-Cy7	Eomes	1:50	Transcription factor
PE	Ror-yt	1:50	Transcription factor
BV421	GATA-3	1:50	Transcription factor
APC	Bcl-6	1:50	Transcription factor
Ex/em 416/451 L34955 APC-Cy7	L/D	1:1000	Live/dead marker

Table 2-7: Tetramer surface staining panel

Fluorochrome	Antibody	Concentration	Purpose
BV570	CD3	1:100	T cell marker
AF700	CD4	1:100	CD4 T cell marker
PB	CD127	1:100	T cell memory marker
FITC	CD57	1:50	Cytotoxic CD4+ marker
PerCP-Cy5.5	CD28	1:50	T cell activation marker
PE-Cy7	CCR7	1:50	T cell memory marker
PE	tetramer	1:100	Tetramer
APC	CD45RA	1:50	T cell memory marker
Ex/em 416/451 L34955 APC-Cy7	L/D	1:1000	Live/dead marker
APC-Cy7	CD14	1:100	Monocyte marker
APC-Cy7	CD19	1:100	B cell marker

Table 2-8: Tetramer transcription factor panel

Fluorochrome	Antibody	Concentration	Purpose
BV570	CD3	1:100	T cell marker
AF700	CD4	1:100	CD4 + T cell subsets
PerCP-Cy5.5	Tbet	1:50	Transcription factor
PE-Cy7	Eomes	1:50	Transcription factor
PE	Tetramer	1:100	Tetramer
FITC	Foxp3	1:50	Transcription factor
Ex/em 416/451 L34955 APC-Cy7	L/D	1:1000	Live/dead marker
APC-Cy7	CD14	1:100	Monocyte marker
APC-Cy7	CD19	1:100	B cell marker

2.9.2 INTRACELLULAR CYTOKINE STAINING

ICS staining was done following *in-vitro* expansion. After the cells were harvested they were left to rest overnight in 1mL RH10 at 37 degrees. Following this they were washed and re-suspended in 100uL RH10, and plated out at between 1-5 x 10^5 PBMCs/well in a 96 well U-bottom plate. Peptides were added to the wells (either a single peptide of interest corresponding to the tetramer sequences, or HCV-NS pools F-M) at a concentration of

1ug/mL. The negative control used was DMSO (1uL/well), and the positive control was PMA and Ionomycin (PMA/I) (at 50 and 500ng/mL respectively). After one hour of stimulation Brefeldin A was added (10ug/mL), and the cells were incubated at 37 degrees overnight. The following morning the cells were washed in dPBS and spun at 450g for 5 mins. Live/dead dye was then added followed by FCS as described above. After another wash in dPBS the cells were incubated in FACS fix solution (36mL dPBS + 1mL 37% formaldehyde) for 20 mins at room temperature in the dark. After this the cells were washed in permeabilsation buffer (as described above), followed by a 20 min incubation also in permeabilisation buffer. The cells were then stained with the full panel of antibodies (both cell surface and intracellular) in permeabilisation buffer for 25 minutes in the dark at room temperature. For the full panel of antibodies used refer to **Table 2-7**. Following this step, the wells were topped with permeabilisation buffer, spun, washed in dPBS, spun again and finally resuspended in dPBS before being run on the LSR II.

Table 2-9: ICS panel

Fluorochrome	Antibody	Concentration	Purpose
BV570	**CD3**	1:100	T cell marker
BV421	**CD4**	1:100	CD4 + T cell subsets
PerCP-Cy5.5	**CD8**	1:100	CD8 + T cell subsets
FITC	**CD57**	1:50	Cytotoxic CD4+ T cell
AF700	**IFN-γ**	1:50	Effector cytokine
PE-Cy7	**TNF-α**	1:25	Effector cytokine
APC	**IL-2**	1:25	Effector cytokine
PE	**MIP-1β**	1:50	Effector cytokine
Ex/em 416/451 L34955 APC-Cy7	**Live/dead**	1:1000	Live/dead marker

2.9.3 FLUORESCENCE MINUS ONE (FMO)

For antibodies where there was no obvious positive and negative population, a fluorescence minus one (FMO) control was used. The full panel minus the antibody of interest was stained, and the negative population of the antibody of interest was used to determine the gating strategy for that antibody. This was used for PD-1 and Foxp3. The PD-1 FMO is shown in **Figure 2-3** as an example. This also demonstrates the basic gating strategy to isolate CD4+ and CD8+ T cells used in all panels.

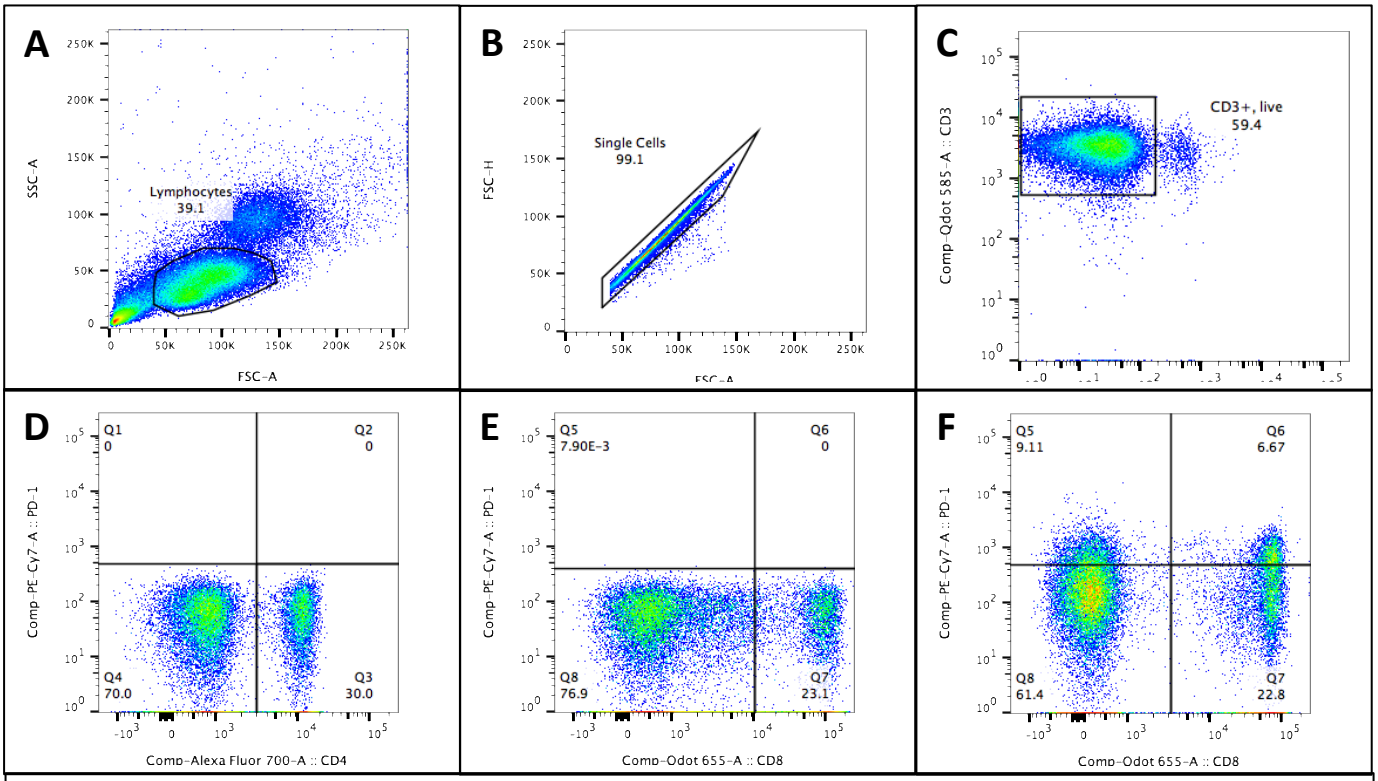


Figure 2-3: Fluorescence minus one (FMO) for PD-1. **(A)** to **(F)** (y-axis followed by x-axis) gating on **(A)** lymphocytes (SSC v FSC-A), **(B)** single cells (FSC-H vs FSC-A) **(C)** CD3 vs live/dead, **(D)** PD-1 vs CD4+, **(E)** PD-1 vs CD8+. **(F)** represents gating of a patient sample (1119 post treatment) demonstrating gating of PD-1 vs CD8+.

2.9.4 TETRAMER STAINING

HCV MHC Class II Tetramer staining was performed predominantly on peptide specific expanded CD4+ T cells following two weeks of culture. Initially the staining was attempted *ex-vivo*, but due to the feeble responses observed this was abandoned. Following harvest at day 13-14 from *in vitro* expansion, the cells were left to rest in 1mL RH10 overnight at 37^0C. The following day the cells were washed in dPBS and stained with live/dead dye and FCS as described previously. Tetramer staining was then done after the tetramers were centrifuged at 4°C (16000g, 4 minutes). The cells were resuspended in 50uL RH10 and 0.5uL of tetramer of interest was added (final concentration 1ug/100mL), and incubated for 60min in the dark at 37°C. Following this the cells were washed in PBS and stained with the remaining cell surface markers (refer to **Table 2-8)** in 50uL PBS at room temperature for 30 mins. Finally, the cells were washed twice and resuspended in dPBS prior to being run on the LSR II.

Table 2-10: MHC Class II tetramer panel

Fluorochrome	Antibody	Concentration	Purpose
BV570	CD3	1:100	T cell marker
AF700	CD4	1:100	CD4 + T cell subsets
BV650	CD8	1:100	CD8 + T cell subsets
PB	CD14	1:100	Dump channel
PB	CD19	1:100	Dump channel
PE	Tetramer	1:100	Tetramer
Ex/em 416/451 L34955 APC-Cy7	L/D	1:1000	Live/dead marker

2.10 REAGENTS

R10 was made using RPMI medium (GibcoBRL 11875-093) plus 10% FCS (Hyclone SH30070.03; heat inactivated by 1hr incubation at 64^{o}C) plus 1% L-glutamine (GibcoBRL 25030-081) and 1% penicillin and streptomycin (pen-strep) (GibcoBRL 15140-122).

RH10 was made using RPMI + L-glutamine + pen-strep as described above, plus 10% heat inactivated human serum (Sigma-Aldrich H4522-100ML).

2.11 ADENOVIRAL VECTOR NEUTRALIZING ANTIBODY TITERS

Anti-adenoviral neutralizing antibody assays were performed on serum samples collected in Oxford at the Okairos laboratory, Napoli, Italy. Recombinant adenoviruses expressing SEAP (secreted alkaline phosphatase) were incubated for 1 hour at 37^{o}C with serial dilutions of sera from volunteers (including a well with no serum). These samples were then added to wells containing human embryonic kidney cell lines (HEK293), incubated for 1h and washed. Expression of SEAP was measured after 24 hours using chemiluminescent substrate (CSPD) from the Phospha-LightTM kit (Tropix Cat No T1016) without heat inactivation. Light emission was monitored 45 minutes after the addition of the SCPD substrate using the Envision 2101 Multi-label reader (Perkin Elmer).

2.12 FACS ANALYSIS

FACS analysis was performed on a BD LSRII machine (Peter Medawar Building, Oxford). Gating and analysis was performed in FlowJo (TreeStar).

2.13 STATISTICAL ANALYSIS

GraphPad prism was used for all statistical analyses and to create all graphs. Non-parametric tests were used: paired (Wilcoxon) T tests were used for matched samples, and unpaired (Mann Whitney) T tests used for unrelated sample comparisons. Correlations were determined using Spearman's rank correlation. Two-tailed p-values were used unless stated, and any p-value <0.05 was considered statistically significant (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$).

CHAPTER 3: DEVELOPMENT OF MHC CLASS II TETRAMERS

3.1 BACKGROUND

3.1.1 CHALLENGES IN IDENTIFYING HCV SPECIFIC T CELLS

The evaluation of antigen specific cells in HCV infection has added to our understanding of HCV pathogenesis, and assessing the phenotype and function of these cells are crucial to vaccine development. However, the ability to study the peptide specific T cell populations has been challenging. In spontaneously resolved and particularly in chronic infection, HCV-specific CD8+ T and CD4+ T cell populations for any given peptide is typically below 1% and 0.001 to 0.1% respectively (Lokhande et al. 2015). This is likely due to a number of factors, including very small memory populations in spontaneous resolution and possible deletion of HCV specific T cell populations in chronic infection (Wherry 2011). Thus the methods used to isolate these rare populations have typically focused on proliferation assays, such as thymidine or CSFE assays, functional assays, such as ICS or IFNγ T cell ELISpot, or by using markers of activation on the T cell surface in response to *in vitro* antigen stimulation, such as CD69 (Mardiney, Brown, and Fleisher 1996), CD154 (Mueller et al. 2010) or OX40 (CD134 + CD25) (Keoshkerian et al. 2012).

These techniques have certain limitations. Firstly, they are each dependant on the evaluation of T cell function, which may be severely impaired in chronic infection. Secondly, these assays may be too insensitive to detect very rare cell populations. Finally, functional assays all involve manipulation of the cell in some way, through either activation or proliferation, and would therefore alter the phenotypic profile of the cell surface

markers or transcription factors from its naïve state, making evaluation of the phenotypic or transcriptomic profile challenging *ex-vivo*.

Another method of detecting HCV specific T cells is to use MHC-peptide multimers (tetramers and pentamers). Multimer complexes consist of four or five (tetramer or pentamer respectively) MHC molecules containing the peptide of interest covalently bound to a streptavidin-biotin complex, tagged with a fluorochrome. Due to this MHC binding the HLA type of the sample must be known, and although not essential, ideally the affinity of the peptide of interest. Multimer assays can be useful in that they can label peptide specific T cell populations without altering the phenotypic profile, and therefore as long as the cell population is sufficiently large, *ex-vivo* phenotypic analysis may be performed.

3.1.2 MHC CLASS II MOLECULE-PEPTIDE BINDING IS BOTH VARIABLE AND PROMISCUOUS

Unlike MHC Class I pentamers in HCV, there has not been a substantial amount of research using Class II tetramers, and as a result there are very few commercially available MHC Class II tetramers. There are two likely reasons for this.

Firstly, as discussed previously, HCV-specific CD4+ T cell populations exist in much smaller numbers and are therefore more challenging to study, particularly *ex-vivo*.

A second reason is that peptide-MHC binding in Class II molecules is both less predictable and promiscuous, and therefore presents challenges in determining optimal peptide and

HLA specificity and commercial tetramer development. This is in contrast to well characterised and predictable binding patterns in MHC Class I-peptide binding complexes. The reasons behind this are as follows. Firstly, peptide degradation in MHC class I antigen presentation involves the use of a proteasome, of which there are only two forms. Both of these forms are well characterised and therefore patterns of antigen degradation are easily predictable. In contrast, the proteases used in the MHC Class II antigen pathways are not well characterised, and as a result the formation of epitopes in this process is not predictable (Nielsen et al. 2010). Secondly, the binding motifs differ between the two molecules. The MHC Class I core binding motif is closed at both ends and therefore can only accommodate 8-10 amino acids, thus the optimal sequences are well defined. On the other hand, the ends of the MHC Class II peptide binding groove are open, and as a result can accommodate variable length and often much longer peptides (Castellino, Zhong, and Germain 1997). Finally, it has been established in the context of other pathogens, such as tetanus toxoid (O'Sullivan et al. 1991), *Plasmodium falciparum* (Sinigaglia et al. 1988) and HIV-1 (Kaufmann et al. 2004) that identical immunodominant CD4+ epitopes display promiscuity and can bind to multiple MHC Class II alleles. This is very different to peptide binding to MHC Class I molecules, which typically displays affinity for a single Class I allele (Rammensee, Friede, and Stevanoviic 1995). These observations present practical challenges in the development of Class II tetramers, and as a result, there are very few commercially available tetramers, and very few studies employing this technology.

However, these observations also have potentially positive implications for vaccine development. The ability of the same immunodominant CD4+ epitope to bind to multiple HLA Class II molecules may be beneficial on a large population scale, as a favourable

response to vaccination may be observed with a diverse combination of HLA types. Furthermore, MHC Class II peptide binding despite sequence variability that has been demonstrated imply that a given sequence used in a vaccine immunogen may be efficacious across different HCV subtypes and even genotypes (Schulze zur Wiesch et al. 2005).

3.1.3 EVALUATION OF CLASS II TETRAMERS IN PREVIOUS STUDIES

To select a panel of Class II tetramers for my experiments, I identified previous studies that developed and employed MHC Class II tetramers in HCV infection.

There are two key papers published that address these issues of variable CD4+ epitope binding and MHC Class II binding promiscuity in HCV infection. The first paper, by Gerlach et al (Gerlach et al. 2005) trialled a set of peptides spanning NS3/4 in 20 patients with either acute or resolved HCV infection. After identifying the most immunodominant epitopes, these epitopes were further mapped to both the minimal and optimal stimulatory sequences using T cell clones and PBMCs. HLA restrictions of these epitopes were also identified, and ranged from 1 to 3 HLA alleles for each epitope studied. Most of the epitopes identified were also highly conserved between genotypes.

The second paper, published by Schulz zur Wiesch (Schulze zur Wiesch et al. 2005) identified immunodominant epitopes in a cohort of spontaneously resolved and chronically infected HCV patients using 20-mer peptides spanning the entire HCV polyprotein. HLA-DR binding assays were then performed on the most commonly

identified peptides, which were correlated with functional assays using patients with various HLA-DR alleles. It was observed that CD4+ Class II epitopes demonstrated a promiscuous binding pattern, and displayed affinity for multiple HLA-DR molecules. Of note, the six most commonly recognised epitopes were able to bind between 7 and 12 different Class II alleles.

Furthermore, a third key paper was identified that employed 8 MHC Class II tetramers in the *ex-vivo* analysis of HCV specific CD4+ T cells in a cohort of patients with acute, chronic and spontaneously resolved HCV infection (Schulze Zur Wiesch et al. 2012).

3.2 AIM

I sought to identify a range of tetramers to be used to identify HCV specific CD4+ T cells of various HLA types. My main aim was to use tetramer assays to identify the magnitude and proliferative capacity of the HCV specific T cell populations, and potentially – if the populations were of sufficient size – to use them for *ex-vivo* phenotypic analysis.

3.3 METHODS

3.3.1 SELECTION

To assemble a panel of tetramers in which to perform my experiments I undertook the following steps. Firstly, where possible, I used a tetramer in which there is published data using the exact tetramer. I then assessed the tetramer peptide sequence in other publications to determine how frequent the epitope was targeted, and only used peptide sequences that were commonly targeted in the study populations. Subsequently I looked at other publications that reported on the binding affinity of the particular peptide with different HLA molecules. This turned out not to be a straightforward process. Although the same immunodominant regions of the Hepatitis C virus appeared in multiple publications, there were slight variations between, and even within publications with regards to both peptide sequences and HLA binding affinity. As discussed in the introduction, this was not unexpected due to the flexible and promiscuous binding patterns of MHC Class II-peptide interactions. For example, one of the tetramers I've used (**Table 3-1;** tetramer 17), has been trialed using this exact sequence as a tetramer (Schulze Zur Wiesch et al. 2012). However, Gerlach, et al (Gerlach et al. 2005) has published three different variations of this sequence – both with DRB1*15 and with other molecules – each with a slightly different 'optimal' and 'minimal' stimulatory sequence. Ultimately due to the peptide binding nature observed in Class II molecules this may have not have made a huge difference. However, as Schulze zur Wiesch et al. (2012) had already trialed the sequence that was used as a tetramer, I ultimately chose this sequence.

There were three tetramers I have used that there was no binding data for. Two of these (**Table 3-1** tetramers 22 and 29) were commercially available from the NIH and so I have trialed them. The third, number 25, was donated by ProImmune for investigational use prior to it being commercially available. The full list of tetramers, including the rationale behind the selection of each can be found in **Table 3-1.**

A commonly used method of selecting MHC Class I tetramers is by using an epitope prediction program. However, although these methods do exist for predicting Class II tetramers (eg TEPITOPE/ProPred), the accuracy and performance prediction remains lower than by using data driven methods (Nielsen et al. 2010)**,** and as such have not been used in the determination of the Class II tetramers I have used.

N	Protein	position	Peptide sequence	HLA restriction	Supporting Evidence
14 (5)	NS4B	1806-1818	TLLFNILGGWVAA*	DRB1*0101	• Schulze zur Wiesch et al. 2012 (exact sequence) • Lucas et al. 2007 • Gerlach et al. 2005 (almost same sequence. Optimal sequence included & HLA restriction)
15 (2)	NS3	1248-1262	GYKVLVLNPSVAATL*	DRB1*0401	• Ulsenheimer et al. 2006 (exact sequence) • Day 2002 – (exact sequence) • Gerlach et al. 2005 (almost exact sequence. 40% cohort response) • Schulze zur Wiesch et al. 2005 (exact sequence except 1aa each end (1247 – 1261)) • Schulze zur Wiesch et al. 2012 (exact sequence)
17 (3)	NS3	1582-1597	NFPYLVAYQATVCARA*	DRB1*1501	• Schulze zur Wiesch et al. 2012 (exact sequence) • Schulze zur Wiesch et al. 2005 (minimal sequence, detected by 30% of cohort • Gerlach et al. 2005 (minimal sequence contained within epitope and with same HLA restriction)
18 (1)	NS3	1411-1425	GINAVAYYRGLDVSV*	DRB1*1501	• Schulze zur Wiesch et al. 2012 (exact sequence) • Gerlach et al. 2005 (minimal and optimal sequence contained within epitope (1406 – 1425)) • Schulze zur Wiesch et al. 2005 (targeted 27% of patients (1411 – 1430))

N	Protein	position	Peptide sequence	HLA restriction	• Supporting Evidence
19	NS3	1535-1551	TTVRLRAYMNTPGLPVC	DRB1*1501	• Schulze zur Wiesch et al. 2005 (1531-1550 – targeted by 36%. Binding data for *15) • Gerlach et al. 2005 (min and opt sequences contained within sequence. Binding data for *15 (02, not 01), recognised by 40%)
20 (4)	NS4B	1771-1790	SGIQYLAGLSTLPGNPAIASL*	DRB1*0401	• Schulze zur Wiesch et al. 2005 (optimal length and HLA specificity contained within sequence, binding data for HLA type, 54% of subject with same peptide) • Schulze zur Wiesch et al. 2012 (exact sequence)
21	NS4B	1773-1790	QYLAGLSTLPGNPAIASL	DRB1*0404	• Schulze zur Wiesch et al. 2012 (exact sequence) • Gerlach et al. 2005 (almost exact sequence with HLA specificity (1775 – 1794), recognised by 55%) • Schulze zur Wiesch et al. 2005 (optimal length (exact sequence), with HLA specificity)
22	NS2	794-810	WPLLLLLLALPQRAYAQ	DRB1*0701	• Commercially available – NIH
24	NS3	1535-1551	TTVRLRAYMNTPGLPVC	DRB1*0701	• Schulze zur Wiesch et al. 2005 (optimal length (minus one aa), same HLA restriction • Gerlach – optimal sequence contained within sequence and HLA restriction
25 (6)	NS5B	2800-2815	VYYLTRDPTTPLARAA**	DRB1*0301	• Donated by ProImmune
29	NS4B	1950-1969	SLTITSLLRRLHNWITSCS	DRB1*0701	• Commercially available – NIH (note this is published as $NS5A_{1957-1975)}$

Table 3-1: List of MHC Class II tetramers used in my experiments. Assigned tetramer number (N), sequence and HLA specificity, and evidence supporting tetramer selection described. *Supplied by both ProImmune, Oxford and the NIH Core Tetramer Facility. **Supplied by ProImmune only. Note: ProImmune supply assigned number in brackets.

3.3.2 SYNTHESIS OF MHC CLASS II TETRAMERS

I sourced the tetramers for my experiments from two suppliers. Firstly, ProImmune in Oxford had tetramers in which they donated for use prior to becoming commercially available (identified as '*' in **Table 3-1**)

The second source was from the NIH Core tetramer facility in Maryland, USA. For tetramers to be synthesised from here an application with evidence of binding for each tetramer had to be provided, which was then reviewed by a panel prior to production. I made a successful application for the production of 10 tetramers. Two of these were commercially available through the NIH, and therefore binding evidence was not necessary. Peptides were obtained from Mimitopes (3-4mg of each, HPLC-purified), from which the yield was divided and sent to both the Core Tetramer Facility (for tetramer synthesis) and to the Peter Medawar Lab (Oxford) to be used in cell lines and for ICS assays.

3.3.3 FACS BASED METHODS

The methods used for this chapter, including PBMC separation, freezing and thawing; short term cell line development and tetramer staining are described in detail in Chapter 2.

3.4 RESULTS

3.4.1 TRIALING PROIMMUNE TETRAMERS

In order to determine if the tetramers were working, I selected two vaccine trial samples (from HCV001 and HCV003) to be used on four of the six tetramers. The trial samples were selected from a trial visit in which there was a positive ELISpot response in the pool in which the tetramer peptide came from. We were also able to appropriately select for HLA restriction (as all vaccine trial volunteers had been HLA typed). The four tetramers trialed were either DRB1*04:01 or DRB1*15:01 restricted, and both volunteers were positive at both of these alleles, therefore all four tetramers could be trialed with each sample. I used an HLA mismatched sample as a negative control. The tetramers and vaccine samples used are summarized in **Table 3-2.** I initially trialed the tetramers on freshly thawed *ex-vivo* samples (**Figure 3-1 B**), however there was very small response, and it was difficult to determine whether there was a true positive population as opposed to background staining.

Proimmune Tetramer no	Vaccine peptide pool	Trial Sample	ELISpot pool response	Trial Sample	ELISpot pool response
1	G	HCV001060 (TW10)	1823 SFU / 10^6 PBMCs	HCV003345 (TW14)	1192 SFU / 10^6 PBMCs
2	F		267 SFU / 10^6 PBMCs		270 SFU / 10^6 PBMCs
3	G		1823 SFU / 10^6 PBMCs		1192 SFU / 10^6 PBMCs
4	H		42 SFU / 10^6 PBMCs		77 SFU / 10^6 PBMCs

Table 3-2: Tetramers and vaccine trial samples used to trial the ProImmune tetramers. Vaccine trial number and vaccine-specific ELISpot result to the vaccine peptide pool corresponding to tetramer sequence shown (for peptide pool description refer to **chapter HCV003).**

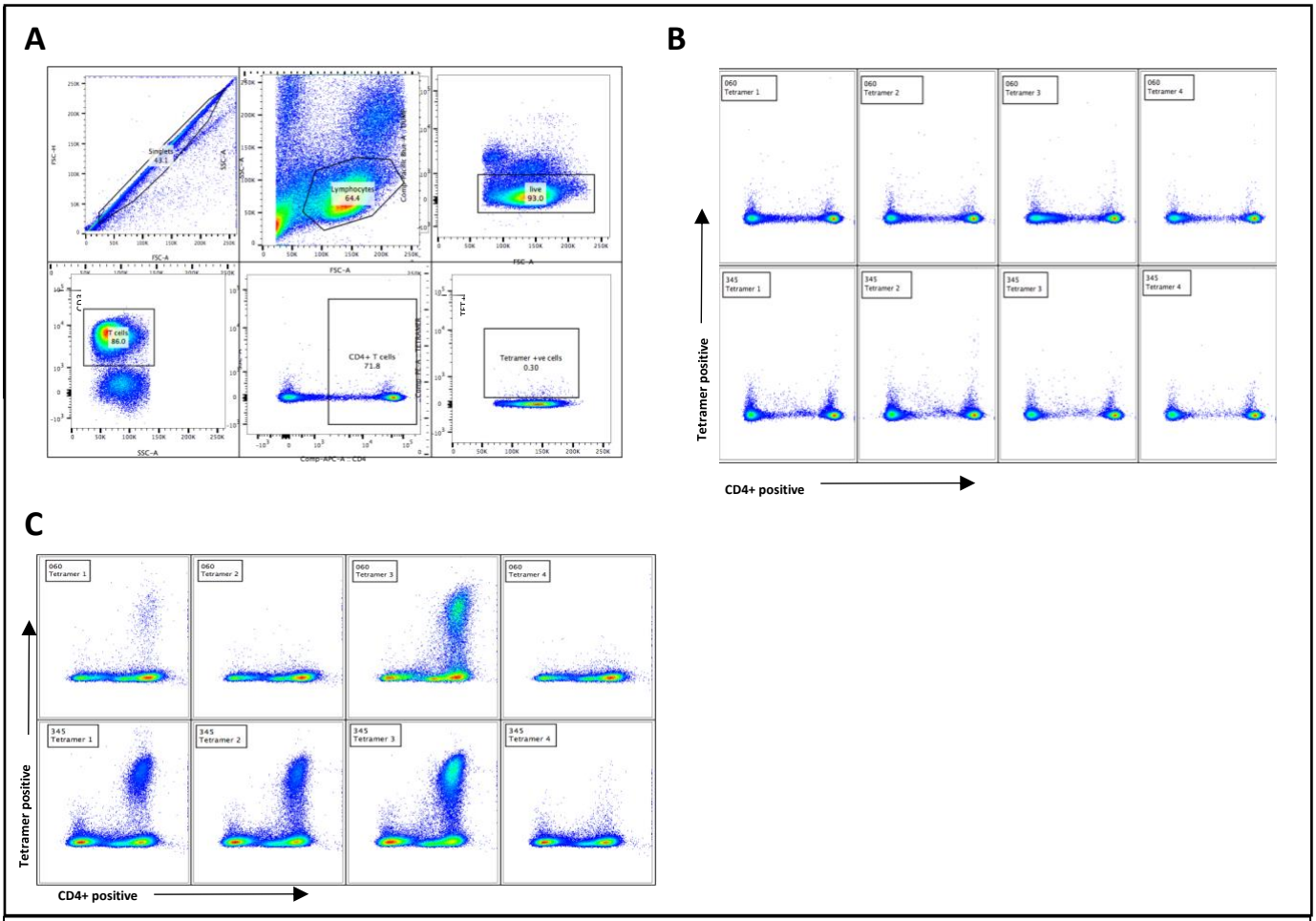


Figure 3-1: ProImmune Tetramer staining with vaccine samples. Staining performed on HCV001060 at trial week 10 and HCV00345 at trial week 14. **(A)** Gating strategy used to identify tetramer positive cells. **(B)** *Ex-vivo* tetramer staining gated on CD3+ lymphocytes. The CD4+ negative population (presumed CD8+ T cells) were used as an internal negative control. **(C)** The same staining following *in vitro* stimulation in a short-term cell line (14 days).

In an attempt to try and identify a true tetramer positive population we grew out short term cell lines with peptide corresponding to the tetramer (described in chapter 2**)**, and then stained again with the tetramer panel following two weeks of culture. For three of the four tetramers, very clear tetramer positive populations could be identified **(Figure 3-1 C** (bottom panel)). The fourth tetramer (#4) showed a very modest tetramer positive cloud (for HCV003345) and little more than background staining (HCV001060). However,

the ELISpot responses in this pool were very small (77 and 42 SFU / 10^6 PBMCs) and therefore a robust response to the peptide may not be expected. HCV001060 also had modest responses to tetramers 1 and 2 despite high magnitude ELISpot responses, however this may be because either the ELISpot responses may have been in response to other peptides in the pool, or, as this trial involved the use of only adenoviral and not MVA vectors – known to induce predominantly CD8+ T cell vaccine responses (Swadling et al. 2014)**,** the ELISpot response may have been largely CD8+ T cell responses. Regardless, these results gave confidence that the tetramers were able to bind HCV specific T cell populations.

The next step was to determine the optimal staining conditions and concentration in which to use the tetramers, and for this I did a titration experiment of the four tetramers using the remainder of the cells from the cell line. For this experiment I chose two tetramers from the previous experiment, one that stained poorly (but still an obvious positive cloud), and one that stained very well. These were tetramer 1 with HCV001060 and tetramer 3 with HCV003345 respectively. The plots for these titrations can be found in **Figure 3-2; A-B**. From these I calculated the staining index and percentage of tetramer positive cells from my samples (**Figure 3-2; C, D)**, and taking into account these values and the efficiency of running experiments and tetramer usage, determined the most efficient method of staining with these tetramers to be 2uL per 50uL, and to stain for 60 minutes.

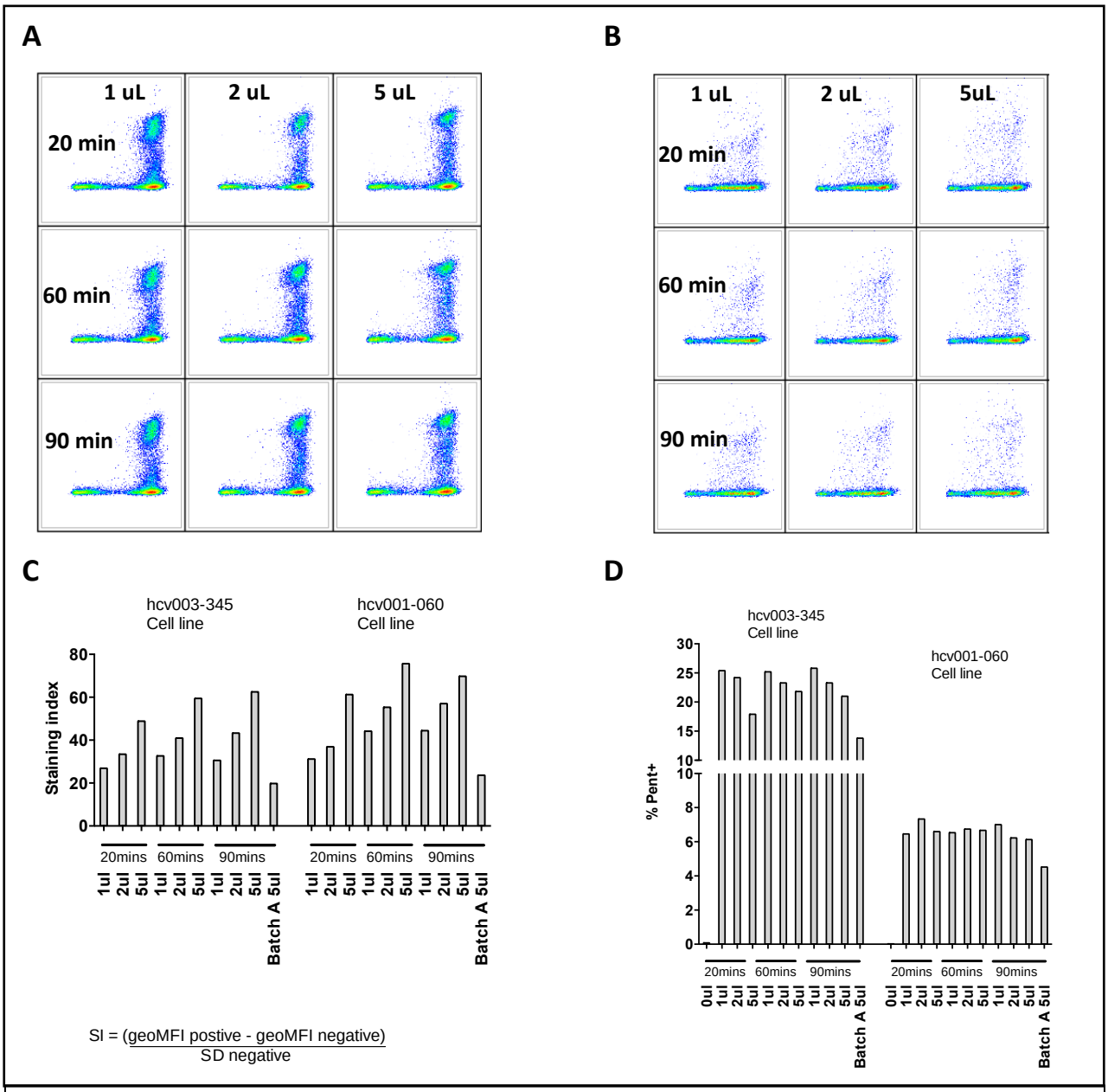


Figure 3-2 MHC Class II tetramer titration experiment. **(A)** HCV001060 (trial week 10) with ProImmune tetramer #1. **(B)** HCV003345 (trial week 14) with ProImmune tetramer #3. Different experiment conditions were trialled for both tetramers (incubation period and tetramer concentration). **(C)** Staining Index and **(D)** percentage of CD4+ tetramer positive cells for each sample.

3.4.2 TRIALING NIH TETRAMERS

Tetramers supplied by the NIH were provided with a staining protocol which was used in all experiments with these tetramers (0.5uL/50uL).

3.4.3 DETERMINING BACKGROUND STAINING

Because the HCV specific tetramer positive CD4+ T cell populations I was trying to identify would likely be very small, it was important to determine the level of background staining and a clear negative cut-off for each tetramer. Each tetramer was trialed with between one and two HLA matched and mismatched healthy individuals. These were HCV001 volunteers at trial screening, and therefore had not been exposed to either HCV infection (HCV antibody and PCR were checked at screening) or HCV peptides. All tetramers demonstrated clean staining, with low levels of background staining (**Figure 3-3)**. This gave confidence that any tetramer clouds in future experiments were true tetramer positive cells and not just background.

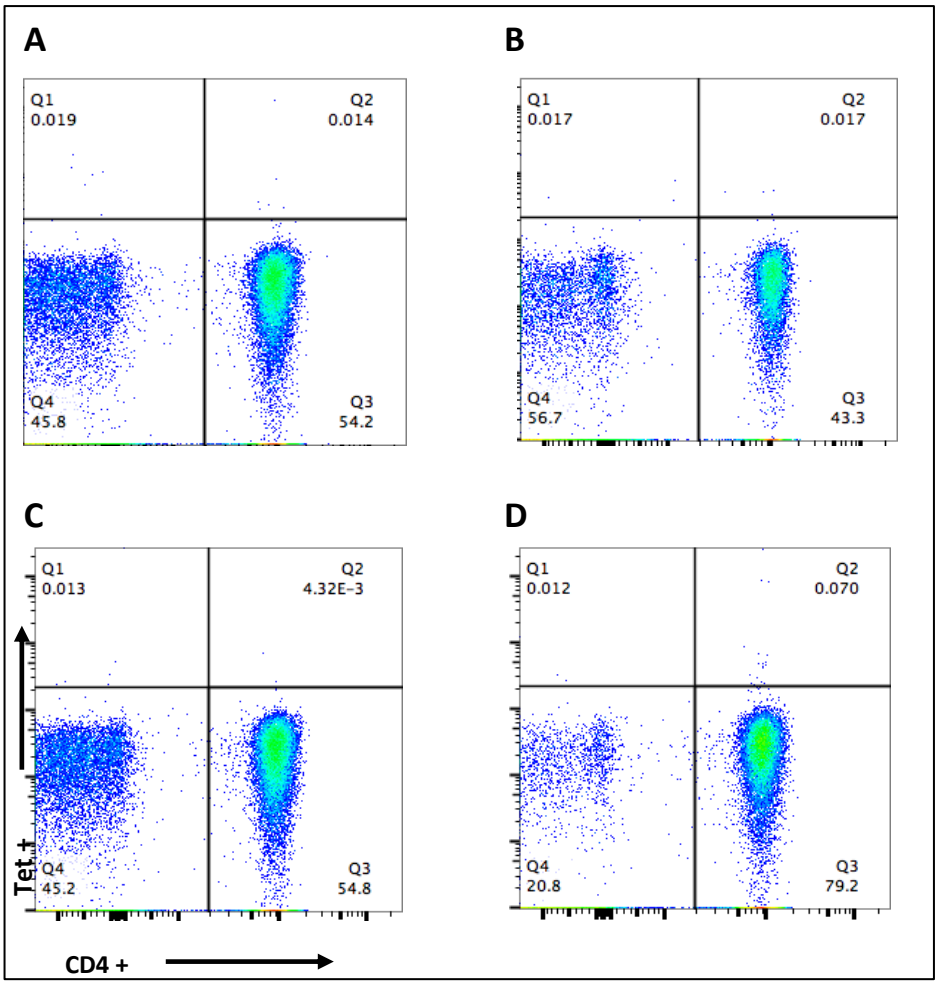


Figure 3-3: Example plots used to determine background level of MHC Class II tetramer staining. (A) tetramer 14 (HLA matched). **(B)** tetramer 14 (HLA mismatched). **(C)** tetramer 15 (HLA matched). **(D)** tetramer 15 (HLA mismatched)

CHAPTER 4: HCV003 TRIAL

4.1 AIM

1. To assess HCV specific T cell responses following re-vaccination with AdCh3NSmut1 / MVA-NSmut

2. Assess HCV specific T cell responses following administration of MVA-NSmut at low doses.

4.2 BACKGROUND

It has been previously shown that a heterologous prime-boost vaccination strategy using viral vectors induce robust HCV specific T cell antiviral responses. Two key Phase I studies in healthy adult volunteers have demonstrated broad, high magnitude and durable HCV specific T cell populations can be produced using viral vectors encoding the non-structural (NS) region of HCV genotype 1b as the HCV immunogen. The first, 'HCV001' (Barnes et al. 2012), used an Ad prime / Ad boost regimen with human Ad6 (Ad6) and Chimpanzee Ad3 (AdCh3). This study demonstrated a very good ability of adenoviral vectors to prime a peptide specific T cell response. However, response to boosting with adenoviral vectors appeared attenuated (**Figure 4-1**). The possible reasons for this are discussed in chapter 1.

The second Phase I vaccine trial, 'HCV003', assessed 10 healthy adults vaccinated with a combination of AdCh3 prime followed by MVA boost – again with both vectors encoding a genotype 1b immunogen – given at weeks 0 and 8 weeks at doses of 2.5×10^{10} vp and 2×10^{8} pfu respectively (Swadling et al. 2014). This showed that the use of MVA rather than

an adenovirus as a boosting vector led to a much higher magnitude T cell response (**Figure 4-1**), and in particular led to induction of a higher proportion of CD4 + T cells than seen with the use of heterologous adenoviral vectors. Additionally, the HCV specific T cells (both CD4+ and CD8+ subsets) that were induced were polyfunctional (**Figure 4-2**), producing significantly higher levels of TNFa, IL-2 and IFNγ than with an adenoviral vector boost. MVA-NSmut was also given to a small number of volunteers as a single vaccination, however no circulating HCV specific lymphocytes could be detected, indicating MVA is not a suitable vector for priming T cells.

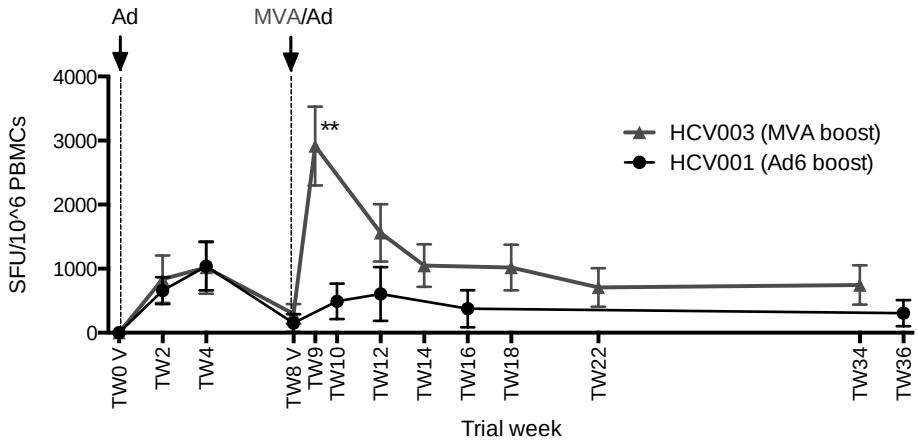


Figure 4-1: T cell magnitude following vaccination with HCV-NS (HCV001 vs HCV003). Total *ex vivo* IFN-γ ELISpot response to the entire NS region of HCV shown over different time points in the trials. Arrows indicate vaccinations. HCV001 (black circles) received AdCh3-NS prime with Ad6-NS boost, and HCV003 (grey triangles) received AdCh3 prime followed by MVA-NS boost. Peak responses for HCV003 (TW9) are significantly higher than peak responses for HCV001 (TW10) (P=0.004). (Swadling et al. 2014). *This work was completed by Leo Swadling and Anthony Brown.*

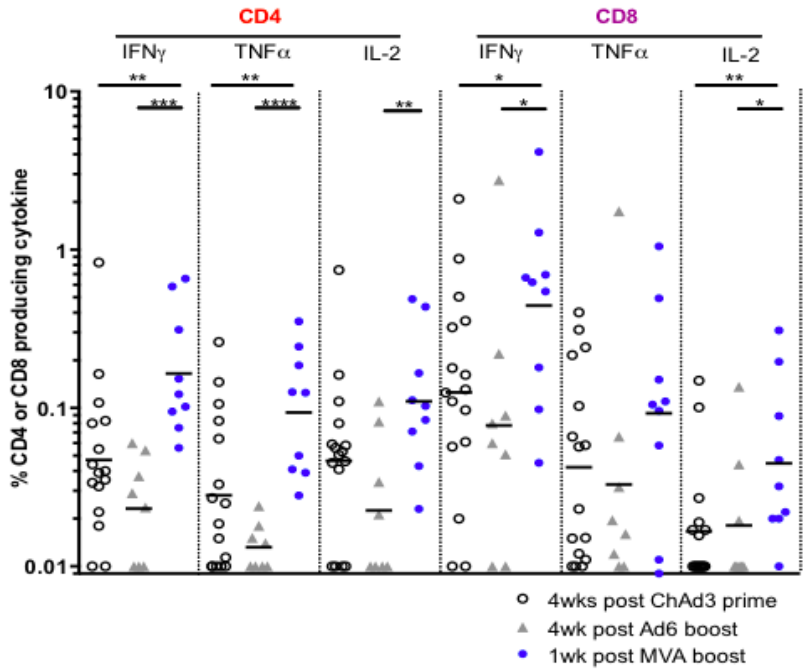


Figure 4-2: Cytokine production of HCV-specific CD4+ and CD8+ T cells following vaccination. A comparison is made of cytokine production by CD4+ and CD8+ T cells at peak time point following vaccination with AdCh3 prime (open circles; TW4, *n*=16); Ad6 boost (grey triangles, TW12, *n*=8) or MVA boost (filled circles, TW9, *n*=9). PBMCs were stimulated with peptide pools F+G+H (NS3-4) or I+L+M (NS5) overnight, and the percentage of cytokine secreting cells were summed with background (DMSO) subtraction. (Swadling et al. 2014). *Completed by Leo Swadling.*

Despite this high peak magnitude of HCV specific T cells in the HCV003 trial, the cell population diminished significantly over time (mean ± SD: 747 ± 690 SFU per 10^6 PBMCs at the end of study visit) (**Figure 4-1**). We sought to develop a strategy to increase the T cell 'set-point'.

We therefore determined firstly whether the HCV specific T cells could be reboosted to raise the memory set-point of the T cell response, and evaluated the optimal timing and vaccine combination in which to achieve this.

To do this we recruited three groups of volunteers (named A3 to A5) to receive additional reboosting vaccination with AdCh3NSmut1 (AdCh3) and MVA-NSmut (MVA-NS) or MVA-NS alone at short (8 weeks), intermediate (32 weeks) or long (average 62, range 39 – 85 week) intervals following the end of the initial prime/boost vaccinations (**Table 4-1**; Groups A3-A5, **Figure 4-3**).

Group	N volunteers	Vaccination and dose
A1	4	TW0: MVA-NS
A2	9	TW0: AdCh3NS; TW8: MVA-NS
A3	5	TW0: AdCh3NS; TW8: MVA-NS; TW16: AdCh3NS; TW24: MVA-NS
A4	4	(recruited from A2 post vacc): TW0: AdCh3NS; TW8: MVA-NS
A5	5	TW0: AdCh3NS; TW8: MVA-NS; TW40 MVA-NS
A6	5	TW0: AdCh3NS; TW8 MVA-NS (2×10^7 pfu)
A7	5	TW0: AdCh3NS; TW8: MVA-NS (2×10^6 pfu)
All AdCh3NS vaccination doses: 2.5×10^{10} vp *All MVA-NS vaccination doses: 2×10^8 pfu unless otherwise stated*		

Table 4-1: HCV003 vaccination and dosing schedule. Groups A3-7 discussed in this chapter. Results from groups A1-A2 discussed in Swadling et al (2014). N=number. TW=trial week.

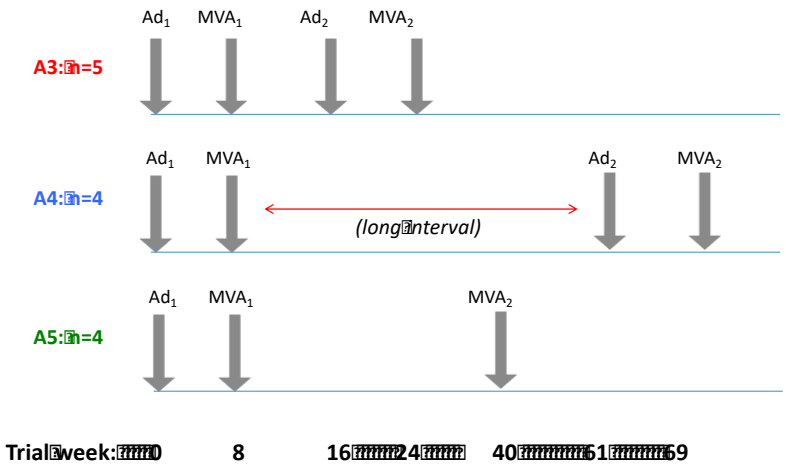


Figure 4-3: Graphical representation of HCV003 groups A3 – A5. A3: short interval AdCh3/MVA re-boost, A4: long interval AdCh3/MVA re-boost, A5: intermediate MVA only reboost.

There are certain technical challenges in manufacturing MVA-NS on a large scale, predominantly due to the constant demand of pathogen free avian cell lines (Jordan et al. 2013), and thus there is a requisite to establish the lowest dose of MVA-NS that can be administered whilst maintaining immunogenicity. Therefore, the final 2 groups of volunteers (groups A6 and A7) were given a single round of prime-boost vaccination with AdCh3 and MVA-NS at weeks 0 and 8 respectively, however the dose of MVA-NS was either one log lower (A6; dose 2 x 10^7 pfu; named 'low') or two logs lower (A7; dose 2 x 10^6 pfu; named 'very low') than the dose given to groups A1-A5.

An earlier group from the same trial will be referred to in the results, and used as a comparison group. This was group A2 (see **Table 4-1**) in which AdCh3 and MVA-NS were

given as heterologous prime-boost vaccinations with an 8-week interval to 9 volunteers at doses 2.5 x 10^{10} vp and 2 x 10^{8} pfu respectively. This work was produced by Anthony Brown and Leo Swadling, and has been published (Swadling et al. 2014).

4.3 HYPOTHESES TO BE TESTED IN THIS CHAPTER:

1. Additional reboosting with heterologous AdCh3 and MVA-NS or MVA-NS alone is safe and well tolerated
2. Additional reboosting with AdCh3 and MVA-NS after a short interval will be less effective than re-boosting after a long interval
3. Additional reboosting with MVA-NS alone is sufficient to enhance the vaccine specific T cell response
4. Administration of MVA-NS at a low dose may successfully boost HCV specific T cells

4.4 CHAPTER SPECIFIC METHODS

4.4.1 VACCINE TRIALS

Vaccine trial recruitment, study visits and treatment of subjects are discussed in detail in chapter 2, however will be briefly re-iterated here. Volunteers were recruited to receive i.m. vaccination with either AdCh3 or MVA-NS into the non-dominant arm. Numbers recruited to each group and vaccinations received are described in **Table 4-1**. Both vaccines encoded the entire non-structural region of HCV genotype 1b BK strain (NS3-

NS5b; approximately 1985 amino acids long). Adverse events were recorded both in a diary card and at clinical review.

4.4.2 IMMUNOASSAYS

The immunoassays performed are fully described in chapter 2. Briefly, freshly harvested PBMCs were stimulated with overlapping peptide pools (F-M) overnight in an IFNγ secreting ELISpot assay. The assay was performed in triplicates. The anti-adeno neutralizing antibody assay was performed in the Okairos laboratory, Italy.

For the remainder of the chapter priming vaccination with AdCh3NSmut1 will be referred to as AdCh3 and boosting vaccination with MVA-NSmut will be referred to as MVA-NS.

4.5 RESULTS

4.5.1 ADMINISTRATION OF ADDITIONAL ADCH3-NS AND MVA-NS IS SAFE AND WELL TOLERATED

In the three groups of volunteers that received additional AdCh3 and MVA-NS or MVA-NS alone (after the initial AdCh3/MVA prime-boost vaccination; groups A3 – A5), additional vaccination resulted in a similar adverse event profile to the first round of AdCh3/MVA-NS. Regarding the AdCh3 vector **(Figure 4-4 A and B)**, after the first round of vaccinations (n=14) 76% of adverse reactions (AE's) were grade I (no disruption to normal activities), 18% grade II (some disruption to daily activities, with or without medication required), and only 6% were considered grade III (significant interruption to activities of daily life, usually

requiring bed rest or absence from work). There was no increased reactogenicity observed when volunteers received an additional AdCh3 vaccination (n=9), with 92% of AE's considered grade I, 8% grade II and nil grade III AE's noted. The three most common AE's noted for the first and second round respectively were local pain, fatigue and headache, and local pain, local warmth and headache.

Regarding the MVA vector, a similar number and severity of adverse events were noted between the first and second round of MVA-NS vaccination (**Figure 4-4 C and D**). There were 68 AE's recorded following primary MVA-NS vaccination, with grade I, II and III being 63%, 34% and 3% respectively. Following repeated MVA-NS administration there were 71 AE's recorded, with 59% grade I, 35% grade II and 6% grade III. Local pain and erythema were the most common AE's noted in both primary and secondary MVA-NS vaccination, followed by local swelling and headache following the first round and fatigue, headache and malaise following the second round. There was one serious adverse event (SAE) occurring in group A5, prior to additional MVA-NS vaccination. This was death following a drug overdose and concluded not to be related to vaccination.

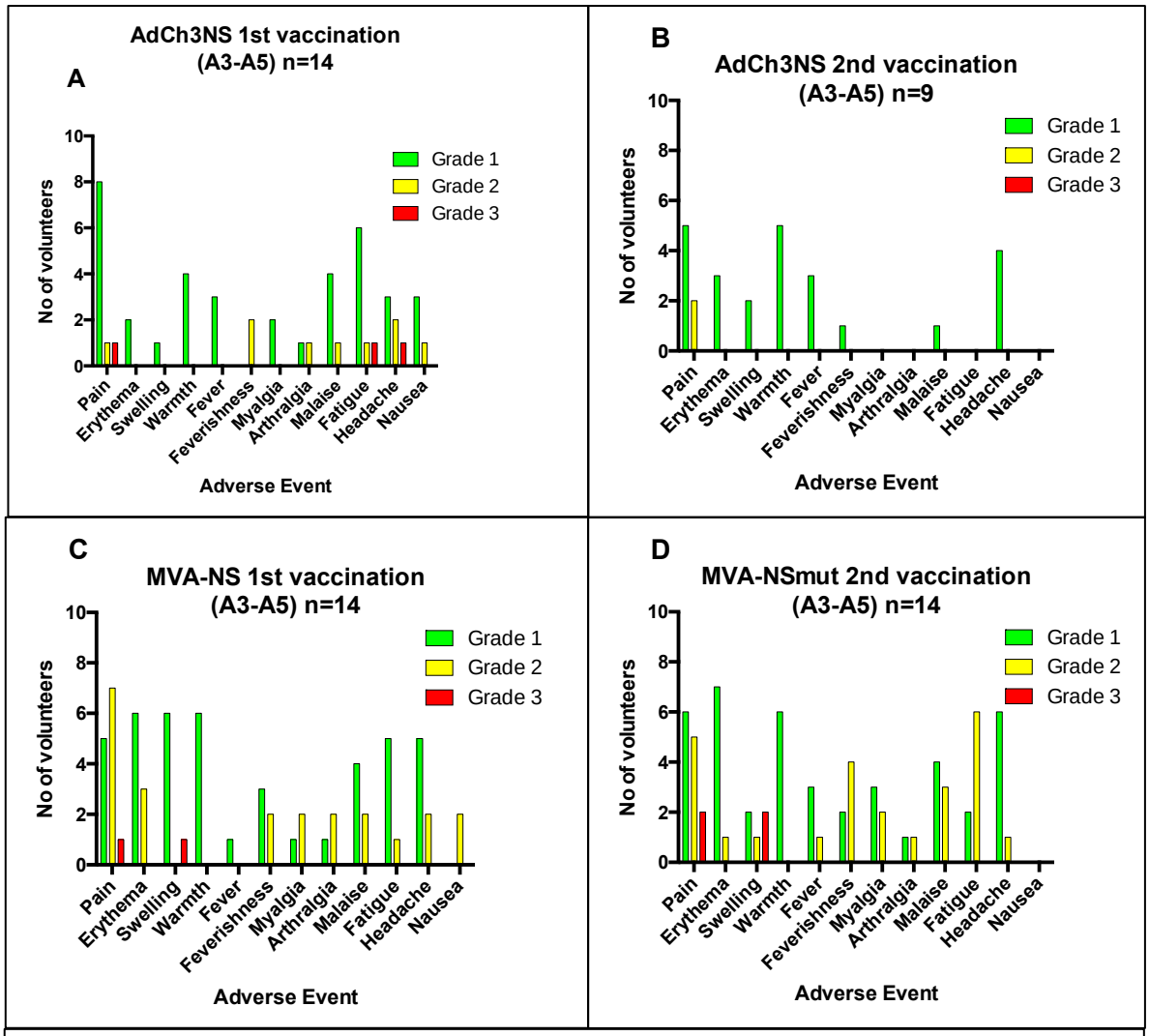


Figure 4-4: Safety data following re-boosting with AdCh3/MVA-NS: Safety data is shown following vaccination with first round AdCh3-NS **(A**; arms A3-A5, *n=14***),** repeated rounds of AdCh3-NS **(B**; arms A3-A4, *n*=9**)**, first round MVA-NS **(C**; arms A3-A5; *n=14*), and repeated rounds of MVA-NS **(D**; arms A3-A5; *n*=14). The number of volunteers for each adverse event (AE) is shown, with only the peak severity of each AE recorded. Each AE is graded from 1 (minimal) to 3 (maximum).

4.5.2 ADDITIONAL RE-BOOSTING OF ADCH3 AND MVA-NS AFTER A SHORT INTERVAL HAS MINIMAL EFFECT ON HCV SPECIFIC T CELL PRODUCTION.

It has previously been demonstrated that heterologous AdCh3/MVA-NS at weeks 0 and 8 optimally induces HCV specific T cells. I sought to answer whether additional doses of these vaccines following a short interval of 8 weeks would further improve immunogenicity.

Five volunteers were recruited to receive reboosting vaccinations after a short interval (**Table 4-1; A3**). These subjects received the initial prime and boost vaccinations AdCh3 (2.5×10^{10} vp) and MVA-NS (2×10^{8} pfu) at weeks 0 and 8, followed by the same vaccine regimen and doses at weeks 16 and 24. They were followed up until trial week 48. Initial prime-boost vaccination induced a similar magnitude of HCV specific T cells compared with that seen previously in group A2. Specifically, the number of SFU's at weeks 0 and 8 were (mean $\pm$ SD) 1039 $\pm$ 1756 and 2292 $\pm$ 1431 SFU per 10^6 PBMCs respectively, with peak numbers occurring one to four weeks following vaccination (**Figure 4-5**).

The second AdCh3 vaccination at week 16 however failed to induce a T cell response at all, and in fact the T cell magnitude actually decreased by 250 SFU per 10^6 PBMCs (mean $\pm$ SD = 821 $\pm$ 1023 SFU per 10^6 PBMCs). The second MVA boosting vaccination at week 24 had a modest effect on peak T cell magnitude (mean $\pm$ SD = 1317 $\pm$ 1641 SFU per 10^6 PBMCs).

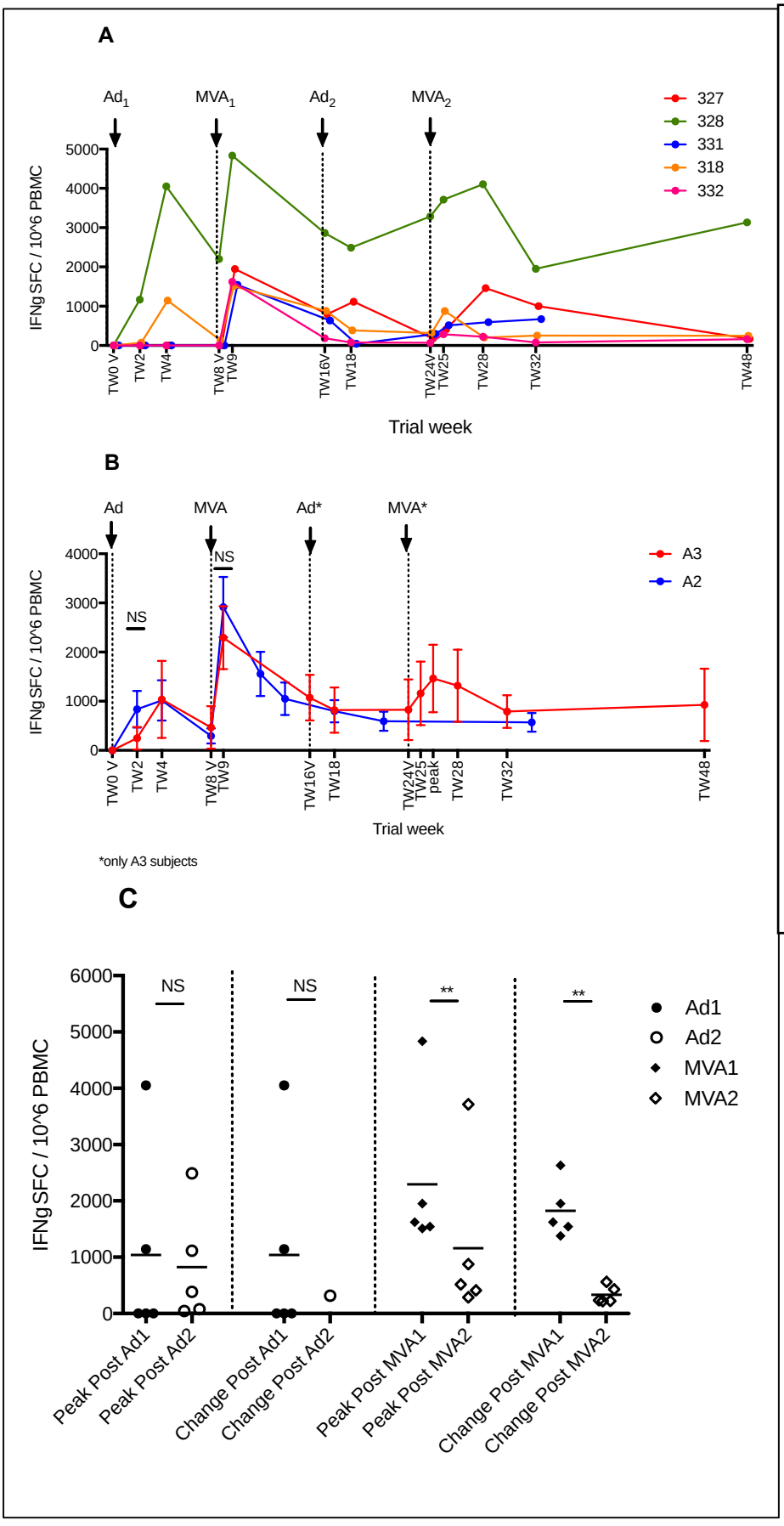


Figure 4-5: Total Magnitude of T cell responses to HCV-NS following revaccination with AdCh3/MVA after a short interval (trial weeks 16 and 24). Total pooled responses to HCV-NS as measured by *ex vivo* IFNγ ELISpot assay (summed positive pools with background (DMSO) subtracted – refer to Materials and Methods). Vaccinations represented by arrows, trial week refers to week since initial vaccination. **(A)** Kinetics of individual responses in subjects receiving additional AdCh3 and MVA at weeks 16 and 24 respectively (volunteer 331 withdrew at TW32). **(B)** Mean T cell magnitude of group A2 (single AdCh3/MVA round) vs group A3 (additional AdCh3/MVA after short interval). **(C)** Comparison of the total HCV-NS response following first and second AdCh3 and MVA. Individual T cell responses (including mean) shown. Peak = peak T cell magnitude following vaccination. Change = change in T cell magnitude following vaccination (peak T cell magnitude (minus) magnitude just prior to vaccination.

One volunteer warrants particular discussion for the robust response generated following both prime and boost vaccination in the first round and MVA boost in the second round. Volunteer 328 generated an ELISpot response of between 4000-5000 IFN-y SFU per million PBMCs in round one of vaccination, which is several fold higher than any other volunteer. This healthy volunteer had never been exposed to HCV antigen prior to the trial. On further analysis, the most likely reason for this was their HLA type. This volunteer had HLA-A*2-11, B*35-62 and DR1*14-13. Published data on previous groups from the same trial (Swadling et al. 2014) have observed high magnitude and broad CD8+ T cell responses in HLA-A*11, A*2 and B*35. It is therefore likely that this individuals high response was a consequence of their particular HLA makeup and capacity to respond to certain HCV epitopes. Although no further analysis was done in this particular individual, it would be interesting to perform further phenotypic studies on this individual.

A comparison of the first and second round of vaccinations was made in two ways, firstly by measuring the peak HCV-specific T cell frequency induced by the vaccine, and secondly by comparing the change in T cell frequency from the time point preceding vaccination to the peak response following vaccination (**Figure 4-5 C**). Following AdCh3 vaccination, similar responses following first and second vaccination were observed by measuring the peak ELISpot response. However, when the response was measured by the change in cell frequency, the T cell magnitude following AdCh3 reboosting dropped from (mean $\pm$ SD) 822 $\pm$ 1027 to -251 $\pm$ 366 SFU per 10^6 PBMCs. This marked drop failed to reach statistical significance however, possibly due to the absence of any T cell induction in 3 of the 5 volunteers following initial AdCh3 vaccination.

A comparison between the first and second round MVA-NS vaccination showed a significantly lower frequency of antigen specific T cells when measured by both peak T cell response following vaccination (p=0.001) and change in T cell response between pre-vaccination T cell frequency and peak frequency following MVA-NS vaccination (p=0.002).

The final visit occurred at trial week 48. 4 of the 5 volunteers were followed up to the last time point, however one volunteer (331) withdrew at the time point earlier (trial week 32). The HCV specific T cell response seen with ELISpot at trial week 48 was (mean $\pm$ SD) 928 $\pm$ 1472 SFU per 10^6 PBMCs. This was compared to the final visit of the group receiving a single round AdCh3/MVA-NS at weeks 0 and 8 (**Table 4-1: Group A2**). In these volunteers, the end of trial (EOT) visit occurred 26 weeks following last vaccination, and the mean T cell frequency for this group was 571 $\pm$ 192 SFU per 10^6 PBMCs (mean $\pm$ SD) (**Figure 4-5 B**). Although the time period between last vaccination and EOT visit differed slightly between the groups (two weeks), no significant difference was observed in the EOT T cell frequency between single round and multiple rounds of AdCh3/MVA-NS (p=0.67).

The breadth of the immune response to the immunogen is also an important measure of the effectiveness of vaccination. In natural infection, a correlate of a successful immune response is one that targets a broad number of peptides, particularly contained within the non-structural proteins (discussed in chapter 1). In this group the breadth of the immune response was measured by determining both the number of positive peptide pools (F-M) and the number of IFNγ secreting T cells in each pool. In order to compare the breadth of the response between single and repeated rounds of vaccination with AdCh3NS and MVA-NS, the T cell frequency in each pool and the number of positive peptide pools were

measured at peak T cell response following each round of vaccination, which occurred one week following MVA-NS administration (i.e. TW9 and TW25 (**Figure 4-6**). The peptide specific T cell frequency in individual pools was greater in every pool following initial vaccination compared with repeated vaccination. This reached statistical significance in pool F (p=0.04), pool G (p=0.016), and pool H (p=0.002). The number of positive pools targeted was also significantly higher at peak response following initial round vs subsequent round of vaccination (p=0.03).

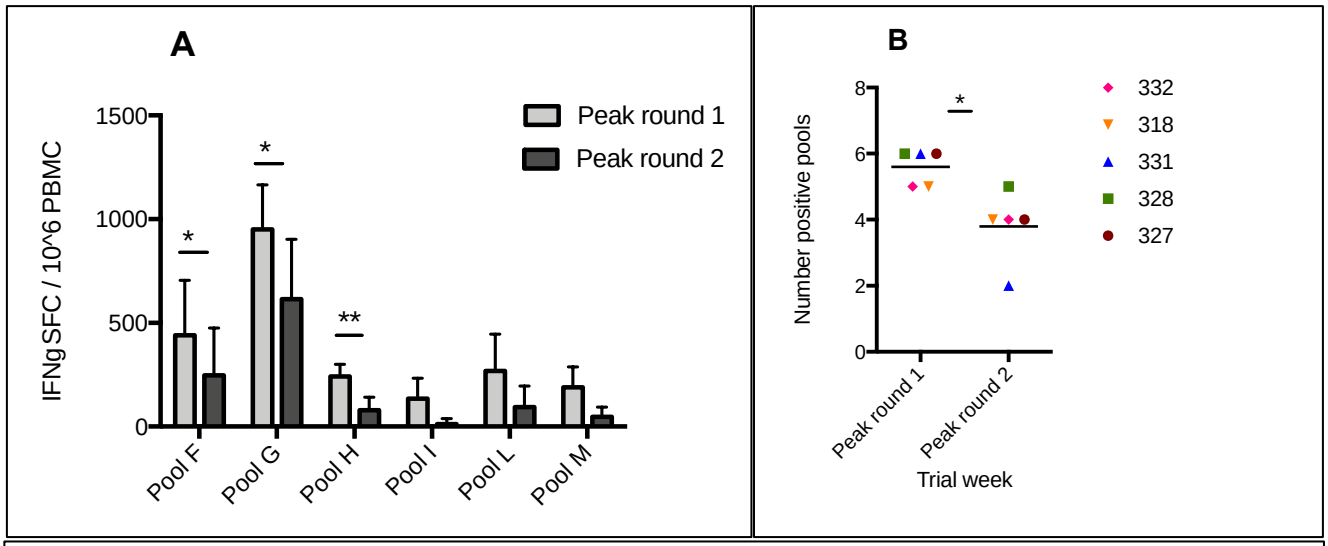


Figure 4-6: Breadth of T cell responses following re-boosting with AdCh3/MVA-NS after a short interval: Comparison of breadth of T cell response between the first and second round of AdCh3/MVA in the same individuals is made by measuring *ex vivo* IFNγ ELISpot T cell magnitude to peptide pools F-M. **(A)** The T cell magnitude to individual peptide pools comparing the first and second round of AdCh3/MVA vaccination at weeks 0 and 8 (TW9; light grey, *n*=5) with subsequent re-vaccination of the same vaccine regimen at weeks 16 and 24 (TW25; dark grey, *n*=5). **(B)** The number of positive peptide pools targeted at trial week 9 vs trial week 25 (*n*=5) (determination of positive pool cut off described in Materials and Methods).

4.5.3 ADDITIONAL RE-BOOSTING OF ADCH3 AND MVA-NS AFTER A PROLONGED PERIOD OF TIME INDUCES A ROBUST HCV SPECIFIC T CELL RESPONSE

To assess the effect of additional vaccination after a longer interval, four volunteers were recruited from Group A2 after the first round of vaccination to receive additional doses of AdCh3 and MVA-NS. All four volunteers had a robust response to initial prime and boost vaccination (mean ± SD = 1104 ± 502 SFU per 10^6 PBMCs and 3863 ± 1884 SFU per 10^6 PBMCs for AdCh3 and MVA-NS respectively) (**Figure 4-7 A and B**). The interval after the first round of vaccinations varied between volunteers, but was on average 62 weeks following initial MVA-NS administration (range 39 to 85 weeks). The doses of AdCh3 and MVA-NS were the same as the first round, and were again given 8 weeks apart. All four volunteers responded to the second AdCh3 vaccine, peaking between two and four weeks following vaccination (mean ± SD = 1288 ± 879 SFU per 10^6 PBMCs). Similarly, all volunteers demonstrated a robust T cell response following the second MVA-NS, with responses peaking between one and three weeks following boost (mean ± SD = 3213 ± 2512 SFU per 10^6 PBMCs) **(Figure 4-7; A and B**).

All four volunteers were followed up until TW32, which was 24 weeks following last vaccination. The mean ELISpot T cell response at the EOT visit was (mean ± SD) 1520 ± 1406 SFU per 10^6 PBMCs. (**Figure 4-7; A and B**).

Volunteer 333 demonstrated a more robust response than other individuals in this group. This individual had HLA-A*11, B*39-57 and DRB1*1. They correspond to volunteer 304 from group A2 (Swadling et al. 2014) and further individual analysis in this group observed

this volunteer to have a high magnitude and broad HCV specific CD8+ T cell response to antigen. Furthermore, in chapters 6 and 7 of this thesis, a tetramer containing DRB1*1 demonstrates robust HCV specific CD4+ T cell responses. Therefore it is likely this individuals HLA makeup has resulted in a high ELIspot response.

A comparison between the initial and subsequent rounds of vaccination was made again by comparing the peak frequency of IFNγ secreting T cells as well as the change in frequency from just prior to vaccination. Both methods of analysis resulted in a similar T cell magnitude, with no statistically significant difference observed. (**Figure 4-7; C**)

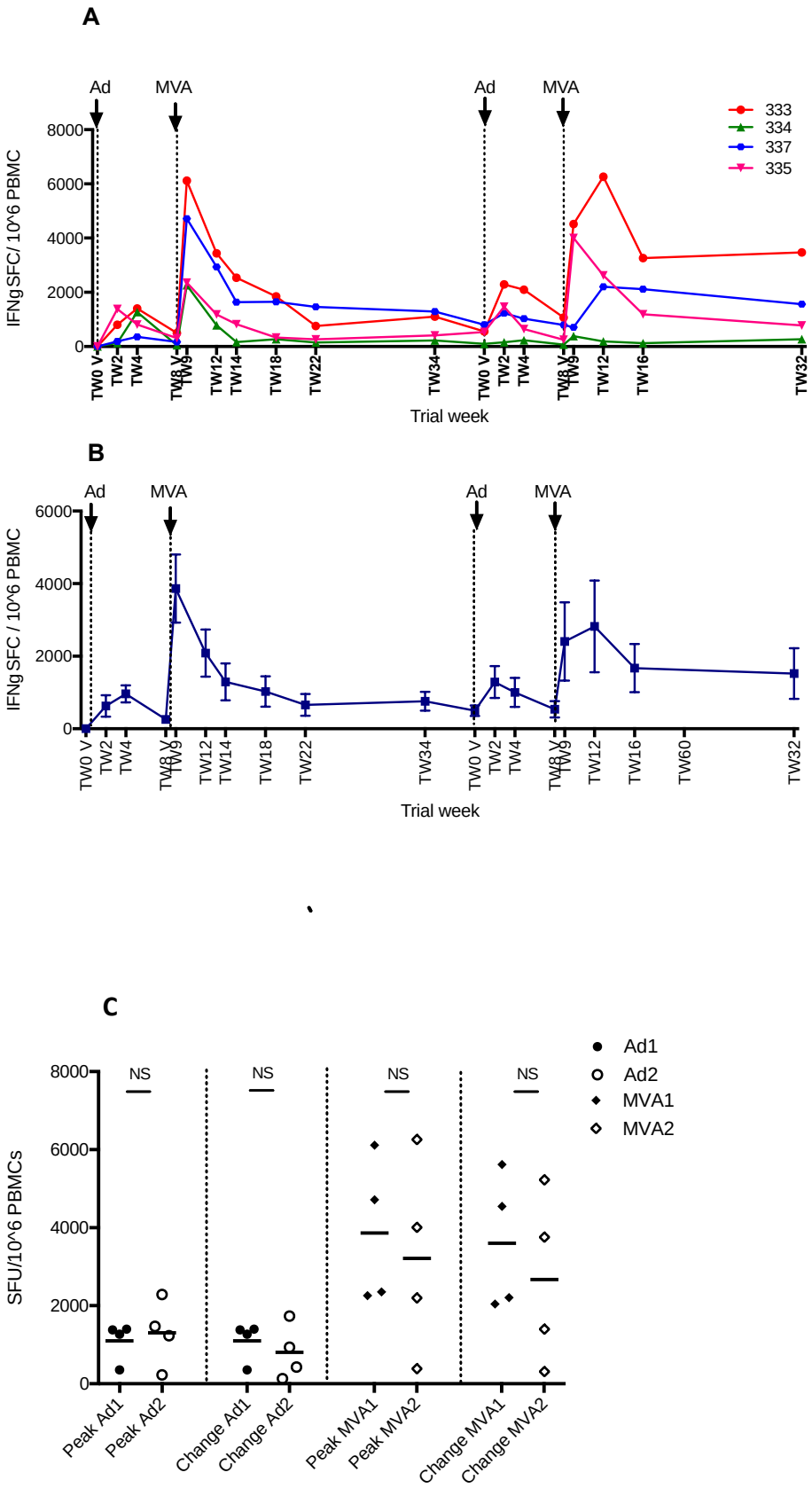


Figure 4-7: Magnitude of T cell responses to HCV-NS following revaccination after a long interval. Total pooled responses to HCV-NS as measured by *ex vivo* IFNγ ELISpot assay (summed positive pools with background (DMSO) subtracted – refer to Materials and Methods). Vaccinations represented by arrows, trial week refers to week since initial vaccination. **(A)** Kinetics of individual responses in subjects receiving additional AdCh3 and MVA-NS following a longer interval (average 62 [range 39-85] weeks) between first and second rounds of AdCh3/MVA. **(B)** Mean response of individuals receiving additional vaccination after a long interval. **(C)** Comparison of the total HCV-NS response following first and second AdCh3 and MVA-NS. Individual T cell responses plus mean shown, with peak magnitude calculated as the largest response following vaccination and change in magnitude calculated from the time point preceding vaccination.

The breadth of the response to HCV-NS was again determined by comparing the frequency of IFNγ secreting T cells in each peptide pool following initial and subsequent vaccination rounds, as well as the number of positive peptide pools at these time points (**Figure 4-8; A-B**). The magnitude of the individual pools varied between the first and second vaccination rounds. Pools F, H and I were all greater following the first round, reaching statistical significance in Pool F (p=0.04). However, the response magnitude trended towards being greater following the second vaccination round in Pools G, L and M (not statistically significant). There was no significant difference between the first and second vaccination round comparing the number of positive peptide pools.

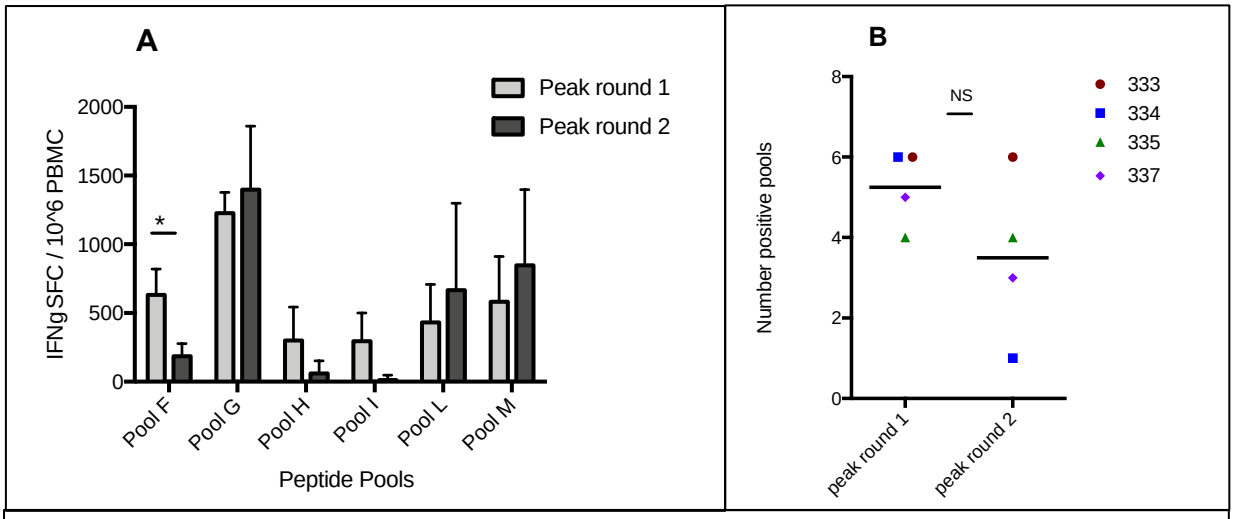


Figure 4-8: Breadth of T cell responses following re-boosting with AdCh3/MVA-NS after a long interval: Comparison of breadth of T cell response between the first and second round of AdCh3/MVA-NS in the same individuals is made by measuring ex vivo IFNγ ELISpot T cell magnitude to peptide pools F-M. **(A)** The T cell magnitude to individual peptide pools comparing the peak response following the first round (light grey; n=4) and second round (dark grey; n=4) of AdCh3/MVA-NS **(B)** The number of positive peptide pools at peak following the first and second round of vaccinations (determination of positive pool cut-off described in Materials and Methods). Paired T tests used.

4.5.4 REBOOSTING WITH MVA-NS ALONE INDUCES A SIMILAR T CELL FREQUENCY TO ADCH3 AND MVA-NS

It has already been shown (Swadling et al. 2014) that MVA-NS when given alone as a priming vaccine is unable to stimulate an immune response. However, to re-stimulate the HCV specific immune response following initial prime-boost, it is unknown whether MVA-NS alone may be sufficient to re-boost the immune response, or whether it would need to be given in conjunction with AdCh3. We sought to answer this question by administering a single dose of MVA-NS following initial round of AdCh3/MVA-NS.

In this group, five volunteers were recruited to receive the same prime and boost regimen as the other groups – AdCh3 (dose 2.5 x 10^{10} vp) and MVA-NS (2 x 10^{8} pfu) at weeks 0 and 8 – with a single additional MVA-NS boost at trial week 40, at a dose of 2 x 10^{8} pfu. The volunteers were then followed for 12 weeks following vaccination until trial week 52. There was a loss to follow up for one volunteer (volunteer 341) prior to the second MVA-NS boost.

The frequency of IFNγ secreting T cells on ELISpot results following initial prime and boost was consistent with previous groups (mean $\pm$ SD = 944 $\pm$ 808 SFU per 10^{6} PBMCs and 4205 $\pm$ 1947 SFU per 10^{6} PBMCs respectively). MVA-NS reboosting at week 40 induced a peptide specific T cell response in all 4 volunteers. Peak ELISpot responses occurred between one and four weeks following vaccination (mean $\pm$ SD = 2388 $\pm$ 1207 SFU per 10^{6} PBMCs) (**Figure 4-9; A and B**).

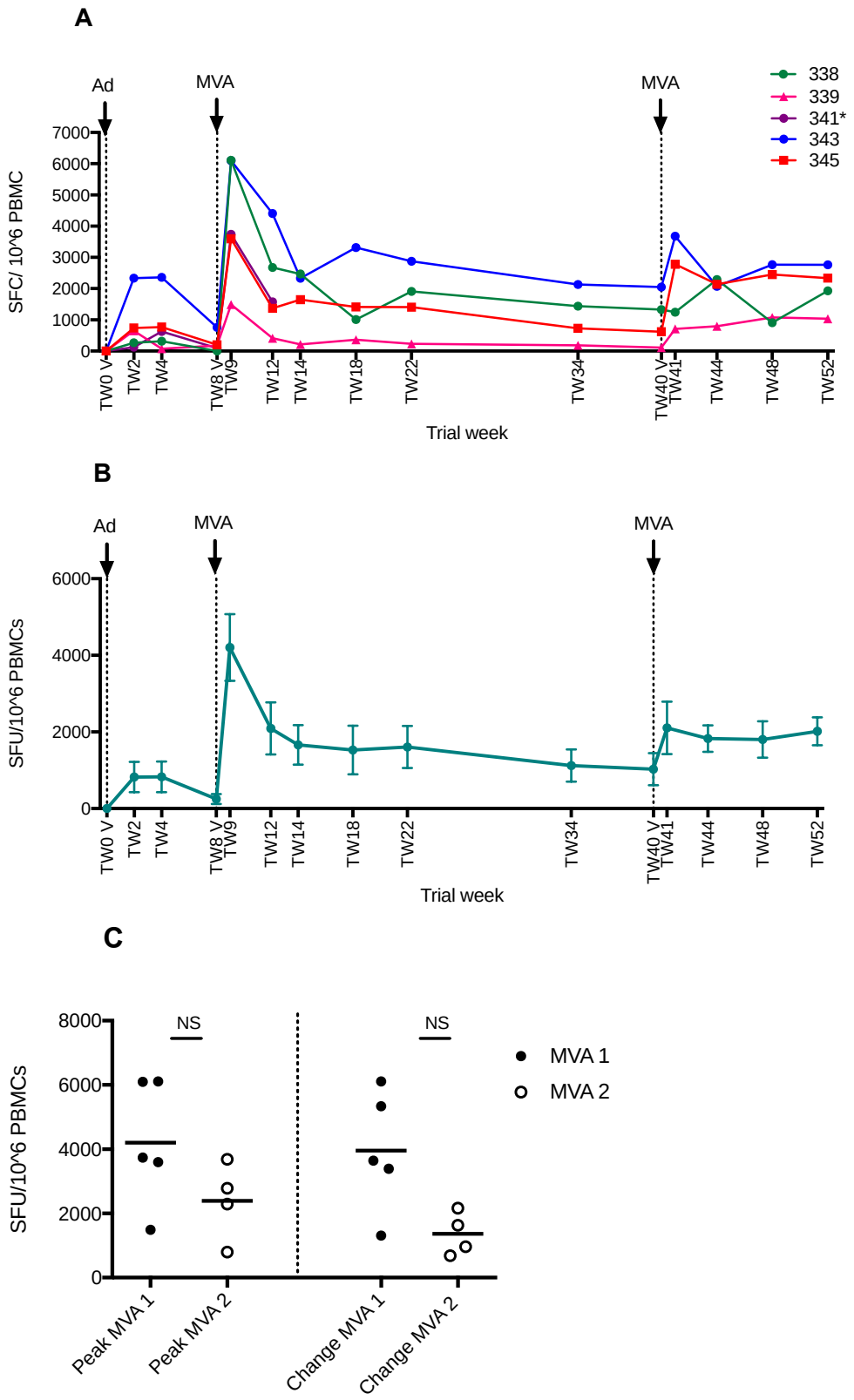


Figure 4-9: Magnitude of T cell responses to HCV-NS following revaccination with MVA only.
Total pooled responses to HCV-NS as measured by *ex vivo* IFNγ ELISpot assay (summed positive pools with background (DMSO) subtracted – refer to Materials and Methods). Vaccinations represented by arrows, trial week refers to week since initial vaccination. **(A)** Kinetics of individual responses in subjects receiving additional MVA at week 40 (*n*=5). *341 lost to follow up after week 12. **(B)** Mean response of individuals receiving additional MVA-NS. **(C)** Comparison of the total HCV-NS response following first and second MVA. Individual T cell responses plus mean shown, with peak magnitude calculated as the largest response following vaccination and change in magnitude calculated from the time point preceding vaccination. Paired T tests used.

As with the short and long interval reboosting groups, a comparison was made between the first and second round of vaccinations (**Figure 4-9; C**). Again, both the peak T cell frequency following MVA-NS and the change in T cell magnitude from pre- to post- MVA-NS were measured. There was no statistically significant difference between the two rounds of vaccination (p=0.07 and 0.08 for the peak and change respectively).

The breadth of response was also measured following the first and second MVA-NS administration (**Figure 4-10; A and B**). In all 6 peptide pools, there was a higher frequency of HCV specific T cells induced by the first vaccination round, which reached statistical significance in 2 pools – F and H (p=0.037 and p=0.036 respectively). There was no significant difference with regards to the number of positive peptide pools following first and second vaccination round.

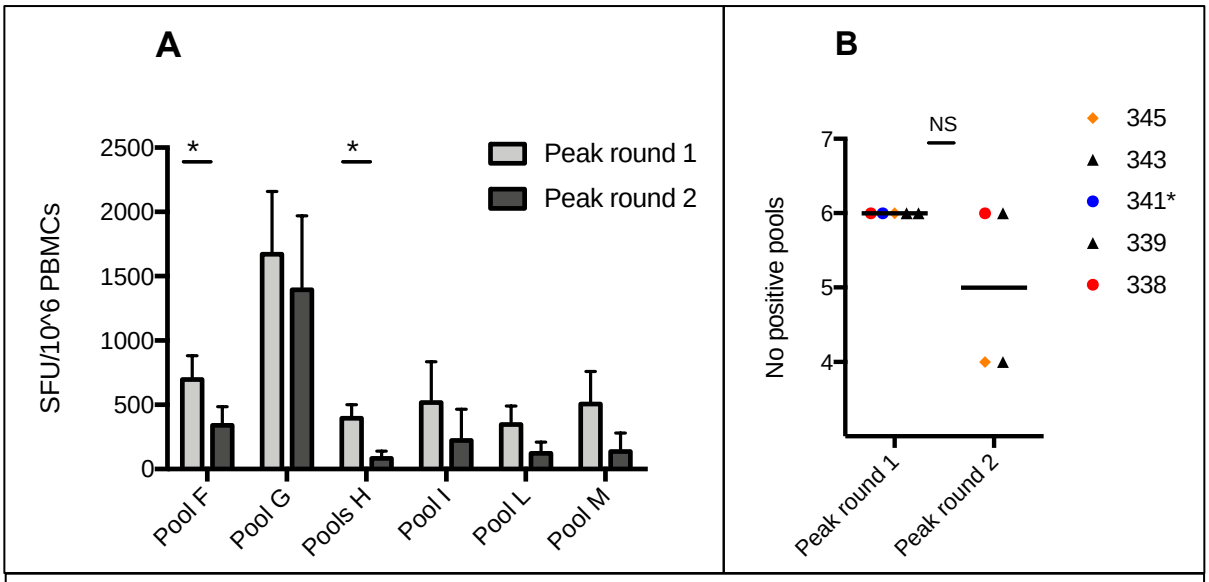


Figure 4-10: Breadth of T cell responses after re-boosting with MVA-NS only. Comparison of breadth of T cell response between primary AdCh3/MVA-NS and secondary MVA-NS alone is made by measuring ex vivo IFNγ ELISpot T cell magnitude to peptide pools F-M. **(A)** The T cell magnitude to individual peptide pools comparing the peak response following the first (light grey; n=5) and second (dark grey; n=4) administration of MVA-NS. **(B)** The number of positive peptide pools at peak following the first and second round of vaccinations (determination of positive pool cut-off described in Materials and Methods). Paired T tests used.

4.5.5 COMPARISON OF THE PEAK AND END OF TRIAL RESPONSES BETWEEN THE ADDITIONAL REBOOSTING GROUPS

A comparison was made between the three groups that received an additional round of vaccination. Specifically, we wanted to determine whether there were any differences between the peak magnitude of the HCV specific T cell response, the breadth of the response at peak, and the HCV specific T cell magnitude at the end of the trial. There were no results that reached statistically significant differences, however this may have been due to the small study population. Therefore, focus has been made on a description of the groups and trends.

The group receiving additional AdCh3 and MVA-NS after a long interval (A4) had the highest number of IFNγ secreting T cells at peak response following the second round of vaccination (occurring 1 to 4 weeks following second MVA-NS). The second highest peak response following additional vaccination was the single MVA-NS administration group (A5), and the smallest peak was in the short interval group (A3) (**Figure 4-11; B**).

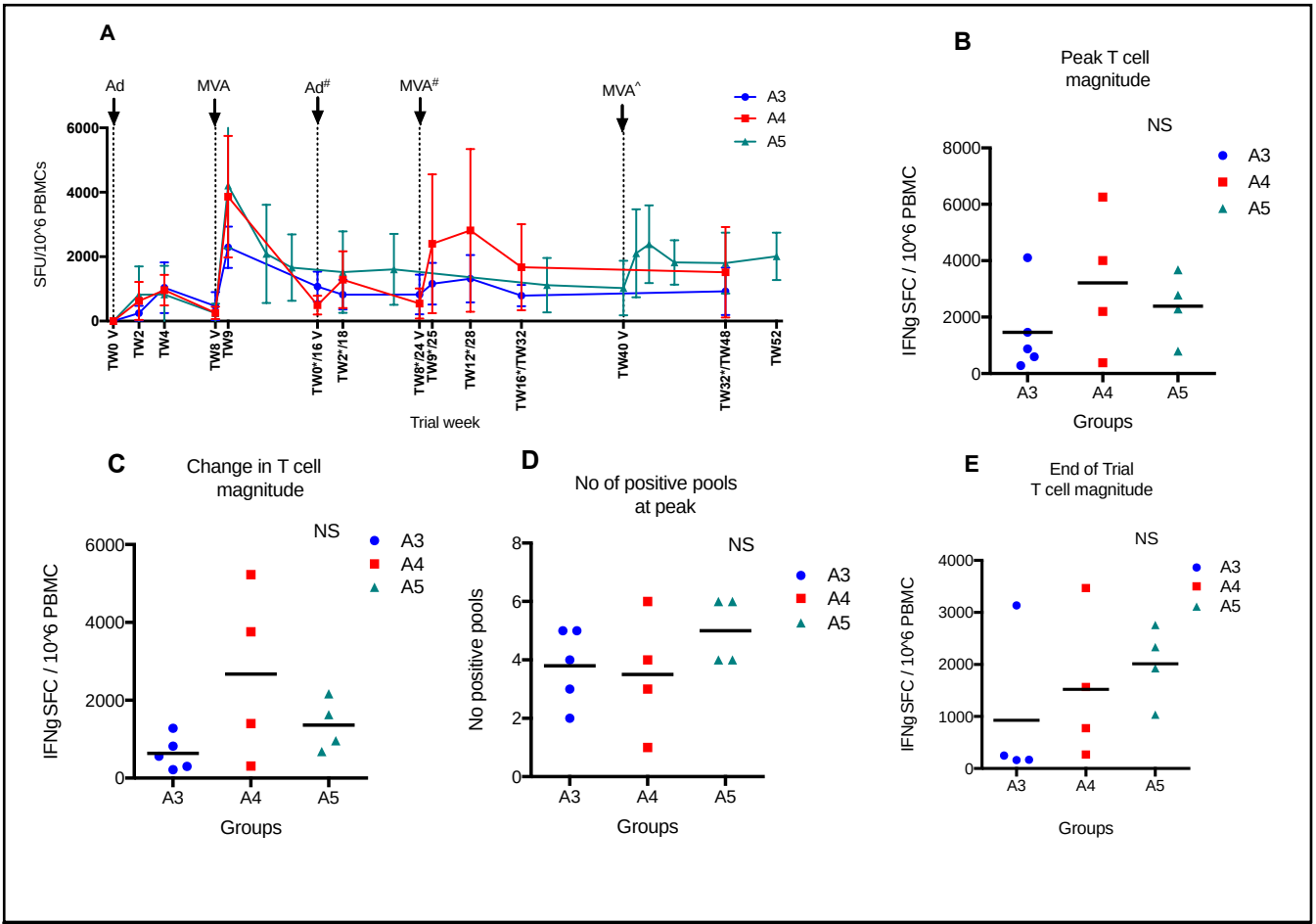


Figure 4-11 Comparison between Groups A3 – A5 (short interval AdCh3/MVA-NS, long interval AdCh3/MVA-NS, MVA-NS only reboost). Reponses measured by *ex vivo* IFNγ secreting ELISpot assay. **(A)** Mean ELISpot responses. #represents AdCh3 and MVA-NS reboosting for Group A4 superimposed on A3 timeline for comparison. ^MVA-NS reboost (Group A5). **(B)** Peak T cell magnitude between Groups A3-5 following reboosting vaccination round **(C)** change in T cell magnitude following second round of vaccination **(D)** number of positive peptide pools following second vaccination round. **(E)** End of trial T cell magnitude for Groups A3-A5. One-way ANOVA used.

Calculating the difference in frequency of IFNγ secreting T cells (peak minus prior to vaccination), the same pattern of results was observed. However, the difference between A3 and A4 was more pronounced, approaching statistical significance (p=0.08) (**Figure 4-11; C**).

A comparison between the breadth of T cell response between the groups was determined by measuring the number of positive peptide pools at peak response following the second round of vaccination. The breadth of the response was comparable between the groups, though was greatest in the single MVA-NS administration group (mean = 5, range 4-6), followed by the short duration (mean 3.8, range 2-5) and finally the long duration (mean 3.5, range 1-6) (**Figure 4-11, D**). However, the group numbers are small and it is difficult to make a detailed comparison beyond a descriptive analysis.

Finally, the end of trial response was compared by measuring the ELISpot T cell magnitude across all the peptide pools at the final visit (**Figure 4-11, E**). The group receiving a single MVA-NS at TW40 (A5) had the highest frequency of IFNγ secreting T cells at this time point, followed by the long interval group (A4). Group A3 (short interval) had the smallest frequency (mean 928 SFU per 10^6 PBMCs). However, it should be noted that the final visit for A5 occurred only 12 weeks following vaccination (TW52), whereas for the other two groups the final visit occurred 24 weeks following vaccination. It could be hypothesized that this observation is time dependent, and it may be expected that the T cell frequency in A5 would continue to contract over time.

4.5.6 THE EFFECT OF NEUTRALIZING ANTIBODIES ON REVACCINATION WITH ADENOVIRAL VECTOR

Next, I sought to understand why a better T cell response is seen following a longer interval between AdCh3/MVA-NS and subsequent vaccinations. One possibility is that the presence of circulating adenovirus-specific antibodies (anti-vector or anti-adeno antibodies) induced by primary adenoviral vector administration could result in an attenuated response to re-vaccination, due to clearance of the adenovirus from circulation by the humoral response before Ad-encoded HCV proteins can be expressed, and that circulating anti-adeno antibody levels are higher after only a short interval. To monitor the potential influence of anti-vector antibodies on revaccination with AdCh3, titers of anti-adeno antibodies were measured at multiple time points throughout the trial. A SEAP assay was used to do this (explained in detail in chapter 2).

The two groups of the trial this is most relevant to are the groups that received additional adenoviral vector administration – A3 and A4. The kinetics of the individual and mean anti-adeno antibodies titers are shown in **Figure 4-12; A-C**. The positive cut-off for anti-adeno antibodies has been previously determined as 300 (McElrath et al. 2008)**.** In the short interval revaccination group (A3), four of the five individuals had a positive antibody titer at the time of receiving AdCh3 re-boost (mean 1466, range 263 – 3821), compared to only one of the four volunteers in the long interval revaccination group (A4) (mean 229, range 18 – 507). However, the antibody titers between the two groups were also markedly different following priming vaccination with AdCh3. All five volunteers in A3 had a positive antibody titer following prime (mean 1455 range 457 – 2645), whilst only one of the four

in A4 had a positive antibody titer at this time point (mean 323, range 28 – 876). It is therefore difficult to accurately draw conclusions between the groups.

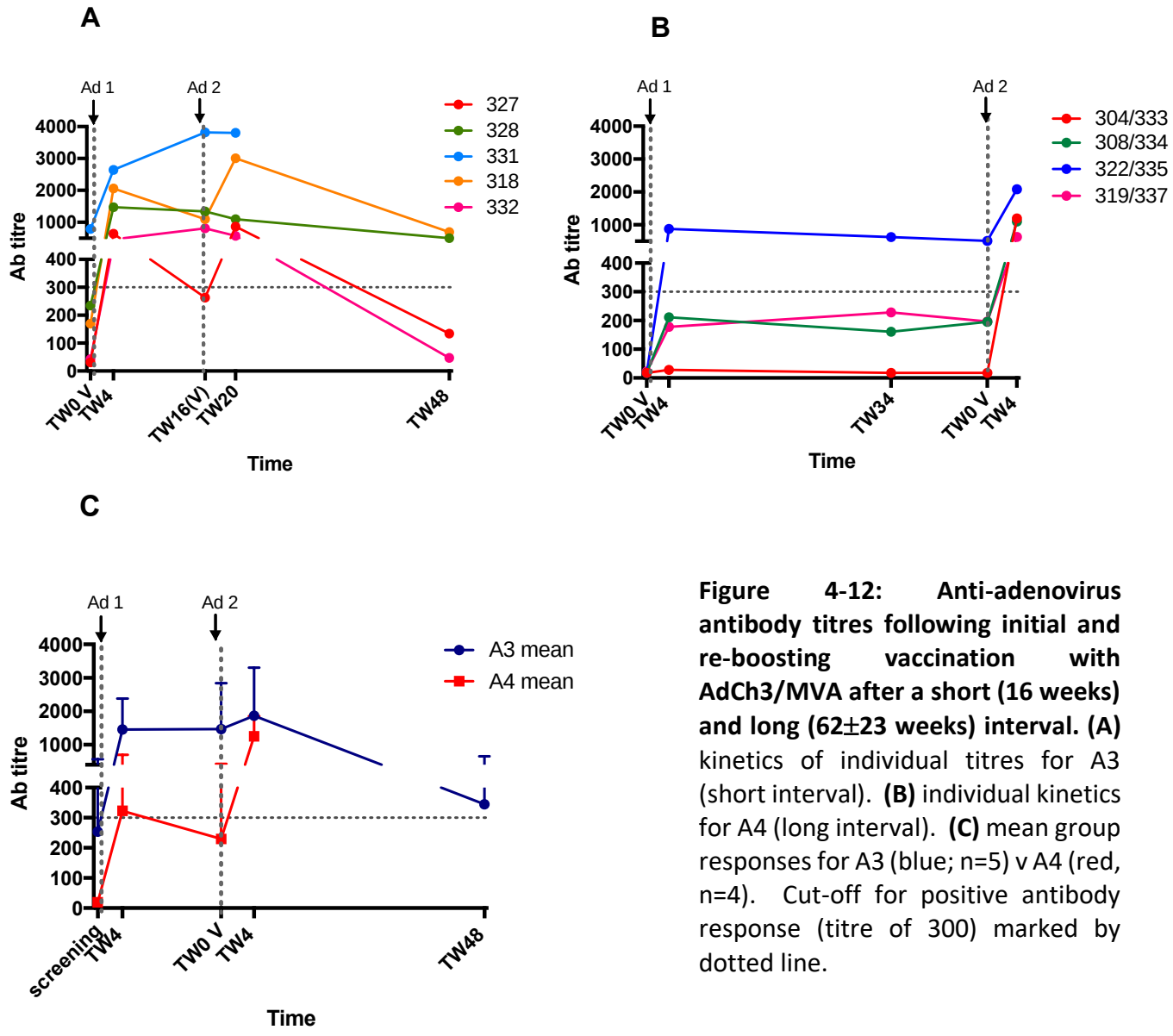


Figure 4-12: Anti-adenovirus antibody titres following initial and re-boosting vaccination with AdCh3/MVA after a short (16 weeks) and long (62±23 weeks) interval. (A) kinetics of individual titres for A3 (short interval). **(B)** individual kinetics for A4 (long interval). **(C)** mean group responses for A3 (blue; n=5) v A4 (red, n=4). Cut-off for positive antibody response (titre of 300) marked by dotted line.

An analysis was done to compare the anti-adeno antibody titer with the T cell magnitude following first and second round of AdCh3 (**Figure 4-13**). No correlation was found with baseline (pre-vaccination) anti-adeno antibody levels with T cell magnitude following priming vaccination (**Figure 4-13; A**), nor with Nab levels at time of AdCh3 re-boost with peak T cell magnitude (**Figure 4-13; B**).

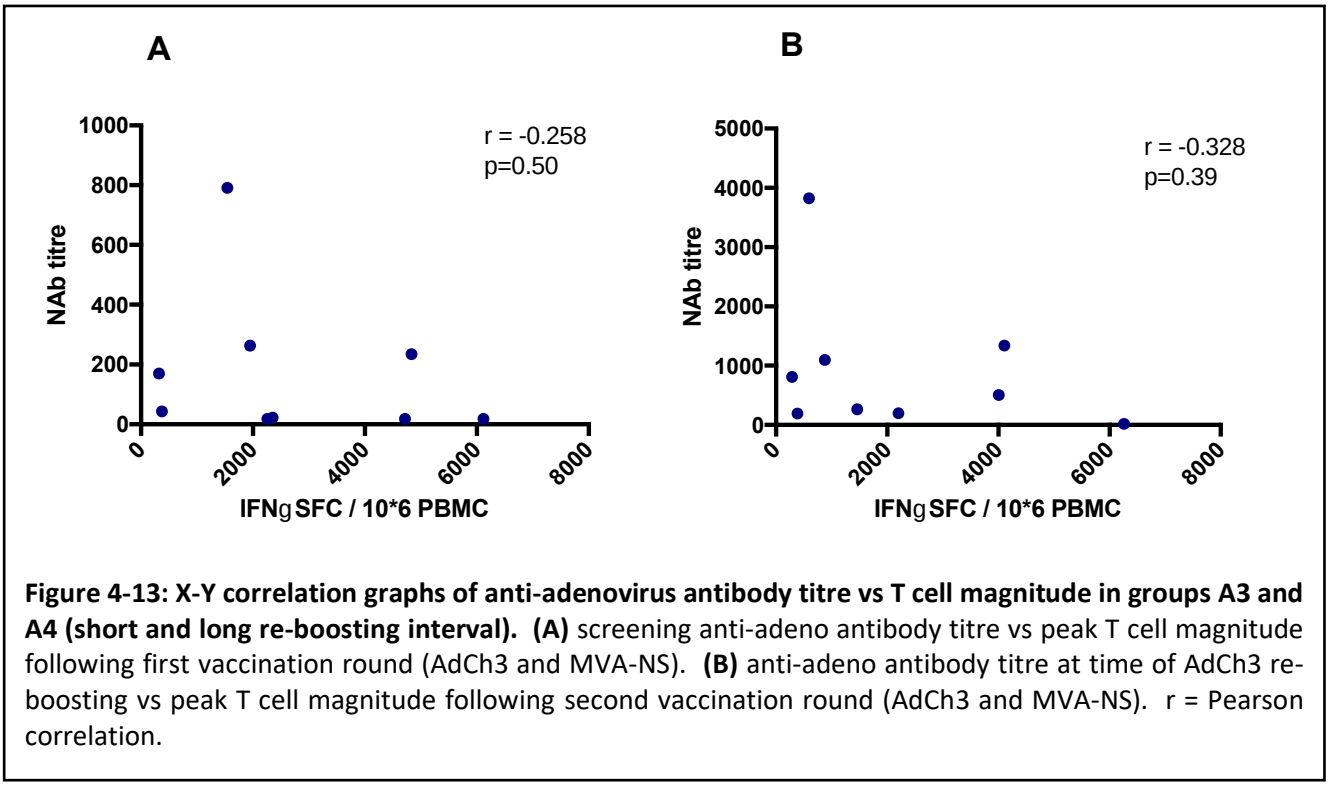


Figure 4-13: X-Y correlation graphs of anti-adenovirus antibody titre vs T cell magnitude in groups A3 and A4 (short and long re-boosting interval). (A) screening anti-adeno antibody titre vs peak T cell magnitude following first vaccination round (AdCh3 and MVA-NS). **(B)** anti-adeno antibody titre at time of AdCh3 re-boosting vs peak T cell magnitude following second vaccination round (AdCh3 and MVA-NS). r = Pearson correlation.

4.5.7 T CELL REACTIVITY TO AD-HEXON FOLLOWING SHORT AND LONG RE-BOOSTING

Another method of assessing anti-vector immunity is to measure T cell reactivity to the adenoviral hexon sequence. Sequence homology of hexon is used to classify human adenoviruses and chimpanzee adenoviruses into different clusters (A-F), and given that Ad5 and AdCh3 are within the same cluster, the Ad5 hexon sequence can be used to assess hexon-specific T cells induced by AdCh3 (Colloca et al. 2012).

T cell reactivity to the hexon sequence was measured at every time point by ELISpot. The kinetics of mean T cell responses following short (A3) and long (A4) reboosting vaccination are shown in **Figure 4-14**. In both of these groups there was a high frequency of hexon specific T cell induction following priming adenoviral vaccination. Following reboosting vaccination with AdCh3 there was a modest response of the hexon specific T cells following a shorter reboost (mean $\pm$ SD = 243 $\pm$ 163 SFU per 10^6 PBMCs). Following longer reboost this trended towards a more robust response but did not reach statistical significance (405 $\pm$ 165 SFU per 10^6 PBMCs) (p=0.18).

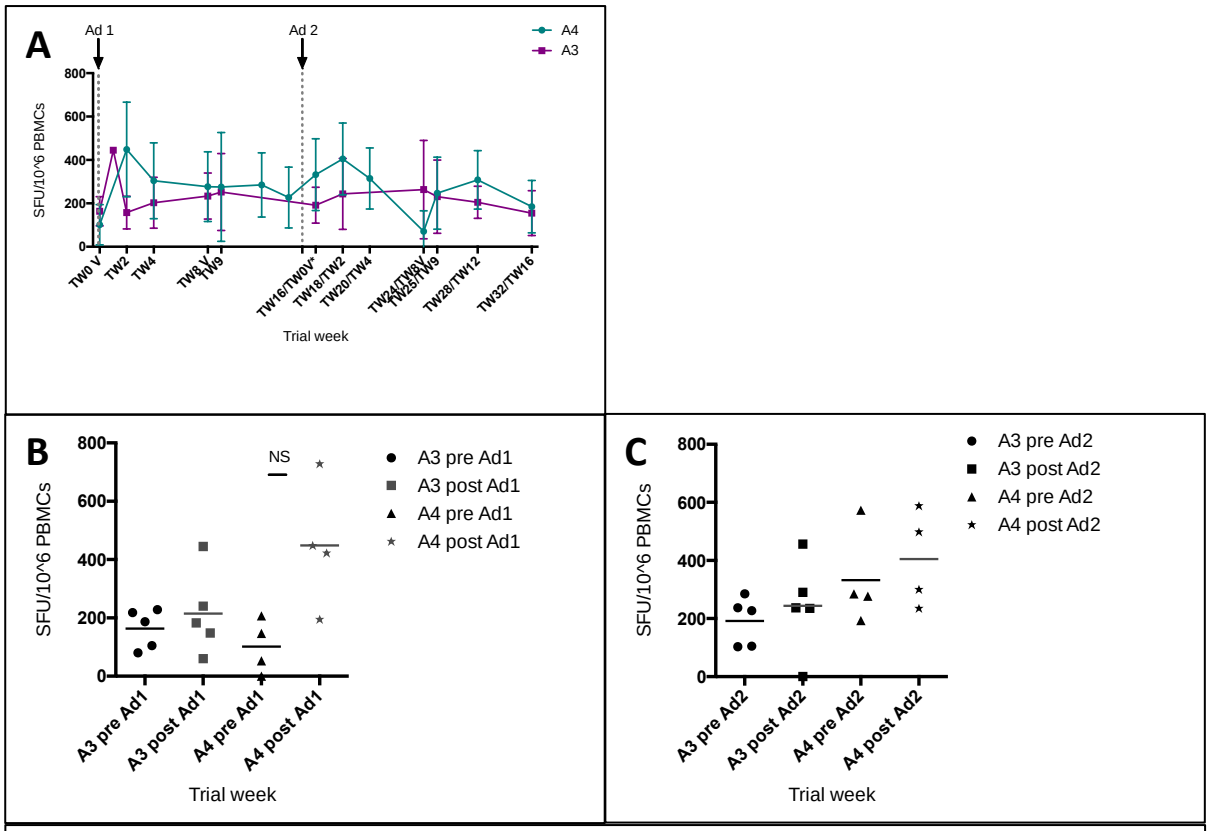


Figure 4-14: T cell reactivity to Ad-5 hexon sequence following short (A3) and long (A4) AdCh3 reboosting. Response measured by *ex vivo* IFNγ ELISpot assay **(A)** kinetics of mean T cell response to hexon. *Timeline of A4 (longer reboost) superimposed on timeline of A3 (shorter reboost) for comparison from this timepoint on. **(B)** T cell magnitude to hexon pre- (just prior to vaccination) and peak frequency post- AdCh3 (2 – 4 weeks) priming vaccination **(C)** T cell magnitude to hexon pre- (just prior to vaccination) and peak frequency post- (2 – 4 weeks) reboosting with AdCh3

4.5.8 ACTIVATION OF NON-HCV SPECIFIC T CELLS WAS NOT OBSERVED

To evaluate the potential of non-specific or 'bystander' activation and expansion of non-HCV specific T cells following vaccination, PBMCs were cultured with influenza (flu), CMV and EBV (FEC) epitopes in parallel with the HCV peptide pools (**Figure 4-15 A-C**).

Although there was a lot of variability in individual responses to these peptides, there was no correlation observed between the frequency of FEC specific IFNγ producing T cells with vaccination time points observed in groups A3 – A5.

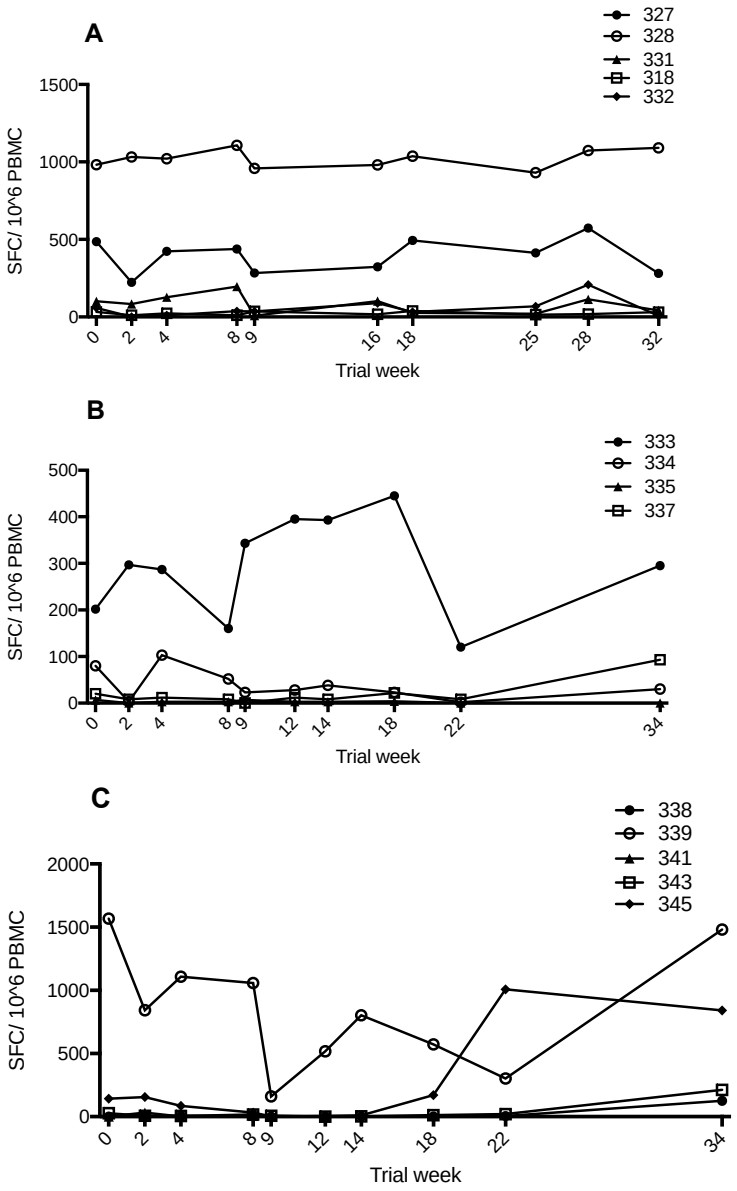


Figure 4-15: Frequency of Flu, EBV and CMV (FEC) specific T cell response at various time points in groups A3, A4 and A5. PBMCs from volunteers were stimulated with a FEC peptide pool and responses were measured at various time points throughout the trial. **(A)** group A3 [n=5], **(B)** group A4 [n=4], **(C)** group A5 [n=5].

4.5.9 HCV SPECIFIC T CELLS ARE ABLE TO BE BOOSTED BY DOSES OF MVA ONE AND TWO LOGS LOWER THAN PREVIOUSLY GIVEN

We next sought to determine whether MVA administered at a lower dose is immunogenic. The rationale for this is largely due to the technical and logistical challenges of manufacturing MVA vectors on a large scale. To answer this, two additional groups of 5 volunteers were recruited to receive MVA at trial week 8 at a low dose (A6; 2 x 10^7 pfu) or very low dose (A7; 2 x 10^6 pfu), following the same dose of AdCh3 as previously given at trial week 0. Both groups were followed up until trial week 32.

The AE profile following priming vaccination with AdCh3 was similar to what has been observed in all other groups. AdCh3 was well tolerated, with 17 AE's in A6 (100% grade I) and 10 AE's in A7 (60% Grade I, 40% Grade II) (**Figure 4-16, A & C**). Following boost with low and very low doses of MVA, very few adverse events were observed **(Figure 4-16, B & D).** Only 11 AE's were experienced in the low dose MVA (all Grade I). There were 7 reported AE's in the very low dose MVA: 2 Grade I, 4 Grade II and one Grade III. No SAE's occurred in either group.

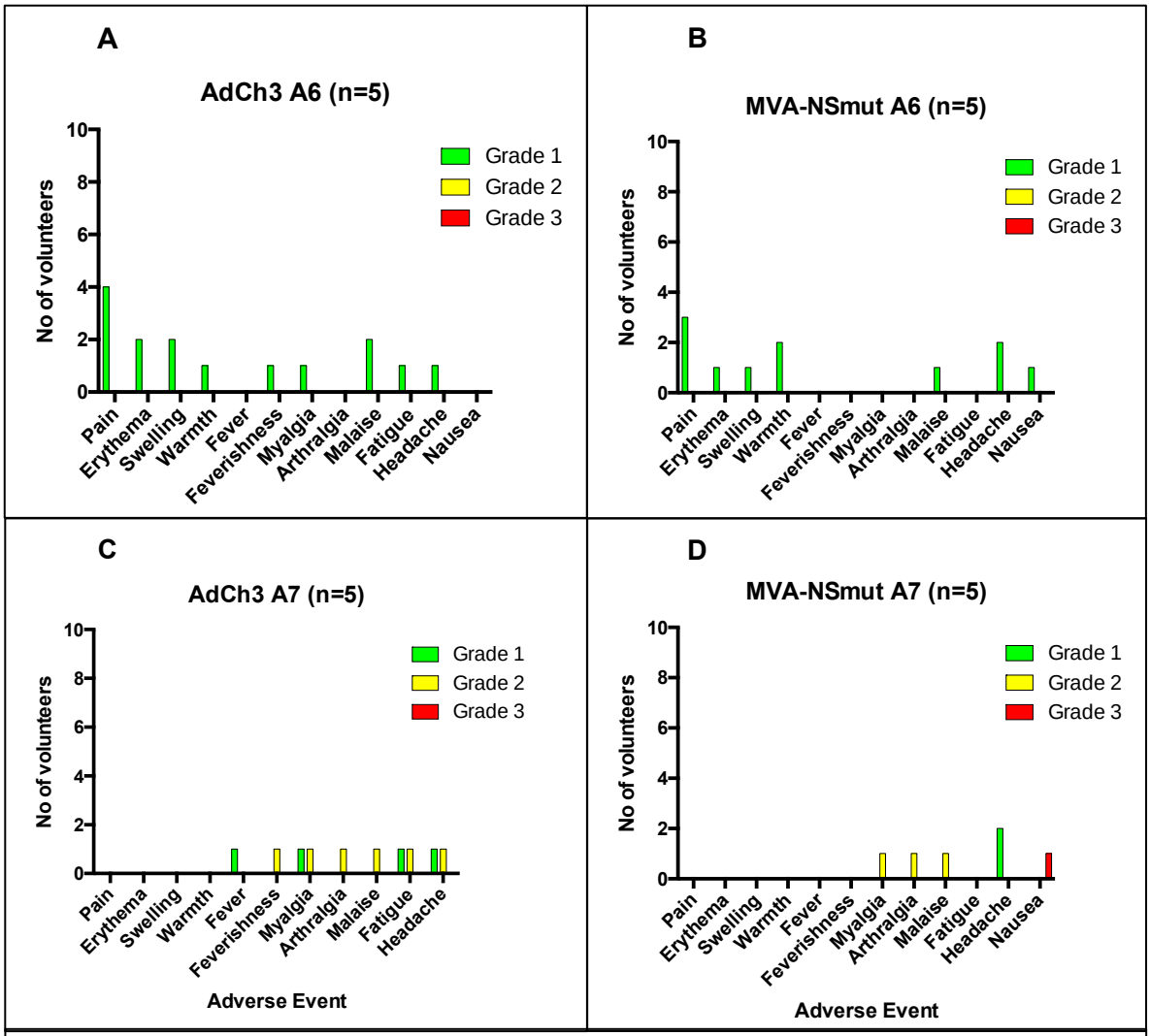


Figure 4-16: Safety data following vaccination with AdCh3/MVA-NS (MVA-NS at low [2x10^8 pfu] and very low [2x10^{7}pfu]. **(A)** prime vaccination with AdCh3 in group A6. **(B)** boost vaccination with MVA-NS (low dose) in A6. **(C)** prime vaccination with AdCh3 in group A7. **(D)** boost vaccination with MVA-NS (very low dose) in A7. The number of volunteers for each adverse event (AE) is shown, with only the peak severity of each AE recorded. Each AE is graded from 1 (minimal) to 3 (maximum).

Immunogenicity was determined by measuring the frequency and breadth of HCV specific T cells using ELISpot assays. In both the low and very low dose MVA-NS groups, initial priming with AdCh3 induced a comparable T cell response to that seen in all other groups (mean $\pm$ SD = 763 $\pm$ 1229 SFU per 10^6 PBMCs and 1283 $\pm$ 834 SFU per 10^6 PBMCs for A6 and A7 respectively). In the low dose MVA-NS group, peak response following MVA-NS administration was 4042 $\pm$ 3396 SFU per 10^6 PBMCs, and occurred at TW9 in all volunteers (one week following MVA-NS boost) (**Figure 4-17 A)**. Peak response following very low dose MVA-NS administration were again comparable, with an ELISpot T cell magnitude of (mean $\pm$ SD) 2083 $\pm$ 1959 SFU per 10^6 PBMCs, also occurring in all volunteers one week following MVA-NS (**Figure 4-17 B)**.

Comparison of the peak responses of the low and very low dose MVA-NS is in **Figure 4-18 A.** An accurate comparison can be made with all volunteers in groups A2 to A5 up to the peak response following initial MVA-NS vaccination, as the vaccination schedule was identical for all volunteers. There was no statistically significant difference comparing either low or very low dose MVA-NS to normal dose at peak magnitude (p=0.4 for low vs normal and p=0.3 for very low vs normal). However, as there were only five volunteers in each of the low and very low dose groups, they may not have been powered enough to reach significance, and it is likely in groups this small only the trends can be observed.

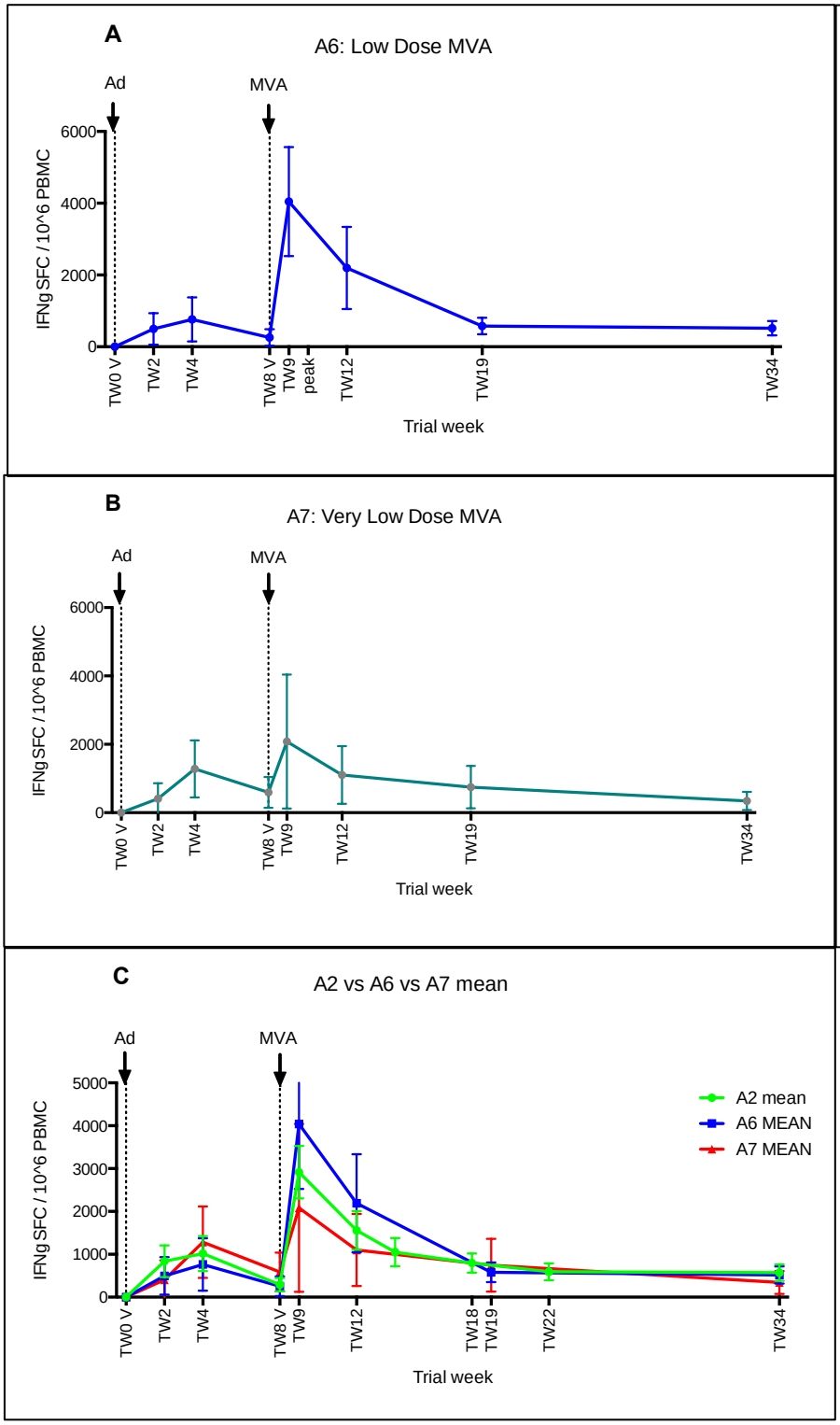


Figure 4-17: Magnitude of T cell responses to HCV-NS following AdCh3 prime and MVA boost of varying doses.

Total pooled responses to HCV-NS as measured by *ex-vivo* IFNγ ELISpot assay to fresh PBMCs. Vaccinations represented by arrows, trial weeks refers to week since initial vaccination. **(A)** Mean T cell response following low dose MVA (dose $2x10^7$pfu) at week 8 (n=5). **(B)** Mean T cell response following MVA boost at very low dose MVA (dose $2x10^6$pfu) at week 8 (n=5). **(C)** Comparison of mean T cell responses in group A2 (AdCh3 week 0, MVA week 8 (dose $2x10^8$pfu) (n=9), A6 and A7.

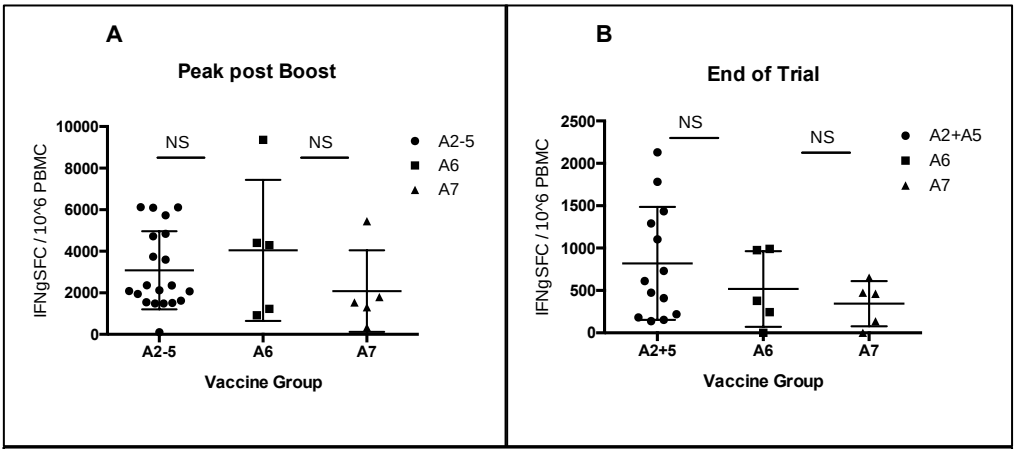


Figure 4-18: Comparison of T cell magnitude between Groups A2-5, A6 and A7 following AdCh3 prime ($2.5x10^{10}$vp) and MVA-NS boost of different doses. Responses measured by *ex vivo* IFNγ secreting ELISpot assays. **(A)** Comparison of groups A2-A5 (MVA-NS dose $2x10^8$ pfu; shown as circles, *n=20*), A6 (MVA-NS dose $2x10^7$pfu; shown as squares, *n=5*) and A7 (MVA-NS dose $2x10^6$pfu; shown as triangles) at peak T cell magnitude following boost**. (B)** Comparison of groups A2 and A5 (MVA dose $2x10^8$ pfu; shown as circles, *n=13*), A6 (MVA dose $2x10^7$pfu; shown as squares, *n=5*) and A7 (MVA dose $2x10^6$pfu; shown as triangles) at trial week 32 (end of trial for A6 and A7).

The end of trial T cell magnitude is also critical, as this will give an indication of the set point of the memory T cell population, and possibly the effectiveness of low dose MVA-NS in a prophylactic setting. A comparison of T cell magnitude at TW32 (EOT for low and very low dose) can only be made with groups A2 and A5, as other groups received additional vaccinations prior to this time point. This is shown in **Figure 4-18 B.** As before, there were no statistically significant differences between the groups (p=0.36 and p=0.15 for A2+A5 vs low and very low dose respectively), however a trend towards a smaller T cell magnitude could be observed with sequential lowering of the MVA-NS dose, which would need to be powered to determine significance. There was also no significant difference observed between the low and very low MVA doses.

4.6 DISCUSSION

The focus of this chapter is aimed at establishing the safety and immunogenicity of the optimal dose, timing and combination of vector vaccines for the prevention of HCV infection.

A prophylactic prime-boost vaccination strategy using viral vectors remains an attainable goal for HCV prevention, and preliminary work using this approach in both HCV patients and healthy volunteers has been trialed to support this. For reasons that are further discussed in chapter 1, an attenuated response was observed in chronic HCV infection (Kelly et al. 2016; Swadling et al. 2016). In healthy volunteers however, a broadly targeted and robust T cell response was observed, which was unprecedented in any HCV vaccine trial to date (Swadling et al. 2014). This preliminary work establishes the groundwork upon which the dosing, timing and combination of vector vaccines can be optimized.

The first question addressed was the safety of administering additional doses of AdCh3 and/or MVA-NS at different time points. The data presented in **Figure 4-4** confirms that additional rounds of vaccination are both safe and well tolerated. A similar reactogenic profile was observed in all volunteers receiving a second round of vaccinations to that observed in the first round, and there were no serious adverse events that occurred relating to additional vector administration.

The two next important questions to consider are whether either the peak magnitude and/or set point of the HCV specific T cell response can be improved by administration of additional vaccinations. These considerations are important both in the context of the

HCV specific immune response as well as the application of AdCh3 and MVA-NS vectors in other settings. A higher peak magnitude of the peptide specific response may be particularly important in the setting of therapeutic vaccines. It is also a plausible hypothesis that a higher peak response will ultimately induce a higher peptide specific memory response. The memory magnitude or 'set-point' is critically important in the setting of a prophylactic vaccination, as this may determine the response of the immune system upon re-exposure to the pathogen.

Three groups of volunteers were recruited to address these questions (A3-A5). It was observed that when additional vaccines were given after a longer interval following initial AdCh3/MVA-NS (average 62 weeks**),** both a higher peak T cell response and a higher set-point were observed compared to when given after a short interval (8 weeks following MVA). Revaccination with MVA-NS alone after an intermediate duration of 32 weeks induced a peak and end of trial HCV-specific T cell set point that was between the short and long interval.

There are two potential hypotheses for the differences observed. The first is that the presence of anti-vector antibodies induced following initial adenoviral vector administration may attenuate the response of additional adenoviral administration (groups A3 and A4), which may then negatively impact the effect of additional MVA-NS administration. Although this may be the case, it was not possible to draw any conclusions in this trial due to the differing titers of the anti-vector antibodies between the two groups at baseline. However, if the presence of anti-adeno antibodies had a significant impact on the response to re-boosting, then it could be expected that the response in group A5

(MVA-NS only reboost) would be higher than both A3 and A4, as these volunteers did not receive additional AdCh3 vaccination.

The other hypothesis is that the HCV specific T cells induced following initial AdCh3 and MVA-NS take several months to fully differentiate, mature and then contract before an optimal response on re-exposure to antigen is observed, and that re-exposure to antigen prematurely will result in an attenuated response. There is evidence to support the continued differentiation of antigen specific T cells with time, with regards to both the functional and memory phenotype of the cell. Swadling et al (Swadling et al. 2014) observed a trend towards more polyfunctional HCV specific T cells with time following AdCh3/MVA-NS administration, with the majority of peptide specific T cells able to produce three or more functional cytokines by trial week 22.

Furthermore, it has been suggested that T cells evolve and contract into different memory subsets with time (Appay et al. 2008; Newell et al. 2012). Three distinct memory subsets have been identified depending on expression of the LN homing marker CCR7 and naïve T cell marker CD45RA. Tcm (central memory; $CCR7^+/CD45RA^-$) home to peripheral lymphoid tissue, have high proliferative capacity and poor effector function. Tem (effector memory; $CCR7^-/CD45RA^-$) circulate through the periphery, and have immediate effector and cytolytic capacity. Finally, Temra (terminally differentiated effector memory; $CD45RA^+/CCR7^-$) re-express the naïve marker CD45RA despite being antigen experienced (Sallusto, Geginat, and Lanzavecchia 2004).

Relative numbers of each subset correlating to protection following vaccination and in natural infection is conflicting. Higher numbers of Tem have been associated with protection in both malarial and SIV challenge studies (Hansen et al. 2011; Reyes-Sandoval

et al. 2011), whereas an increased magnitude of Temra had an inverse correlation with symptom score in H1N1 pandemic influenza (Sridhar et al. 2013). Although the evidence is not clear regarding the T cell memory phenotypes in protective responses, and in fact a protective phenotype may be pathogen specific, these studies are proof of principle that T cell memory differentiation into different subsets may play a role in a protective response. T cell memory subsets induced by HCV vaccination will be explored further in chapter 7.

In HCV003 group A2, the 10 volunteers that received initial AdCh3/MVA-NS prime-boost vaccination progressed towards a Tem/Temra phenotype with time (Swadling et al. 2014). It is a plausible hypothesis then, that the HCV specific T cells may be more optimally boosted after full contraction of the T cell response has occurred, which may explain the more robust response to re-boosting vaccination.

Regarding the results currently available, it is arguable that the ability of the HCV specific T cell populations to respond to antigen re-exposure is more a determinant of time (and the necessity to differentiate and contract) rather than the combination of AdCh3/MVA-NS vs MVA-NS alone. The three groups receiving additional re-boosting vaccination had a linear trend of both peak T cell magnitude and memory set-point with time, irrelevant of additional adenoviral vector administration.

Further functional and phenotypic analysis of the HCV-specific T cell populations as a function of time will need to be performed in these three groups to determine if there is

any correlation with both the functional and T cell memory phenotype and ability of the cell populations to respond to further antigen stimulation.

MVA has proven to be an attractive vector for use in both prophylactic and therapeutic settings. It is safe, tolerable and highly immunogenic, and is currently being investigated for vaccination against several pathogens and malignancies, including malaria (Hodgson et al. 2015), tuberculosis (McShane et al. 2004), prostate cancer (Amato and Stepankiw 2012) and cervical intraepithelial neoplasm (Corona Gutierrez et al. 2004). However, there are certain challenges with MVA manufacture. The production of the vector requires a continual supply of chick embryo fibroblasts derived from pathogen free flocks, and the maintenance of specialized animal facilities and elaborate husbandry including strict isolation of the flocks, vigorous control of any personnel, food and air that comes into contact with the animals, and intense pathogen screening of the animals (Jordan et al. 2013). This process is technically challenging and may not be sustainable, particularly if it were to be on a large scale and/or were to be implemented in parts of the developing world.

It is believed that because the vector is highly attenuated, it must be given at sufficiently high doses for T cell induction (Coulibaly et al. 2005). We sought to determine whether in fact MVA-NS could be administered at a dose of one or two logs lower and still stimulate a HCV specific immune response following AdCh3 priming. We found that both low and very low doses of MVA-NS were able to boost the HCV specific response, although both the peak and end of trial T cell magnitude was progressively lower (though not significantly so) with each dose reduction. It is still unknown what T cell magnitude will induce a protective response *in vivo*, and thus difficult to determine the efficacy and potential

applications of these results. However, the observation that an extremely small dose of MVA-NS can boost the T cells at all is promising, and the results may be useful in the application of MVA-NS in other chronic infections and cancer immunotherapy.

One important question that has not been addressed in these studies but has significant implication for the successful implementation of these vaccines is their ability to develop protective T cell responses to different HCV genotypes. As discussed in chapter one, there is significant heterogeneity between HCV genotypes and subtypes. This has been addressed in previous work in this vaccine trial (Swadling et al. 2014). In 9 individuals in group 2 of this trial, ELISpot analysis was performed with peptides derived from genotypes 1a, 3a and 4a. Responses were generated in about 60% (1a) and 30% (3a and 4a) of those generated in genotype 1b. The breadth of response was comparable between genotypes.

4.7 PITFALLS AND LIMITATIONS

There are a number of limitations in the trial that should be noted. Firstly, the number of volunteers in each group were very small (between 4 and 5), and although trends and descriptive analyses could be observed between groups, power to detect significant differences in groups was limited. Nonetheless, there was minimal loss to follow up in all groups.

Secondly, there were potential limitations regarding the use of ELISpot assays. Although is a widely used and well validated assay, it only measures a single cytokine (IFNγ). Other important cytokines involved in T cell activation (such as IL-2 or TNFα) and cytotoxic T cell activity (such as granzyme A / B and perforin) are not captured.

Thirdly, one important question not answered is the cross-reactivity of the vaccine to other HCV genotypes. However, this has been previously addressed in an earlier group of the vaccine trial (A2, n=9) by Swadling et. al (Swadling et al. 2014).

Finally, as was addressed in the introduction, the humoral and innate immune response to HCV are likely to be important mechanisms in the host response against the virus. The design of these experiments did not allow for either of these arms of the immune system to be analysed.

4.8 CONCLUSION AND FUTURE WORK

In summary, the work presented in this chapter assesses strategies in the optimization of HCV viral vectored vaccination. Re-boosting of the HCV specific T cell response can be achieved, but it is likely to be more effective following a longer period of time. Furthermore, MVA-NS can be effectively administered as a boosting vaccination using very low doses, which has potential applications for the use of this vector in other settings.

Further phenotypic and functional assessment of the remaining stored PBMCs in groups A3-A7 will be undertaken to assess both the quality of T cell and memory phenotype induced after re-boosting and boosting at a lower dose. Furthermore, the evaluation of AdCh3/MVA-NS as a prime-boost vaccination strategy to prevent HCV infection has entered Phase I/II efficacy trials, the results of which are expected to be available in 2018 (further discussed in chapter 6).

CHAPTER 5: CO-ADMINISTRATION OF HCV AND HIV CANDIDATE VACCINES (PEACHI-04 TRIAL)

5.1 AIMS

The primary aim of this study is to assess the safety and tolerability of co-administration of HCV and HIV-1 prime-boost vaccinations: AdCh3NS / ChAdV63.HIV and MVA-NS / MVA.HIV intramuscularly in healthy adult volunteers.

The secondary aim is to evaluate the cellular immune response generated by simultaneous administration of the above vaccines in healthy adult volunteers, by assessing the antigen specific T cell populations in various immunological assays.

5.2 BACKGROUND

A prophylactic vaccination strategy targeting prevention of both HIV-1 and HCV infection simultaneously is an attractive goal. There is a high global prevalence of co-infection with HIV-1 and HCV, and those infected with both viruses often have a poorer prognosis than individuals with either virus alone (discussed in details in chapter one). The target populations for both infections overlap, due to the similar risk factor profile and modes of transmission. Effective treatment for both infections is available, however they are not accessible to many who require it (Ford et al. 2012) are required life-long (HIV-1) or are unaffordable to most (HCV). Furthermore, dual treatment with antiretrovirals and peg-IFN can be associated with a higher side-effect profile, drug interactions, and hepatotoxicity (Shepard, Finelli, and Alter 2005). Although these adverse events are

expected to diminish with DAA therapy, complex drug interactions can still occur and certain combinations should be avoided (Soriano et al. 2017). For these reasons, a combined vaccination strategy aimed at prevention of both infections is a desirable goal.

There is a significant body of evidence to suggest that the immune response to both HIV-1 and HCV is a predominantly cellular rather than humoral adaptive immune response (further discussed in Chapter 1). Prime-boost vaccination strategies employing adenoviral and MVA vectors have been shown previously to induce robust T cell responses in humans to a number of pathogens, including both HCV and HIV-1 (Ewer et al. 2016; Green et al. 2015; Hayton et al. 2014; Sheehan et al. 2015; Swadling et al. 2014). Considering the immunogenicity of the vaccines to date and the justifications for prevention of both viruses, trialing these vectors together is a rational approach to developing a dual vaccination strategy for HCV and HIV-1 co-infection.

A potential challenge to developing a co-administration vector strategy is the possibility that simultaneous delivery of either distinct immunogens or distinct viral vectors may result in an attenuated immune response compared with either immunogen or vector alone. This concept of immune interference is complex and incompletely understood, and there has been much research over the past two decades into determining the possible mechanisms behind the phenomenon. Reciprocal viral inhibition has been reported in numerous studies. In one such study of HCV and HBV co-infection, patients who were HBV surface antigen positive were significantly more likely to be HBV DNA negative if they were HCV RNA positive, and likewise those that had HCV positive liver biopsies and were HBV DNA positive were all seronegative for HCV RNA (Pontisso et al. 1993). Similar observations

of viral inhibition with HBV and HCV co-infection have been noted elsewhere (Sato et al. 1994). The mechanisms behind this phenomenon are incompletely determined, however it has been suggested that acute HCV and HBV co-infection activate more pathways in the innate response compared to mono-infection with either virus alone, resulting in a more vigorous innate response and suppression of both viruses. (Pontisso et al. 1993). It should be noted however that these studies observed viral suppression in the context of replication competent viruses, which may induce a different host response to that observed in replication deficient viruses, such as chimpanzee adenovirus or MVA vectors.

The impact of viral interference on replication defective viruses is unclear. A study by Betts et al (Betts et al. 2012) assessed administration of Ad5 and MVA both expressing the tuberculosis immunogen Ag85A in a single vaccine preparation to mice, and observed comparable immunogenicity to single administration of either vector, but reduced immunogenicity when compared to heterologous Ad5 prime-MVA boost. However, a similar study by Yashima et al. (Yashima et al. 2010), trialing co-administration of Ad-5 and MVA vectors encoding an HIV immunogen to mice observed conflicting results. Here antigenic interference was observed, with the suppression of HIV specific effector T cell responses by Ad-5 and MVA co-administration when compared to either vaccination alone.

The concept of antigenic competition and immunodominance is another concept that may be relevant to the co-administration of HCV and HIV immunogens. The theory of immunodominance suggests that there is a hierarchy of T cell epitopes that will be presented in an immune response to any given pathogen, with the most immunodominant

epitopes being preferentially selected (Larke et al. 2007). The T-cell hierarchy is influenced by the number and length of peptides, prior antigen exposure, naïve T cell repertoire, HLA type, and pathogen factors (Yewdell and Bennink 1999). This theory postulates that whilst the most immunodominant epitopes will be presented, other subdominant, though capable epitopes, may not be selected for antigen presentation (Holst et al. 2015).

In the context of dual administration of an HIV and HCV immunogen, the increased antigen load may potentially cause competition for antigen uptake from the same host antigen presenting cell, and furthermore, competition for the same machinery and pathways for antigen processing and presentation within the cell (Sedegah et al. 2004).

These concepts of antigen competition, immunodominance, and immune hierarchy have been explored and challenged over the past several years. Although there have been no human studies to date addressing these questions, various animal studies have been done, which both support and refute the hypothesis. Sedegah et al (Sedegah et al. 2004) trialed 9 DNA plasmids encoding malarial antigens both in combination and separately in mice, and observed significant suppression of both antibody and T cell responses with pooled compared with individual vaccines. More relevant to the field of vector vaccines was a study by Draper et al **(**Forbes et al. 2011) trialing multiple malarial antigens in a human Ad-5 and MVA vector given as prime-boost vaccinations to mice. Significant antigenic interference and suppression of the antigen specific T cell response was observed when the immunogens were combined compared with individual administration. However, this suppression was minimized when the immunogens were given at separate anatomical sites, indicating a potential local inhibitory effect. On the other hand, a study by Gabitzsch

(Gabitzsch et al. 2015) observed no inhibition of antigen-specific CD4+ or CD8+ T cell induction with administration of 3 human Ad-5 constructs encoding different tumour associated antigens (TAAs) when given to mice.

We sought to determine whether two vector vaccines containing different immunogens could be safely administered simultaneously without compromising the tolerability and/or immunogenicity of either vaccine administered alone. To answer this question, we designed a clinical trial recruiting volunteers into one of three groups:

1) <u>Group 1:</u> to receive only the HCV prime-boost vaccines (AdCh3NSmut1 [dose 2.5 x 10^{10} vp] and MVA-NSmut [dose 2 x 10^{8} pfu]).

2) <u>Group 2:</u> to receive only HIV prime-boost vaccines (ChAdV63.HIVconsv [dose 5 x 10^{10} vp] and MVA.HIVconsv [dose 2 x 10^{8} pfu]).

3) <u>Group 3:</u> to receive simultaneously AdCh3 + ChAdV63 [dosed as above] and MVA-NSmut + MVA.HIVconsv [both doses 1 x 10^{8} pfu].

Because of the potential local immune competition between the vectors (Forbes et al. 2011), the co-administration group received the candidate prime and boost vaccines in separate arms: all HCV vaccines were given in the left arm and all HIV vaccines in the right. This is the first study to date assessing co-administration of serologically distinct adenoviral vectors and multiple MVA vectors encoding different immunogens in humans.

For the remainder of the chapter the vaccines will be referred to as follows: AdCh3NSmut1 (AdCh3), MVA-NSmut (MVA-NS), ChAdV63.HIVconsv (ChAdV63) and MVA.HIVconsv (MVA.HIV).

5.3 HYPOTHESES

1. Co-administration of adenoviral vectors of serologically distinct serotypes is safe and well tolerated
2. Simultaneous priming with serologically distinct adenoviral vectors encoding different immunogens does not attenuate the breadth or magnitude of the antigen specific T cell response to either immunogen compared with separate administration
3. Heterologous boosting vaccination with two MVA vectors encoding different immunogens does not impair the breadth or magnitude of peptide specific T cell response to either immunogen

5.4 CHAPTER SPECIFIC METHODS

A detailed description of methods can be found in chapter 2 however, key methodologies will be briefly described here.

5.4.1 VACCINE TRIALS

Healthy adult volunteers were recruited to groups 1 to 3 to receive i.m. vaccination. Numbers recruited to each group, vaccinations received and trial duration are summarised in **Figure 5-1.**

5.4.2 IMMUNOASSAYS

Freshly harvested PBMCs were collected at every visit and stimulated with peptide pools corresponding to either the HCV immunogen (**Appendix 2-1)** or the HIV immunogen (**Appendix 2-2**) in an IFNγ secreting ELISpot assays.

5.5 RESULTS

5.5.1 SCREENING AND ENROLMENT

In total 48 healthy volunteers were screened and 33 were enrolled in the study. 2 volunteers withdrew: PEA-04012 (group 1, replaced) and PEA-04041 (group 3, not replaced). PEA-04012 was not included in the final analysis.

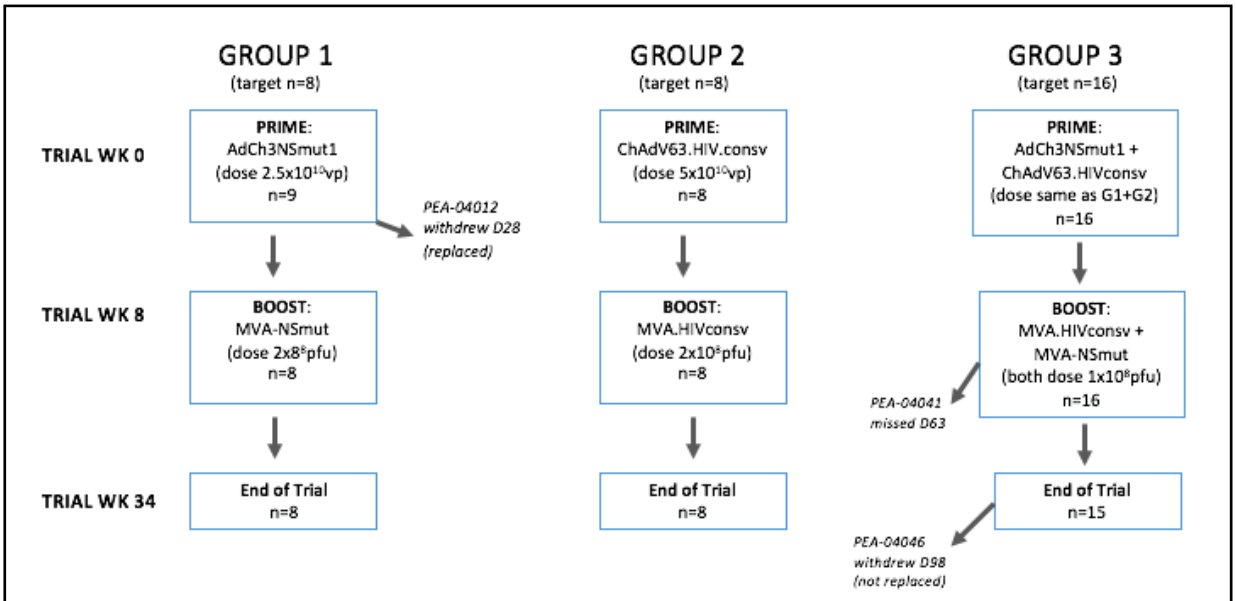


Figure 5-1: PEACHI-04 trial. Flow diagram illustrating recruitment, follow up and vaccination dose and schedule. WK=week. N=number.

5.5.2 CO-ADMINISTRATION OF TWO ADENOVIRAL VECTORS OF DISTINCT SEROTYPES IS SAFE AND WELL TOLERATED

There were no SAE's or SUSAR's that occurred throughout the trial period. All vaccines were generally well tolerated. The number of AE's in each group that were considered likely related to vaccination are summarized in **Figure 5-2.** In total there were 307 adverse events: 82 in group 1, 85 in group 2 and 140 in group 3. The MVA vector was more reactogenic than either chimpanzee adenoviral vector. Following AdCh3/ChAdV63 vector administration, 74% of AE's were considered grade I (mild, with no impact on daily activities), 18% grade II (moderate) and only 8% were considered grade III (severe, with significant impact on daily activities and usually requiring bed rest). In comparison, following MVA-NS administration, only 49% were considered grade I, whilst 34% were grade II and 17% considered grade 3.

The majority of AE's resolved within 48 hours (41% within 24 hours, 32% within 48 hours). Of the remaining, 13% of AE's resolved within 72 hours, and 14% resolved within 96 hours. Pain was the most commonly reported AE to last for beyond 48 hours.

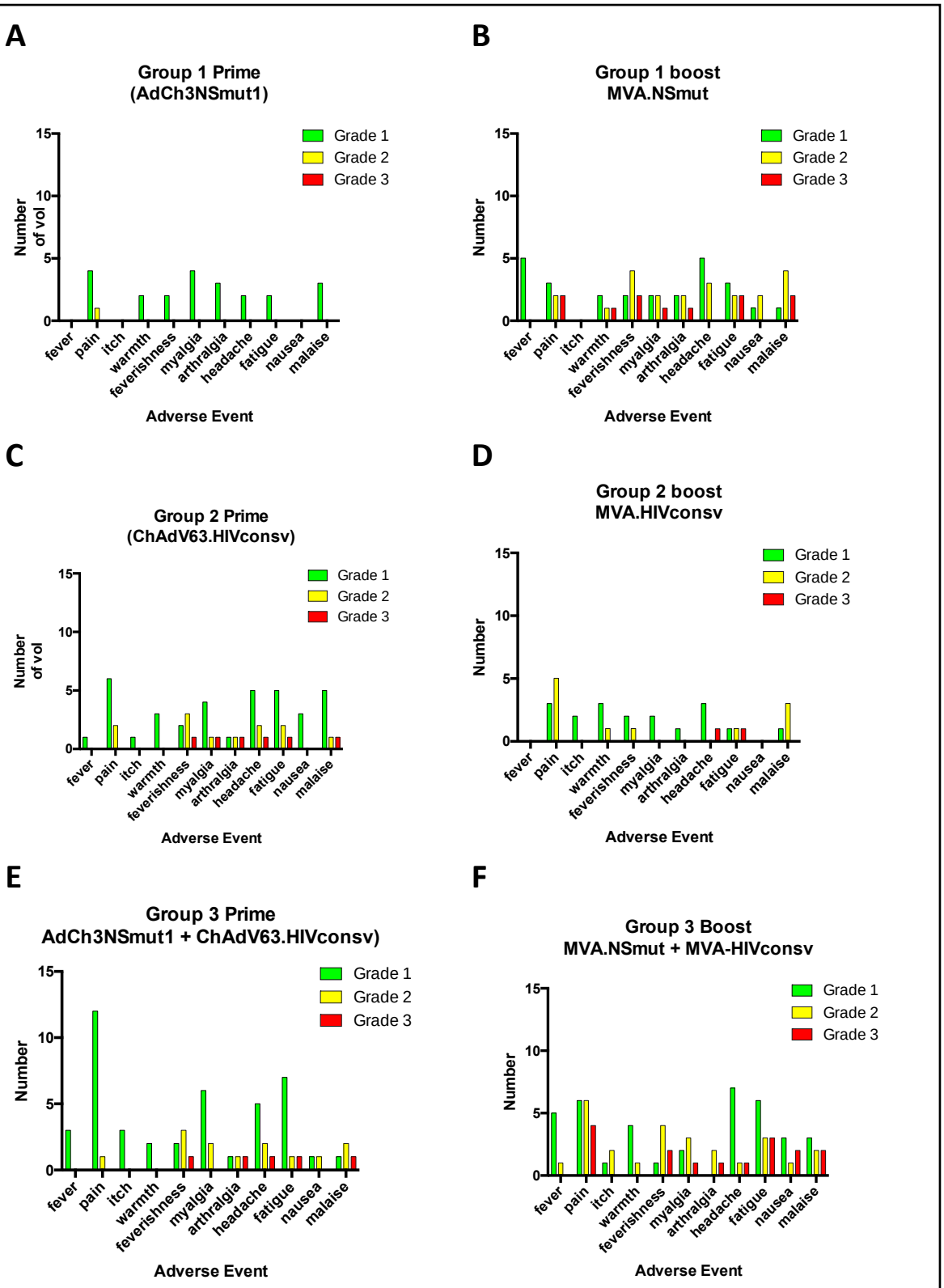


Figure 5-2: Solicited Adverse Events (AE's) for Groups 1-3 following vaccination. AE recorded as peak severity, with grade 1 = mild, grade 2 = moderate and grade 3 = severe. **(A)** and **(B)** group 1 AE's following prime with AdCh3 and boosting with MVA-NS respectively. **(C)** and **(D)** shows group 2 AE's following prime vaccination with ChAdV63 and MVA.HIV respectively. **(E)** and **(F)** group 3 prime and boost vaccination with AdCh3 + ChAdV63 and MVA-NS + MVA.HIV respectively. No SAE's or SUSARs occurred.

Although the above data has established that vector co-administration is safe, an important question is whether co-administration of adenoviral vectors of different serotypes is more reactogenic than single administration. One way of answering this is to look at the number and severity of each AE in the single vs co-administration groups. In all volunteers receiving single adenoviral vaccination (Groups 1 and 2; total n=16), there were 77 AE's; and the proportion that were Grade I, II and III were 75%, 17% and 6% respectively. A very similar number and severity of AE's was observed in the volunteers receiving dual vaccination (Group 3; n=16). Here there were 61 AE's; of which 70% were Grade I, 21% Grade II and 8% Grade III **(Figure 5-3)**.

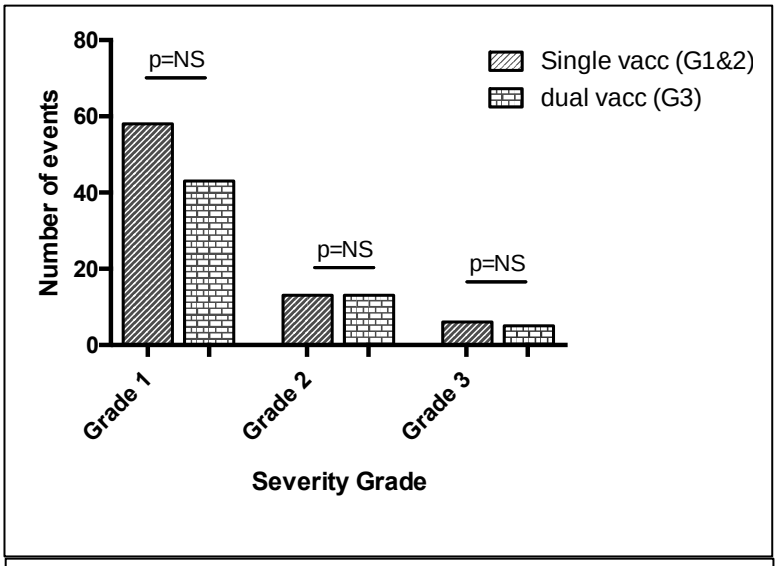


Figure 5-3: Number of all solicited adverse events (AE's) based on severity grading in PEACHI-04. Comparison between groups 1 and 2 (striped columns; n=16 total) with group 3 (boxed columns; n=16). Highest severity for a given AE recorded (between grade I (mild) to grade 3 (severe). Comparison made using unpaired t-test.

However, this analysis does not take into account the duration of each AE, which may be a significant factor in the impact of an AE on an individual. To address this, the 5 most common AE's were selected and compared between the groups with regards to both their severity and duration. For each of these adverse events, the duration (number of days from 0 to 4) and severity (Grade 0 to III) were multiplied together to give an overall score between 0 and 12 (**Figure 5-4)**

Using this analysis co-administration with chimpanzee adenoviral vectors did not result in increased reactogenicity compared with either vector alone (**Figure 5-4; top)**. Interestingly group 2 (single ChAdV63) was significantly more reactogenic than group 1 (single AdCh3) for pain (p=0.04), headache (p=0.001) and fatigue (p=0.02), and group 3 (co-administration) for fatigue (p=0.048).

Assessment of MVA vector vaccination showed no difference between groups 1, 2 and 3 (**Figure 5-4; bottom)**. This was not unexpected, as group 3 volunteers received each MVA vector at half the dose as given in groups 1 and 2 ($1x10^8$pfu vs $2x10^8$pfu respectively).

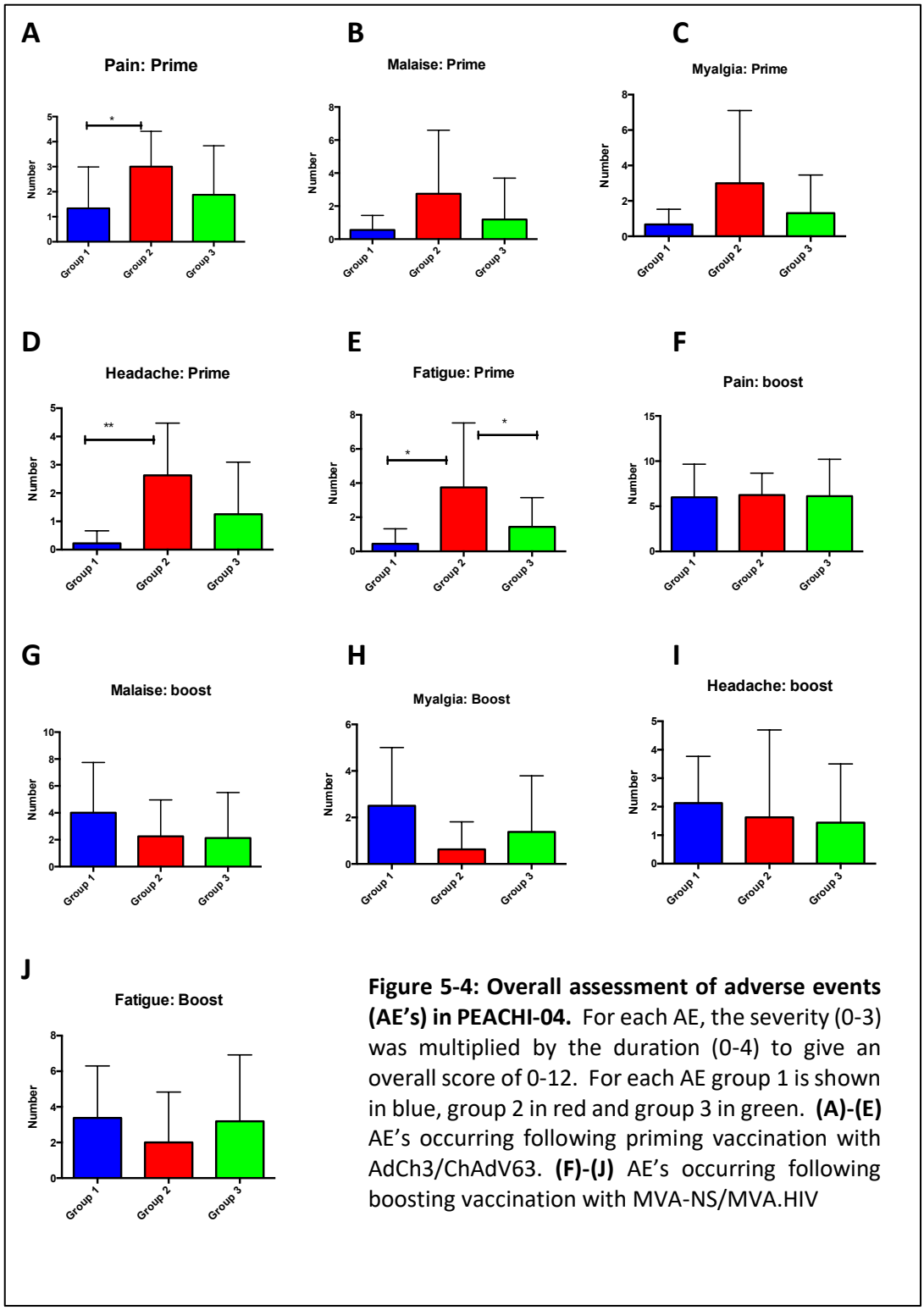


Figure 5-4: Overall assessment of adverse events (AE's) in PEACHI-04. For each AE, the severity (0-3) was multiplied by the duration (0-4) to give an overall score of 0-12. For each AE group 1 is shown in blue, group 2 in red and group 3 in green. **(A)-(E)** AE's occurring following priming vaccination with AdCh3/ChAdV63. **(F)-(J)** AE's occurring following boosting vaccination with MVA-NS/MVA.HIV

5.5.3 CO-ADMINISTRATION OF SEROLOGICALLY DISTINCT ADENOVIRAL VECTORS DOES NOT NEGATIVELY AFFECT THE MAGNITUDE OF THE ANTIGEN SPECIFIC T CELL RESPONSE TO EITHER VECTOR ALONE.

To assess the potential antigenic competition of co-administering two distinct adenoviral vectors containing different immunogens, we measured the magnitude of the HCV-NS and HIV.consv specific T cell induction after vaccination using an IFN-γ ELISpot assay (further described in chapter 2). We administered AdCh3 alone (Group 1; n=8); ChAdV63 alone (Group 2; n=8) or simultaneous AdCh3 and ChAdV63, given in either arm (Group 3; n=16) at week 0.

Priming vaccination with AdCh3 alone induced HCV specific T cell induction in all but one volunteer (PEA-04005). T cell magnitudes peaked between 2 weeks (57% of volunteers) and 4 weeks (43% of volunteers) following vaccination (peak mean $\pm$ SD = 608.5 $\pm$ 374 SFU per 10^6 PBMCs) (**Figure 5-5 A, E).**

Following co-administration of AdCh3 and ChAdV63, there was a robust induction of HCV specific T cells to the HCV-NS immunogen (peak mean $\pm$ SD = 1184 $\pm$ 869 SFU per 10^6 PBMCs) (**Figure 5-5 C, E)**. Antigen-specific T cells were induced in all 16 volunteers and occurred between 2 weeks (18.7% of volunteers and 4 weeks (75% of volunteers) following vaccination, with the exception of PEA-04026, where peak ELISpot magnitude occurred 8 weeks following prime. Although there was a higher peak response following priming vaccination observed in group 3 (simultaneous), this difference in peak response did not reach statistical significance (p=0.09) **Figure 5-6**.

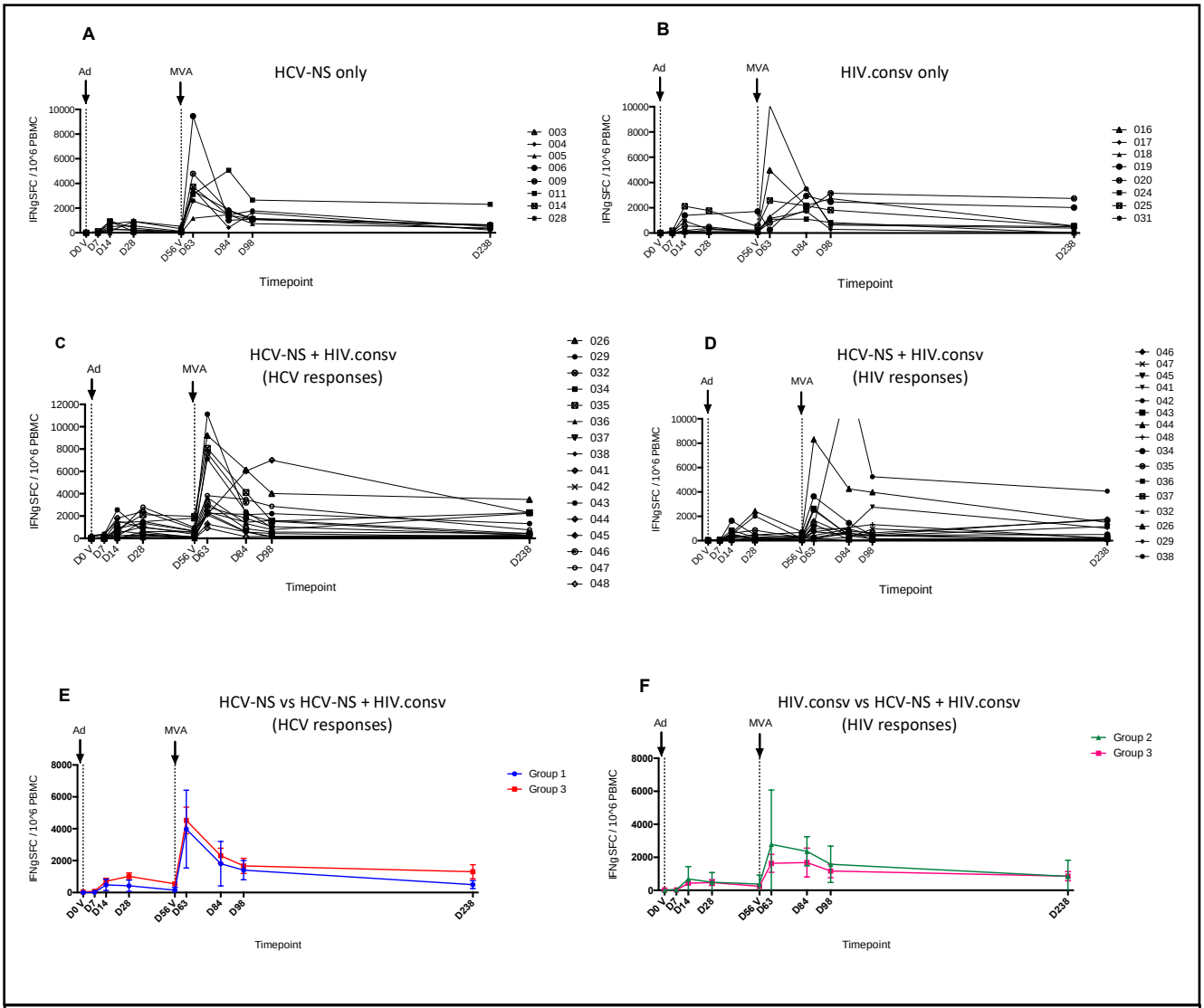


Figure 5-5. Magnitude of antigen specific T cell responses to HCV-NS and HIV.consv following AdCh3-NS/MVA-NS and ChAdV63.HIV/MVA.HIV both alone and in combination: (A to F) Total *ex vivo* IFNγ secreting ELISpot responses to immunogens HCV-NS and HIV.consv following vaccination (calculated by summed positive peptide pools and corrected for background, refer to Materials and Methods). Vaccination indicated by arrows, D=number of days following priming vaccination. **(A)** Individual response kinetics for group 1 volunteers (HCV-NS only, n=8). **(B)** individual kinetics for group 2 volunteers (HIVconsv only, n=8). **(C)** and **(D)** Individual kinetics for Group 3 volunteers (n=16); **(C)** HCV-NS responses and **(D)** HIVconsv responses (042 peak response 13,394 SFU/10^6PBMCs). **(E)** and **(F)** represent mean responses; **(E)** HCV-NS responses for Group 1 vs Group 3, **(F)** HIVconsv responses for Group 2 vs Group 3. *Note PEA-04012 (group 1) withdrew and not included in analysis.*

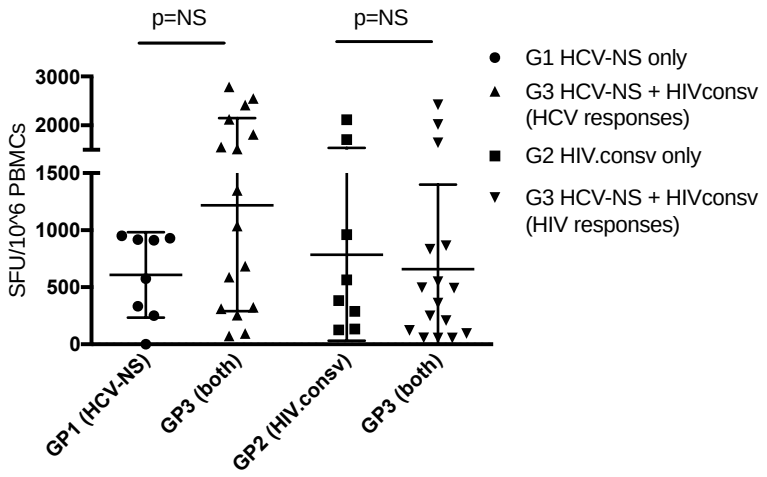


Figure 5-6: ELISpot magnitude to HCV-NS and HIVconsv post prime comparing single and dual administration of HCV and HIV vaccines. Peak response (between 2-8 weeks post vaccination) following priming with either AdCh3 alone (circles), ChAdV63 alone (squares), or both AdCh3 and ChAdV63 to HCV-NS (up triangles) and HIV.consv (down triangles).

Priming vaccination with ChAdV63 alone induced HIV specific T-cell responses in all 8 volunteers (**Figure 5-5 B, F)**. T cell magnitude peaked between 2 weeks (62.5% of volunteers) and 4 weeks (25% of volunteers) following vaccination, with the exception of 1 volunteer who peaked at week 8 (PEA-04019). Peak antigen specific T cell magnitude following prime in group 2 were (mean $\pm$ SD) 785 $\pm$753 SFU per 10^6 PBMCs.

Following simultaneous AdCh3 and ChAdV63 vector administration in group 3, the magnitude to the ChAdV63.HIV immunogen was not statistically different to group 2 (p=0.7; **Figure 5-6**). Antigen specific T cells were induced in all 16 volunteers, peaking between 2 weeks (62.5% of volunteers) and 4 weeks (31% of volunteers) following

vaccination, with the exception of PEA-0401 peaking at week 8. Peak T cell magnitude was (mean ± SD) 780 ± 774 SFU per 10^6 PBMCs.

In summary, the timing and magnitude of the peak T-cell response to either immunogen following priming was unaffected by co-administration of these adenoviral vectors.

5.5.4 THE BREADTH OF T CELL RESPONSE TO HCV-NS AND HIV.CONSV WERE NOT IMPAIRED FOLLOWING CO-ADMINISTERED WITH A DIFFERENT ADENOVIRAL VECTOR AND IMMUNOGEN.

The breadth of the antigen specific T cell induction for both immunogens was assessed in IFNγ ELISpot assays using overlapping peptides spanning the immunogens and arranged into peptide pools F-M (HCV-NS) and 1-6 (HIV.consv) (described in detail in chapter 2).

A comparison of the breadth of T cell response to HCV-NS in all volunteers between single and dual administration is shown in **Figure 5-7 A**. No significant difference was observed between groups 1 and 3. Peptide Pool G was the most immunogenic in both groups at all time points (mean ± 95% CI = 282 (81 – 484) and 624 (355 – 892) IFNγ SFU per 10^6 PBMCs for groups 1 and 3 respectively). These pools correspond to NS3, which is known to contain many highly immunogenic epitopes for both CD4+ and CD8+ T cells (Lucas et al. 2007), and is in keeping with previous studies using this immunogen (Barnes et al. 2012; Swadling et al. 2014).

Analysis of the breadth of the T cell response to HIV.consv following priming vaccination was made between groups 2 and 3 (**Figure 5-7 B)**. The magnitude of T cell response to individual peptide pools was not impaired following co-administration, with no statistically significant difference observed in any individual pool. Peptide pool 3 induced the highest magnitude (corresponding to Pol Clade C), and is in keeping with pre-clinical and clinical studies using this immunogen (Kelly et al. 2016; Borthwick et al. 2014; Letourneau et al. 2007).

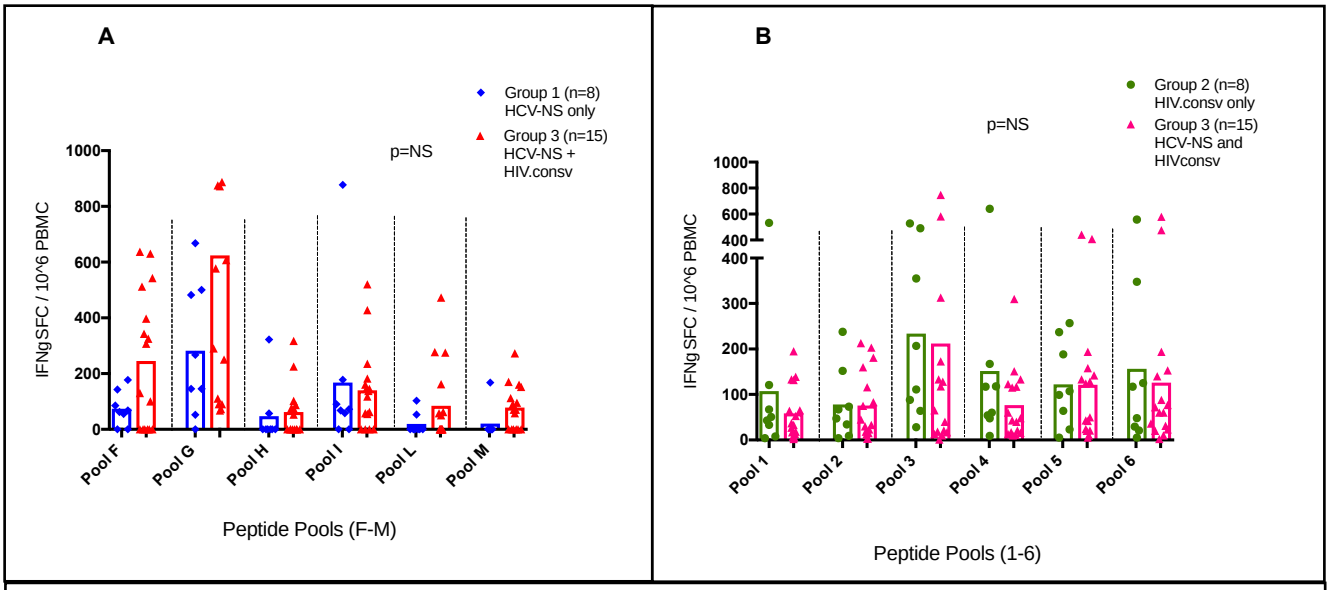


Figure 5-7: Breadth of T cell response to HCV-NS and HIV.consv following priming vaccination for single vs co-administration of AdCh3 and ChAdV63. Comparison of breadth of T cell response is made by measuring ex-vivo IFNγ ELISpot T cell magnitude to peptide pools F-M (HCV-NS) and pools 1-6 (HIVconsv). **(A)** Following priming with AdCh3 alone (group 1; black diamonds) or both AdCh3 and ChAdV63 (group 3; grey triangles). **(B)** Following priming with ChAdV63 alone (group 2; black circles) or both ChAdV63 and AdCh3 (group 3; grey triangles)

5.5.5 NO ANTIGENIC COMPETITION WAS OBSERVED FOLLOWING ADMINISTRATION OF MULTIPLE IMMUNOGENS USING MVA VECTORS

Boosting vaccination with MVA vectors containing HCV-NS (group 1), HIV.consv (group 2) or both immunogens (group 3) were administered to volunteers 8 weeks following priming vaccination. Again the magnitude and breadth of the immunogen-specific T-cell response was assessed *ex vivo* using the peptide pools described earlier in IFNγ ELISpot assays.

The magnitude of the HCV specific T cell induction following boosting with MVA-NS alone was several fold higher than induction with prime (mean ± SD = 4223 ± 2440 SFU per 10^6 PBMCs) **(Figure 5-5: A, E)**. This was of similar magnitude to that observed previously (Swadling et al. 2014). Peak T cell magnitude occurred 1 week following vaccination for all except one volunteer (PEA-04011 at 4 weeks following vaccination). The T cell magnitude to HCV-NS was not reduced when co-administered with MVA containing HIV.consv (mean ± SD = 5297 ± 3153 SFU per 10^6 PBMCs) (**Figure 5-5 C, E; Figure 5-8)**.

Similarly, a robust HIV specific T-cell induction was observed following boosting with MVA.HIVconsv, and was of similar magnitude in both the single and dual MVA administration groups (**Figure 5-5 B, D, F; Figure 5-8)**. Following MVA.HIVconsv alone (Group 2), the mean ± SD ELISpot magnitude was 3590 ± 2893 SFU per 10^6 PBMCs, with peak magnitudes occurring 1 to 4 weeks following MVA vaccination. This magnitude was comparable following dual administration (mean ± SD = 2577 ± 3621) (p=NS).

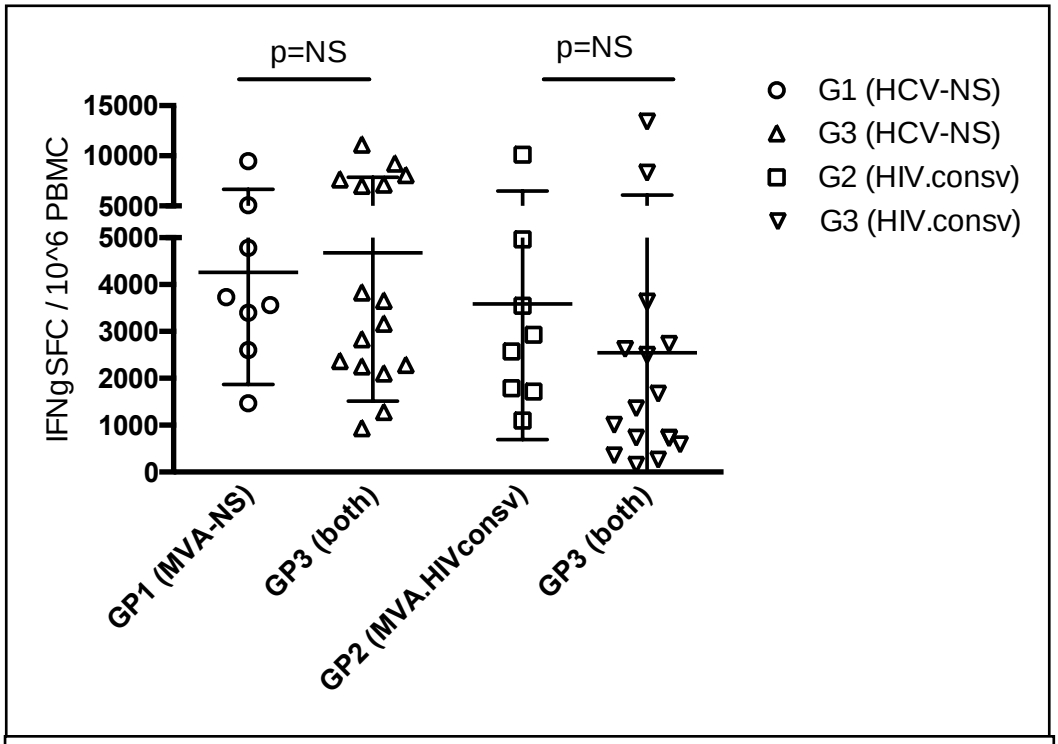


Figure 5-8: ELISpot magnitude to HCV-NS and HIVconsv post boost comparing single and dual administration of HCV and HIV vaccines. Peak T cell magnitude (between 1-4 weeks post vaccination) following boosting with either MVA-NS alone (circles), MVA.HIV alone (squares), or both MVA-NS and MVA.HIVconsv to HCV-NS (up triangles) and HIV.consv (down triangles)

The breadth of the response to both immunogens following boosting was assessed in the same way as discussed previously. A broad T cell response was observed to HCV-NS following boosting with both single and dual MVA administration (**Figure 5-9 A**). Similarly to that observed following priming vaccination, Pool G induced the highest T cell magnitude followed by Pool F in both groups 1 and 3. Likewise, a comparable breadth of response was seen to HIV.consv following single and co-administration, with the highest T cell response in Pool 3, and consistent magnitudes seen across the peptide pools (**Figure 5-9 B)**. No statistical difference was observed in any peptide pool between single and dual administration for either HCV-NS or HIV.consv.

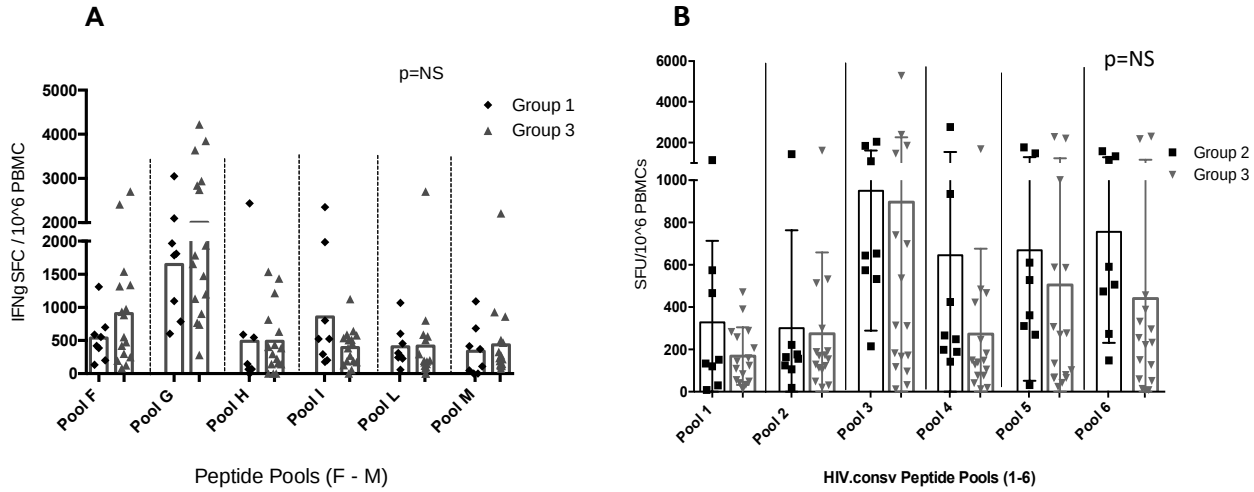


Figure 5-9: Breadth of T cell response to HCV-NS and HIV.consv following boosting vaccination for single vs co-administration of MVA-NS and MVA.HIVconsv. Comparison of breadth of T cell response is made by measuring ex-vivo IFNγ ELISpot T cell magnitude to peptide pools F-M (HCV-NS) and pools 1-6 (HIVconsv). **(A)** Following boosting with MVA-NS alone (group 1; black diamonds) or both MVA-NS and MVA.HIV (group 3; grey triangles). **(B)** Following boosting with MVA.HIV alone (group 2; black circles) or both MVA.HIV and MVA-NS (group 3; grey triangles)

In summary, no significant difference was observed in the magnitude, timing, or breadth of the peak vaccine-induced T-cell response following boost comparing single or co-administration of either the HCV or HIV immunogen.

5.5.6 END OF TRIAL ANTIGEN SPECIFIC T CELL BREADTH AND MAGNITUDE

All except one volunteer was followed up until the last trial visit occurring 34 weeks post-prime vaccination. The magnitude and breadth of the peptide specific IFNγ secreting T cell response to each immunogen was measured at the final time point **(Figure 5-10 A-C**). A robust magnitude was observed at the final time point in all groups. Similar T cell magnitudes were observed in groups 1 and 3 to the HCV-NS immunogen (mean ± SD = 701 ± 668 and 1041 ± 1094 SFU per 10^6 PBMCs respectively, p=NS). Likewise there were comparable T cell magnitudes to HIV.consv in groups 2 and 3 (mean ± SD = 846 ± 991 and 852 ± 1096 SFU per 10^6 PBMCs respectively, p=NS).

A broad T cell response across the peptide pools was also observed to HCV-NS and HIV.consv. Similar to what was observed following both prime and boost vaccination, the most immunogenic peptide pools were pool G in the HCV immunogen (corresponding to NS3) and pool 3 in the HIV immunogen (corresponding to Pol clade C). No significant difference was observed in the magnitude of any individual peptide pool between the groups at the end of the trial.

The number of positive peptide pools to the HCV-NS immunogen was calculated at peak post prime, peak post boost and end of trial **(Figure 5-10 D)**. In all except one volunteer (PEA-04005), peptide specific T cells were induced to at least one peptide pool at each time point. The average number of positive pools was highest at the peak time point following boosting vaccination (mean 5.6 positive pools for both groups, range 5-6 (group 1) and 3-

6 (group 3). This range was more variable at the end of the trial (average 3.6 (range 2-6) and 1.9 (range 1-6) number of positive pools for groups 1 and 3 respectively). This analysis was not done for the HIV.consv, as there is not yet an established cut-off for what constitutes a positive T cell response to a single peptide pool.

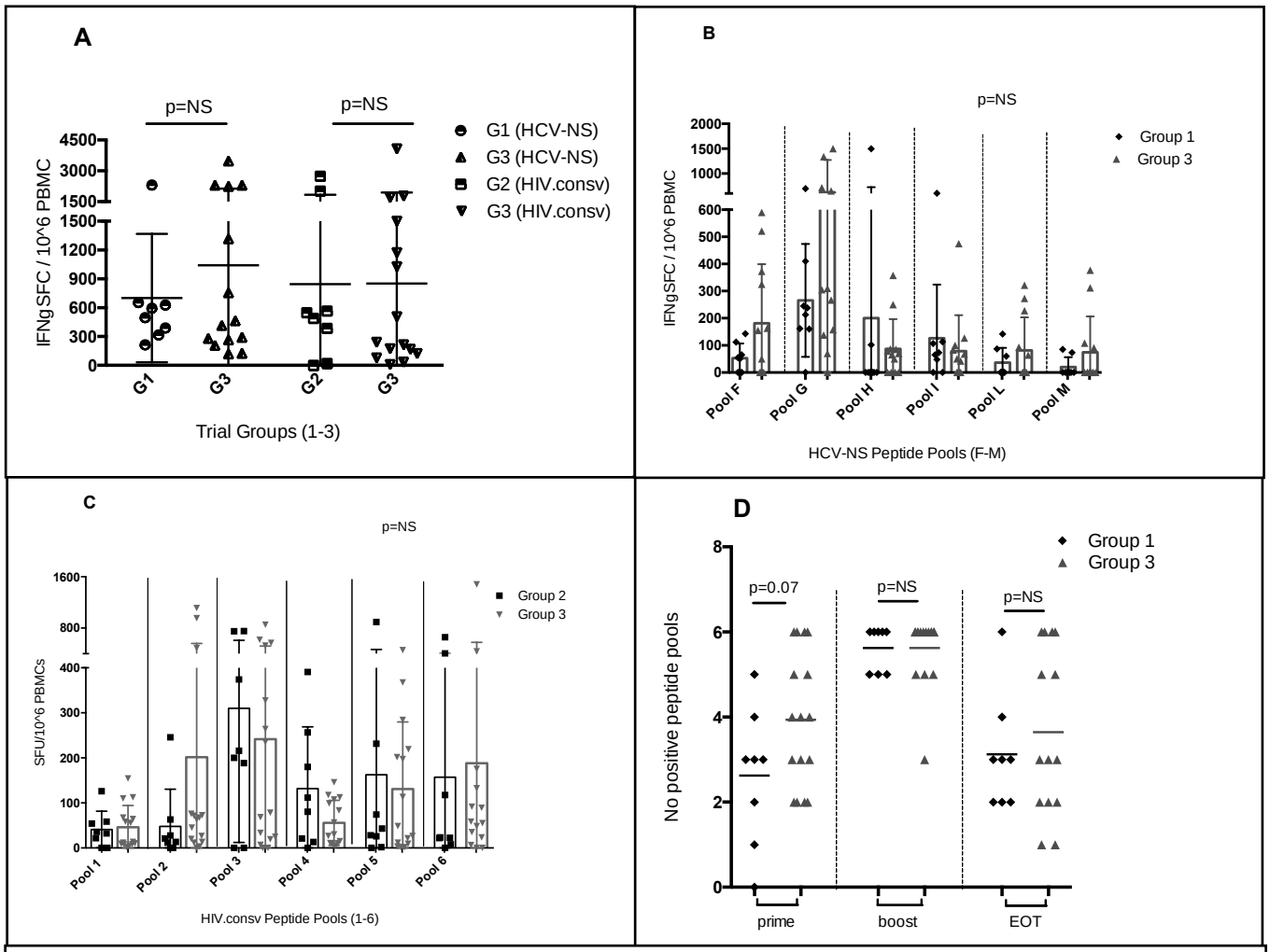


Figure 5-10: Magnitude and Breadth of T cell response to HCV-NS and HIV.consv at the end of trial visit. (A) Comparison of response magnitude (sum of positive peptide pools corrected for background) to HCV-NS in group 1 (circles) and group 3 (up triangles); and to HIV.consv in group 2 (squares) and group 3 (down triangles). **(B)** and **(C)** Comparison of breadth of T cell response made by measuring *ex-vivo* IFNγ secreting ELISpot T cell magnitude in individual peptide pools. **(B)** Comparison between groups 1 (black diamonds) and 3 (grey up triangles) to peptide pools F-M (HCV-NS) **(C)** Comparison between group 2 (black squares) and group 3 (grey down triangles) to peptide pools 1-6 (HIV.consv). **(D)** Comparison of the number of positive peptide pools (F-M) for HCV-NS between groups 1 and 3 following prime, boost and EOT.

5.6 DISCUSSION:

In this chapter data from a first in man study trialing the simultaneous administration of two serologically distinct adenoviral vectors for the prevention of HCV and HIV-1 infection is presented. It provides proof of principle that different vectors can be safely co-administered, and importantly, that immunogenicity to each vector is not impaired.

The safety and tolerability of any vaccine is of critical importance, and assessment of this is indeed the primary aim of all Phase I studies. The 4 candidate vaccines trialed in this study have all previously demonstrated excellent tolerability in pre-clinical and human studies to date (discussed previously). It was not known, however, if combining the vectors may compromise either the safety or AE profile of the vaccines. For each vector, doses were chosen based on the balance between toxicity and immunogenicity measured in pre-clinical studies performed in mice and macaques, as well as available data from human trials. For the chimpanzee adenoviral vectors, the doses in the co-administration group remained the same to single administration. Aside from the pre-clinical data suggesting this to be safe, chimpanzee adenoviral vectors have been administered to humans at a dose of 10^{11}vp with good tolerability, which supported the use of the full doses in the group 3 volunteers. For the MVA vaccines in group 3, the vector doses were halved based on the known increased reactogenicity observed with MVA compared to adenoviral vectored vaccination, with the resulting total vector dose administered in group 3 identical to groups 1 and 2.

There were no safety concerns observed following co-vaccination with either chimpanzee adenoviral or MVA vectors, with no SAE's or SUSARs occurring during the trial period in

any group. The tolerability of the vaccines between the groups were compared in terms of the severity, duration, and number of each AE. Following co-administration with both AdCh3/ChAdV63 and MVA-NS/MVA.HIV these parameters were all comparable to those experienced following single administration. Interestingly, a greater AE profile was observed following single administration with ChAdV63 (group 2) compared with either single AdCh3 or a combination of the two.

As discussed in the introduction, two major concerns with the co-administration of distinct vectors as well as the co-administration of distinct immunogens were the potential effects of viral interference (in the case of administering two distinct chimpanzee adenoviruses) and the potential of antigenic competition between the HCV-NS and HIV.consv immunogens. To address these, the magnitude, breadth, and durability of the vaccine induced T cell responses for each immunogen was compared between single and dual administration.

The peak T cell magnitude following priming and boosting vaccination, as measured by the number of IFNγ-secreting spot forming units in an ELISpot assay, was not diminished to either immunogen following co-administration. Similarly, the breadth, as determined by T cell responses to overlapping peptide pools spanning the entire immunogens, was not impaired with either co-administration of distinct adenoviral vectors, nor co-administration of distinct immunogens in separate MVA vectors. A broad T cell response, comparable in all groups, was observed to both immunogens following priming vaccination, boosting vaccination and at the end of the trial. Finally, the durability of the antigen specific T cell response to the immunogens was assessed by measuring T cell

magnitude 34 weeks following initial vaccination (26 weeks after boost). At this time point a similar magnitude was observed in both single and dual administration groups to both HCV and HIV-1 vaccines.

These observations are in contrast to published studies assessing dual vector administration. Co-administration of replication defective Ad5 and MVA encoding the same HIV immunogen in mice was observed to cause suppression of the magnitude of antigen specific CD8+ T cells when compared to single administration, as measured by MHC Class I tetramer positive populations and IFNγ secretion in ICS assays (Yashima et al. 2010). In this study it was suggested that the inhibitory effect induced by the viruses was likely due to an unidentified secretory factor released by the virus infected cells. They also hypothesized that the increased number of viral proteins present in co-vector administration induced a larger number of effector T cells, thereby decreasing the relative percentage of effector CD8 + T cells against limited epitopes (Yashima et al. 2010).

There are at least two hypotheses to explain why no viral inhibition was observed by the co-administration of adenoviral vectors in our study.

Firstly, Yashima et al. co-administered Ad and MVA vectors, whereas the current study administered 2 adenoviral vectors of different serotypes. Co-administration of two very distinct viruses (adenovirus and vaccinia virus) may induce an entirely different host response compared with the co-administration of 2 distinct adenoviruses.

Secondly, it has been shown (Harro et al. 2009) that adenoviral vectors are retained at the site of local infection and are not systemically disseminated. As the vaccines were administered in separate anatomical sites (separate arms), the virally infected cells would likely drain to different lymph nodes, and thus are unlikely to come into close proximity during induction of the cell mediated response to each immunogen.

This hypothesis was explored in a study trialing prime-boost Ad5/MVA vectors expressing multiple malarial antigens in mice (Forbes et al. 2011). When the different vectors expressing different antigens were co-administered, CD8+ T cell T cell responses were significantly diminished. However, if the same antigens were administered in vectors at different anatomical sites (in this case the ears), this effect was minimized. A similar study by Sedegah et al. (Sedegah et al. 2004) assessing multiple antigens given as either a mixture or separately in DNA plasmids in mice also observed antigenic suppression in the mixture but not when given separately. Forbes et al. hypothesized this may be due to enhanced killing of the APC when it is presenting multiple antigens by previously primed CTLs to those particular antigens, resulting in reduced antigen expression, presentation, and ultimately attenuated secondary T cell response to the antigen (Forbes et al. 2011). In the present study it is extremely unlikely that the HCV-NS and HIV.convs immunogens would be phagocytosed and presented by the same APC, and thus this potential cytotoxic effect to dual antigens by previously primed CTLs is likely to be minimal.

There are some aspects of antigenic interference that the immunoassays performed to this point have yet to completely address. The concept of immune hierarchy suggests that there is a hierarchy of responses to epitopes, determined by both pathogen factors and host factors (Yewdell and Bennink 1999). The ELISpot assays performed to this point have

assessed T cell responses to pools of peptides containing approximately 80 (for HCV-NS) or 30 (for HIV.consv) peptides of 15mer length. To more accurately determine if a hierarchical response occurred, and more precisely if any subdominant epitopes were not presented following co-vaccination, a more detailed analysis of the breadth of T cell responses would need to be performed. One way of assessing this would be to assess the T cell magnitude to 'minipools' (8-10 peptides) and individual peptides following priming and boosting vaccination. These analyses are planned, however, a lack of sufficient cell numbers to perform such detailed analysis and responses of magnitudes approaching the limit of detection for immune assays may limit this analysis.

A further important consideration is the quality and function of the T cells induced by vaccination. A robust antiviral response is likely to involve polyfunctional CD4+ and CD8+ T cells, capable of secreting multiple effector cytokines, and having an effector phenotype. Furthermore, the subset of memory cells induced by vaccination may determine the strength and size of the T cell response on antigen re-exposure (discussed further in chapters 4 and 7). It is unknown whether these functional and phenotypic characteristics of the CD4+ and CD8+ T cells are compromised following co-vaccination with multiple vectors and immunogens. We hypothesise based on the ELISpot results seen thus far that an alteration of the phenotype and function will not occur. However, further immunoassays, including ICS and FACS based phenotyping of MHC Class I and Class II tetramer positive cells are planned**.**

5.7 CONCLUSION AND FUTURE WORK

There is an unmet global need for the development of prophylactic vaccines to prevent HCV and HIV-1 infection, and in particular a vaccine strategy targeting simultaneous prevention of both viruses would be beneficial.

The correlates of a protective vaccine to prevent either HCV or HIV-1 infection are imprecisely defined. Both viruses present enormous challenges to vaccine development based on sophisticated mechanisms to subvert the host immune response, such as inefficient T cell priming and chronic antigen exposure, resulting in exhausted and dysfunctional T cell responses; evading immune detection in latent reservoirs, or high mutability leading to viral escape. Furthermore, in the absence of an acceptable challenge model in healthy volunteers, true correlates of protection cannot be directly measured, but only inferred from *ex vivo* and *in vitro* immunological assays. However, the induction of functional T cells that produce high magnitude, broad, and durable peptide specific responses are likely to be important aspects of a successful immune response to either virus. Using ELISpot assays on freshly isolated PBMCs, we have been able to measure these parameters and observed no reduction in either the breadth, magnitude, or durability of antigen specific T cell responses to dual administration of either distinct vectors or distinct immunogens. Although further immunogenicity assessment needs to be done to determine if the function and phenotype of the T cells are not impaired by dual vaccination, based on the data currently available, it appears that neither the safety nor immunogenicity of either vaccine is impaired following co-administration. This has important implications for future research using multiple vectors and immunogens in both therapeutic and prophylactic applications in infections and cancer.

In addition to further immunogenicity FACS based studies planned in the current PEACHI-04 trial, a trial is currently underway assessing AdCh3 and MVA-NS given as a prime-boost strategy to HIV-1 positive patients in Switzerland and Ireland. The results of this trial will give valuable insight into the induction of HCV specific T cells in the setting of chronic HIV-1 infection.

CHAPTER 6: A COMPARATIVE ANALYSIS OF HCV SPECIFIC T-CELL RESPONSES IN THE SETTING OF CHRONIC HCV, SPONTANEOUS VIRAL RESOLUTION, VECTOR VACCINATION AND HCV CURE.

6.1 AIM

To assess the extent of immune recovery in chronic HCV infection following viral cure with DAA therapy as measured by proliferative, functional and phenotypic changes, and to compare these responses to HCV vaccination and spontaneous HCV clearance.

6.2 BACKGROUND

Until recently, the mainstay of treatment for HCV infection has been with interferon based regimes – predominantly peg-IFN plus ribavirin. These treatments were long (usually 26 weeks), of variable efficacy (achieving long term viral clearance in only 50% of infected persons), and associated with intolerable side effects. Exogenous IFNα alters the landscape of the host innate and adaptive system by mechanisms imprecisely defined (Chung et al. 2008), making interrogation of host immunity challenging both during and post treatment. The development of directly acting antiviral reagents (DAA's) over the past few years has revolutionised treatment of HCV. They have allowed for clearance of HCV with IFN-free regimes, and have led to very high cure rates (SVR of over 90%) in genotype 1 infections, until now the hardest genotype to treat (Everson et al. 2015; Lawitz et al. 2014).

However, an essential unanswered question is the state of the host immune system after IFN free HCV cure. This question is important for a number of reasons. Firstly, many patients remain at risk of re-infection, and it is vital to understand the immunity that

persists in cured patients, and how they may respond immunologically to re-exposure to the virus. Secondly, on a global economic scale, the current exceptionally high costs of these drugs necessitate the most efficient use of DAA's in clinical settings where they will have the highest impact. Thirdly, the subgroup of patients with DAA mediated cure of HCV at risk of re-infection may be prime candidates to receive a preventative HCV T cell vaccine. It is important therefore to establish the extent of immune recovery and if or how their immune systems may respond to a vaccine immunogen of either the same or different viral genotype. Finally, the study of IFN-free viral cure gives a unique opportunity to understand broader questions regarding immunity following cure of a chronic infection. Chronic infections with persisting pathogens such as viral hepatitis B and C, *Mycoplasma* and intestinal helminths affect billions of people. However, detailed immunological interrogation following recovery is challenging due to either the difficulty in curing the infection, or the logistic and economic challenges of treating and studying the infections in the developing world. The study of rapid viral cure with DAA's allows the opportunity to interrogate in detail alterations of the immune system that persist following HCV clearance, in particular the extent of immune recovery. The insights gained from this will be relevant for other persisting pathogens.

The concept of T cell exhaustion is central to this chapter, and although discussed in depth in chapter 1, it will be briefly reviewed again. T cell exhaustion refers to the hierarchical loss of effector functions that occur over time following chronic antigen stimulation, and has been observed in numerous chronic infections including HCV (Bowen and Walker 2005; Lechner et al. 2000). T-cell exhaustion is characterised by the loss of effector functions, such as: IL-2, TNFα and IFNγ production, and eventually can result in the deletion of

antigen specific cell populations (Fuller and Zajac 2003; Virgin, Wherry, and Ahmed 2009; Wherry and Ahmed 2004). T cell exhaustion is believed to be mediated by inhibitory cell surface markers, including PD-1 (Petrovas et al. 2006), immunosuppressive cytokines such as IL-10 and TGFβ (Paladino et al. 2006), CD4+CD25+Foxp3+ regulatory T cells (Boettler et al. 2005), and modulation of transcriptional factor expression, including Blimp-1, eomes and Tbet (Jensen et al. 2008; Wherry et al. 2007). It is incompletely understood whether removal of chronic antigen stimulation reinvigorates functional T cells, however the recovery of T cell function following PD-1 pathway blocking suggests at least partial response may be restored (Barber et al. 2006).

As mentioned above, patients cured of HCV who remain at risk of re-infection are prime candidates to receive preventative HCV vaccination. Prophylactic vaccination using AdCh3/MVA-NS vectors remains a realistic goal following broad, polyfunctional, and cross-reactive responses generated in healthy volunteers (Swadling et al. 2014). However, it remains to be established whether DAA mediated cure reinvigorates T cells that will respond to vaccination. AdCh3/MVA-NS vaccination induced attenuated antigen specific responses in patients receiving IFN therapy, at time points immediately following and 14 weeks following IFN therapy (Kelly et al. 2016). These blunted responses may have been due to either ongoing effects of IFN or inadequate viral clearance. An alternative hypothesis however, is that the HCV specific T cells were exhausted, and despite many patients having undetectable viral loads at the time of vaccination, T cell functional recovery had not occurred following viral clearance, and vaccination was therefore unable to elicit a robust immune response. It is imperative that a detailed analysis of HCV specific

T cells occurs following DAA therapy to identify if these patients may be amenable to prophylactic vaccination.

Due to the array of functional and phenotypic changes T cells undergo on the pathway to exhaustion, there are a number of mechanisms that may be employed to assess T cell recovery. Using the model of hierarchical loss of function observed in both mouse and human models (Gallimore et al. 1998; Wherry 2011), a proposed method to assess recovery is the measurement of T-cell proliferative capacity, production of effector cytokines such as TNFα, IFNγ, MIP-1β and IL-2, the assessment of inhibitory receptors and transcription factor expression, and the presence of regulatory T cell subsets.

Furthermore, spontaneous viral resolution provides a model of natural protection to HCV infection. T cell phenotype and function following DAA mediated cure will therefore be compared to T cell function in spontaneous clearance, to aid in the assessment of T cell recovery following treatment.

Finally, HCV viral vectored vaccination enables the analysis of healthy T cell responses to HCV antigen in a controlled setting, and has been established to induce broad, polyfunctional and high magnitude T cell responses. They therefore serve as another suitable comparison to assess recovery of T cell function. Additionally, a direct comparison of vaccine induced T cell responses to those seen in spontaneous viral resolution will give insight as to how similar AdCh3/MVA-NS vaccination is to the 'gold standard' of spontaneous viral resolution.

6.3 HYPOTHESES

1. *Ex-vivo* MHC class II tetramer specific CD4+ T cell populations may be detected following DAA-mediated viral cure, but will not be detected in chronic HCV infection
2. The proliferative potential of HCV specific CD4+ T cells is partially restored following DAA mediated HCV clearance
3. Effector cytokine production is partially restored in CD4+ and CD8+ T cells following DAA mediated HCV cure
4. The bulk CD4+ regulatory T helper cell population declines following HCV clearance with DAA therapy
5. Phenotypic changes suggestive of the reversal of exhaustion are seen in bulk CD4+ and CD8+ T cells following DAA treatment
6. The proliferative and functional capacity of vaccine-induced HCV specific T cells are analogous to those seen in spontaneously resolved HCV infection

6.4 CHAPTER SPECIFIC METHODS

Full details of the methods used in this chapter can be found in chapter 2, however, chapter-specific methods will be reviewed briefly here.

6.4.1 RECRUITMENT

Both chronic HCV patients undergoing DAA treatment and individuals with spontaneously resolved HCV infection (SR's) were recruited from the John Radcliffe Hospital, Oxford. Patients receiving DAA therapy donated blood prior to (range 0 to 52 weeks) and post (0

to 26 weeks) treatment, and SR's gave a single blood donation. Patient and SR characteristics can be found in **Table 6-1** and **Table 6-2** respectively.

Vaccine trial samples were obtained from the HCV003 trial (refer to chapter 4). Samples were selected based on appropriate HLA matching and positive ELISpot responses.

Vaccine samples selected are summarised in **Table 6-3.**

6.4.2 IMMUNOASSAYS

The development of peptide specific cell lines, plus tetramer, ICS and bulk phenotyping assays are described in chapter 2**.** The peptide pools used are found in Appendix 1**.**

All patients (n=21), spontaneous resolvers (n=10) and vaccine samples (n=5) were included in all analyses. From here onwards the groups will be referred to as follows: patients pre-DAA therapy = pre-DAA, post DAA therapy = post-DAA, spontaneous resolvers = SR, HCV003 vaccine volunteers = vaccinees.

6.5 RESULTS

6.5.1 PATIENT COHORT

Baseline characteristics for the patients included in the study are found in **Table 6-1.** 21 patients were included on the basis of 1) successful viral clearance with DAA therapy and 2) successful blood collection (for PBMC isolation) both prior to commencing and at the end of DAA treatment. The average patient age was 59 years (range 44 – 74). 6 patients

were genotype 3, the rest were genotype 1. Only 4 patients were treatment naïve, and of the remaining 17, all had received at least one course of peg-IFN + ribavirin, and 6 had also failed treatment with a first-generation protease inhibitor. All patients included had chronic HCV infection of at least 10 years duration, and 60% of patients had liver cirrhosis. All patients had undetectable HCV RNA before the end of treatment. Pre-treatment samples were collected either at treatment day 0 or before treatment (range 0-52 weeks). Post treatment samples were taken on average 6 weeks following the end of treatment (range 0-26 weeks).

The SR and vaccinee cohort characteristics are found in **Table 6-2** and **Table 6-3.** Vaccine trial samples were healthy adults identified from the HCV003 trial (refer to chapter 4). All vaccinees received AdCh3 and MVA-NS vaccination at doses of 2.5×10^{10} vp and 2×10^{8} pfu at weeks 0 and 8 respectively.

N	Age	Geno	Cirrhosis present	Previous treatment	Current Treatment	HCV PCR negative	1st time point	2nd time point	HLA DR
261	59	1b	Y	IFN, PI	S + L + riba	TW4	3mth pre	3mth post	DRB1*01, *03
383	68	1	N	IFN	S + L + riba	TW8	11mth pre	1mth post	DRB1*07
402	49	1	Y	IFNx2, PI	S + L + riba	TW6	7mth pre	3mth post	DRB1*01
890	60	1	Y	IFN	S + L + riba	TW4	6mth pre	1mth post	DRB1*03, *07
895	65	1b	Y	IFNx2, PI	S + L + riba	TW8	1mth pre	3mth post	DRB1*15
913	55	1a	Y	IFN	S + L + riba	TW8	6mth pre	3mth most	DRB1*04, *15
996	59	1	Y	IFNx2, PI	S + L + riba	TW4	12mth pre	1mth post	DRB1*04
1119	59	1a	Y	Nil	S + L + riba	TW4	12mth pre	1mth post	DRB1*01
191	65	1	Y	IFNx2, PI	S + L + riba	TW4	4mth pre	6mth post	DRB1*07
185	63	3	N	Nil	S + L + riba	TW8	48mth pre	0mth post (EOT)	DRB1*07, *15
202	61	1b	N	IFN	O + P + R + D	TW8	0 mth pre (SOT)	0mth post (EOT)	DRB1*07
722	64	3	Y	IFN	S + V	TW8	0 mth pre (SOT)	0mth post (EOT)	DRB1*01, *15
736	51	3	N	IFN	S + IFN + riba	TW8	6mth pre	3mth post	DRB1*03, *15
820	51	1a	N	IFN	S + L + riba	TW8	0 mth pre (SOT)	0mth post (EOT)	DRB1*04
950	64	1a	N	Nil	S + L + riba	TW8	0 mth pre (SOT)	1mth post	DRB1*04
984	61	1a	Y (T)	IFN	S + L + riba	TW4	0 mth pre (SOT)	0mth post (EOT)	DRB1*01
1164	74	1	Y	Nil	S + L + riba	TW4	6mth pre	0mth post (EOT)	DRB1*03, *04
1215	55	3a	N	IFN	S + IFN + riba	TW4	6mth pre	3mth post	DRB1*04
238	55	3	N	IFN	S + IFN + riba	TW4	1mth pre	0mth post (EOT)	DRB1*03, *07
780	64	1	Y	IFN, PI	S + L + riba	TW8	6mth pre	3mth post	DRB1*15
1087	44	3	N	IFNx2	S + V	TW2	12mth pre	3mth post	DRB1*07

Table 6-1: Characteristics of patients undergoing DAA therapy. N=cohort number. Geno=genotype. Y=yes, N=no. T=liver transplant. IFN=interferon therapy. Riba=oral ribavirin. PI=protease inhibitor (teleprevir, boceprevir). S=sofosbuvir. L=ledipasvir, V=velpatisvir, O=ombitasvir, P=paritaprevir, R=ritonavir, D=dasabuvir. TW=treatment week. EOT=end of treatment. SOT=start of treatment.

Cohort number	Year of HCV Transmission	HLA DR	Tetramer used
SR3	Unknown	DRB1*01, *04	14, 15, 20, 21
SR4	1980's	DRB1*03, *15	17, 18, 19, 25
SR8	Unknown	DRB1*07	22, 24, 29
SR9	1970's	DRB1*07	22, 24, 29
SR10	1980's	DRB1*15	17, 18, 19
SR11	Unknown	DRB1*04	15, 20, 21
SR13	Unknown	DRB1*15	17, 18, 19
SR14	2000's	DRB1*07, *15	22, 24, 29, 17, 18, 19
SR16	1990's	DRB1*01, *07	14, 22, 24, 29
SR17	1990's	DRB1*15	17, 18, 19

Table 6-2: Spontaneous resolver cohort. Recruited based on HCV Ab positivity. Negative HCV PCR and absence of HCV treatment confirmed prior to enrolment.

HCV003 trial number	TW	ELISpot response (SFU/10^6 PBMCs)	HLA DR	Tetramer used
HCV003333 (333)	9	4515	DRB1*01, *07	14, 22, 24, 29
HCV003335 (335)	12	2648	DRB1*04, *15	15, 17, 18, 19, 20, 21
HCV003337 (337)	32	1579	DRB1*07	22, 24, 29
HCV003339 (339)	48	1077	DRB1*04	15, 20, 21
HCV003343 (343)	18	3313	DRB1*01	14

Table 6-3: Vaccine trial volunteers. TW=trial week. ELISpot=Enzyme-linked immunospot assay. SFU=spot forming units.

6.5.2 EX-VIVO ANALYSIS OF TETRAMERS

Ideally the analysis of tetramer positive HCV specific CD4+ population would occur on *ex-vivo* samples, as this would be a better reflection of the *in vivo* population characteristics. Furthermore, accurate phenotyping of the tetramer positive cells can only be done on the cells in their resting state, as any stimulation of the cells may change their cell surface and

transcriptional factor profile. However, it has widely been reported (Bowen and Walker 2005; Missale et al. 1996; Thimme et al. 2001) that chronic HCV patients have extremely low magnitude or undetectable HCV specific CD4+ T cell populations *ex vivo*, making identification and analysis challenging. To confirm this, we tested the tetramers on a small number of patients *ex vivo* (**Figure 6-1**). As expected, no tetramer positive populations could be identified, and therefore all further tetramer staining was performed on samples following expansion in short term cell lines.

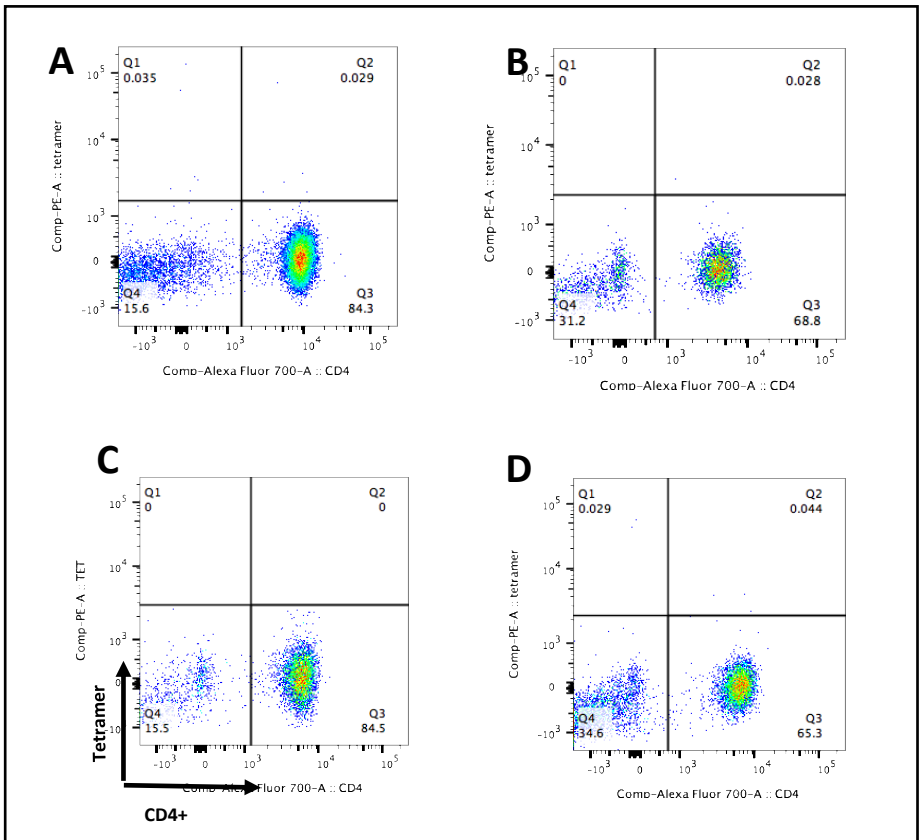


Figure 6-1: *Ex-vivo* tetramer staining of PBMCs using MHC Class II tetramers gating on CD4+ T cells. (A) 261 pre-DAA, tetramer 14. **(B)** 185 post-DAA, tetramer 17. **(C)** 1164 pre-DAA, tetramer 15. **(D)** 238 post-DAA, tetramer 24.

6.5.3 ASSESSMENT OF PROLIFERATIVE RECOVERY OF CD4+ T CELLS FOLLOWING DAA MEDIATED CURE

A reduction in the proliferative capacity of HCV-specific T cells occurs during chronic HCV infection (Gerlach et al. 1999), and it is important to assess the potential of these cells to regain this functional parameter following viral clearance with DAA therapy.

The proliferative capacity of HCV specific CD4+ T cells in the pre-DAA, post-DAA, SR and vaccine groups was assessed using a panel of MHC Class II CD4+ tetramers. Following HLA typing, thawed PBMCs were cultured with peptides that corresponded to HLA matched tetramers (refer to chapter 2).

Representative tetramer plots for the 4 comparison groups are shown in **Figure 6-2 A-H**. Each individual sample was also stained with the full panel minus the tetramer to assess if there was spillover in the fluorescence channel used to assess the tetramers. No background fluorescence was observed (data not shown), therefore, I calculated the background as described in chapter 3, using HLA matched and mismatched healthy controls.

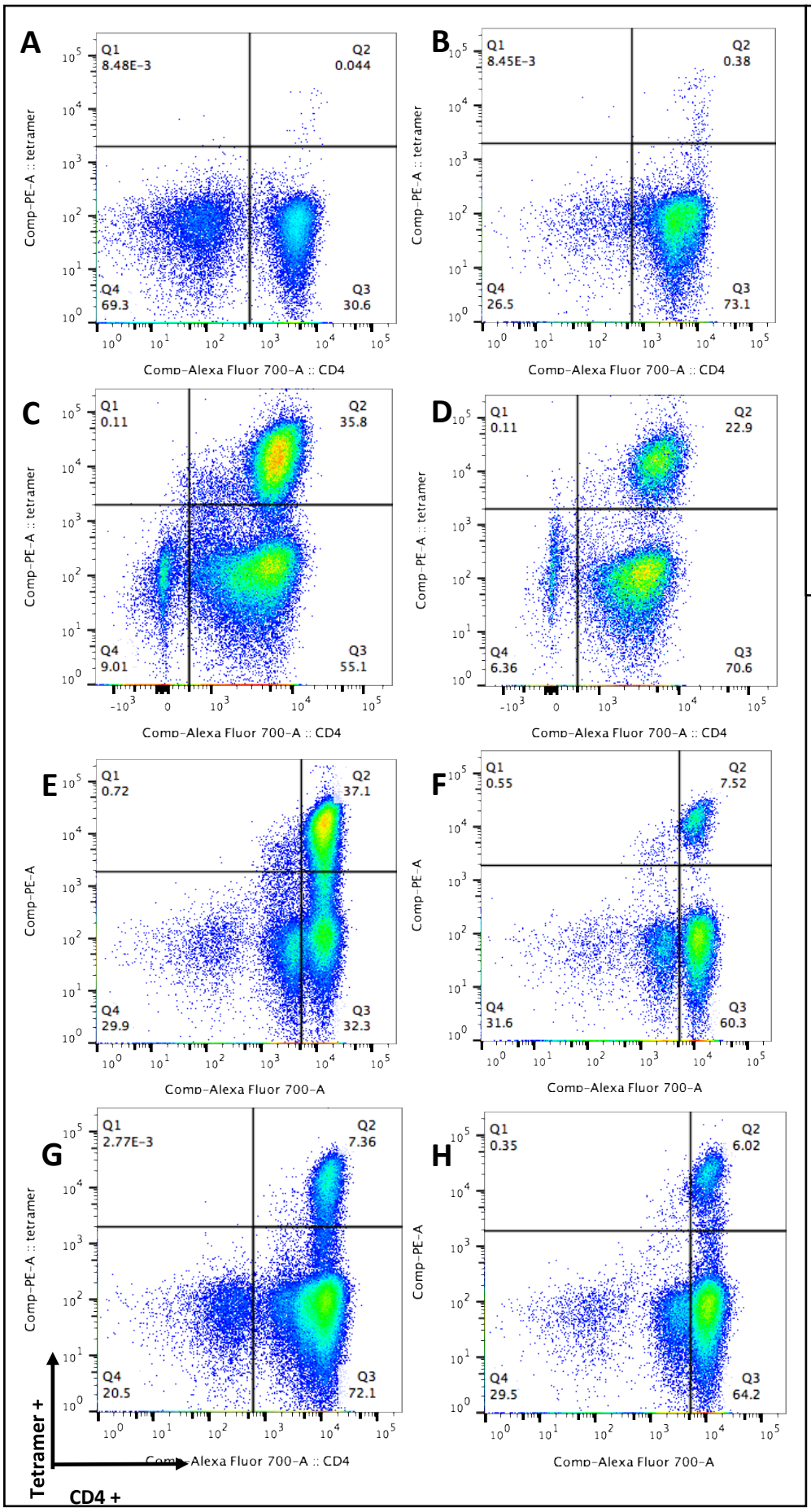


Figure 6-2: Representative MHC Class II tetramer stains on CD4+ cells following culture with peptide. (A) and **(B)** Patient 984 with tetramer 14 (pre-DAA/post-DAA respectively). **(C)** and **(D)** patient 895 with tetramer 19 (pre-DAA/post-DAA respectively). **(E)** vaccine volunteer 333 (TW9) with tetramer 14. **(F)** vaccine volunteer 335 (TW12) with tetramer 18. **(G)** spontaneous resolver SR 11 with tetramer 21. **(H)** spontaneous resolver SR 17 with tetramer 17.

The total percentage of CD4+ T cells that were tetramer positive following culture with tetramer specific peptide for each group are shown in **Figure 6-3.**

A comparison was made between the four groups of the tetramer positive CD4+ T cells following cell culture. There was a variable number of tetramers used with each patient (between 1 to 6) due to the various HLA types between samples and available tetramers. Therefore, in order to represent each individual equally, only the highest magnitude tetramer for each sample was chosen for analysis.

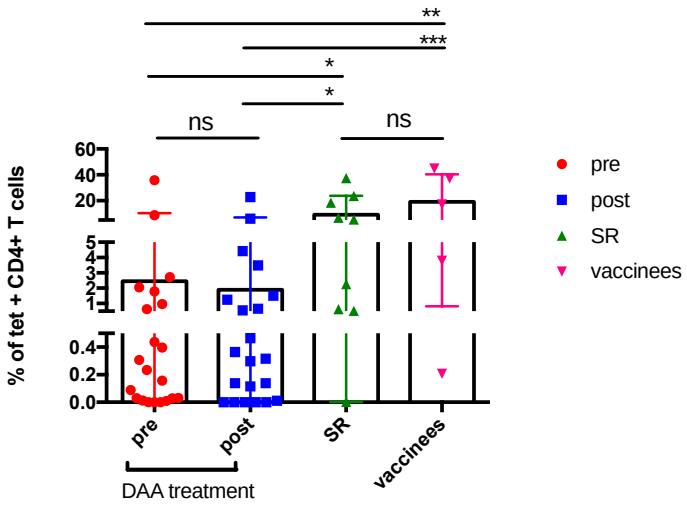
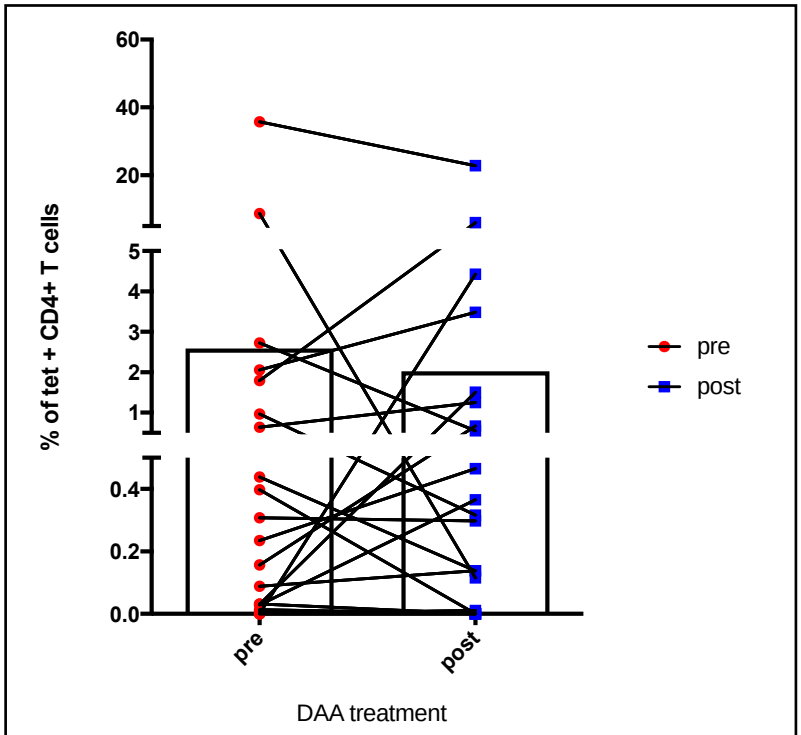


Figure 6-3: Comparison of tetramer positive CD4+ T cells following culture. PBMCs were cultured with peptide matching tetramer sequence for 14 days. Pre: patients prior to commencing DAA therapy (red circles). Post: patients following therapy (bue squares). SR: HCV spontaneous resolvers (green triangles). Vaccine: vaccine trial samples, following AdCh3/MVA with HCV-NS (pink triangles). Mean ± SD shown. Paired (pre-/post-) and unpaired (SR/vaccine) T tests used to determine significance (paired for pre- and post-DAA, unpaired for rest).

In the patient groups, the pre-DAA mean percentage (using the largest tetramer population) of tetramer positive CD4+ T cells was 2.59% (95% CI range 0.0 to 6.16%). This did not change significantly following therapy. At the post treatment time point (average 6 weeks, range 0 – 56 weeks), the average percentage of tetramer positive CD4+ T cells cells was 2.03% (95% CI range 0.0 to 4.33%).

In comparison both the SR's and vaccinees demonstrated vigorous proliferative capacity. Both groups had significantly higher percentages of tetramer positive cells in comparison to both pre- and post-DAA samples. The average percentage of tetramer positive CD4+ T cells in the SR group was 10.57% (95% CI range 0.48 – 20.65%) (p=0.047 and p=0.014 for pre-DAA and post-DAA respectively). The difference in magnitude of the tetramer positive CD4+ T cells in the vaccinees vs patients was even more pronounced, with an average tetramer positive CD4+ T cell population in the vaccine group of 20.63% (95% CI range -3.98 – 45.24%) (p=0.0026 and p=0.0005 for pre-DAA and post-DAA respectively). There was no statistically significant difference between the SR and vaccinee group.

I next sought to analyse the pre-DAA and post-DAA groups in more detail, with the aim of identifying trends or differences between the two groups. **Figure 6.4** shows the tetramer positive CD4+ T cell magnitude of individual pre- and post-DAA samples with lines identifying the change in magnitude following treatment. Interestingly there is no clear trend when the group is looked at as a whole.



F**igure 6.4: Comparison of tetramer positive CD4+ T cells pre-DAA vs post-DAA therapy**. PBMC's cultured with peptide matching tetramer sequence for 14 days. Pre = pre-DAA therapy (red circles), post = post-DAA therapy (blue squares). Mean shown.

I then identified subgroups which either a two-fold increase or decrease in tetramer positive CD4+ T cells following DAA mediated cure with the aim of analysing any significant differences within these subgroups according to the parameters listed in **Tables 6.4 & 6.5**. In the subgroup of patients that had a greater than two-fold increase in frequency of tetramer positive CD4+ T cells following DAA treatment (n=8), there were no significant differences with regards to patient age, genotype, presence of cirrhosis, or expression of listed cell surface / transcription factors markers (**Table 6.4**). Likewise, in the subgroup of patients that had a greater than two-fold decrease in the frequency of tetramer positive

CD4+ T cells following DAA-mediated treatment (n=6), there were also no significant differences in the parameters listed (**Table 6.5**).

	<2-fold increase	>2-fold increase	P-value
Age (average)	58.6y	60.6y	p=0.52
Genotype 1	9 (70%)	6 (75%)	
Genotype 3	4 (30%)	2 (25%)	
Cirrhosis present	6 (46%)	6 (75%)	
Neg RNA <8 wks	5 (41%)	6 (75%)	
Tbet	0.95	-0.53	p=0.57
Eomes	-2.3	-1.61	p=0.75
PD-1	1.1	2.7	p=0.34
Foxp3	-0.74	0.47	p=0.35

Table 6.4: Subgroup analysis of patients with >2-fold increase of tetramer positive CD4+ T cells vs <2-fold increase following DAA mediated HCV cure. PD-1, Tbet, eomes and Foxp3 represented as change in expression in post-DAA – pre-DAA for individual samples, with average of individual sample changes represented. Unpaired T-tests used for significance

	<2-fold decrease	>2-fold decrease	P-value
Age (average)	59.3y	59.3y	p=>0.99
Genotype 1	11 (73%)	4 (66%)	
Genotype 3	4 (27%)	2 (33%)	
Cirrhosis present	9 (60%)	3 (50%)	
Neg RNA <8 wks	8 (53%)	3 (50%)	
Tbet	-0.45	1.7	p=0.47
Eomes	-0.7	-3.83	p=0.11
PD-1	-0.47	-2.92	p=0.29
Foxp3	-0.16	-0.89	p=0.57

Table 6.5: Subgroup analysis of patients with >2-fold decrease of tetramer positive CD4+ T cells vs <2-fold decrease following DAA mediated HCV cure. PD-1, Tbet, eomes and Foxp3 represented as change in expression in post-DAA – pre-DAA for individual samples, with average of individual sample changes represented. Unpaired T-tests used for significance

The individual tetramers were compared **(Figure 6-5A-E)**. DRB1*0101 restricted tetramer 14 [$NS3_{1806-1818}$ TLLFNILGGWVAA] induced the most robust HCV specific T cell response across all four groups (total n=14). The average tetramer positive CD4+ T cell population for this tetramer ranged from 2.09% (post-DAA) to 41% after culture (vaccinees). In comparison, T-cell populations binding tetramers 22 ($NS2_{810-1027}$ WPLLLLLALPQRAYAQ] and 29 [$NS5A_{1957-1975}$ SLTITSLLRRLHNWITSCS] were rarely detected. Tetramer 22 (n=19) and tetramer 29 (n=19) binding T-cells were undetectable in all but one sample each (patient 383 pre-treatment, 0.006% and SR 9, 0.008%).

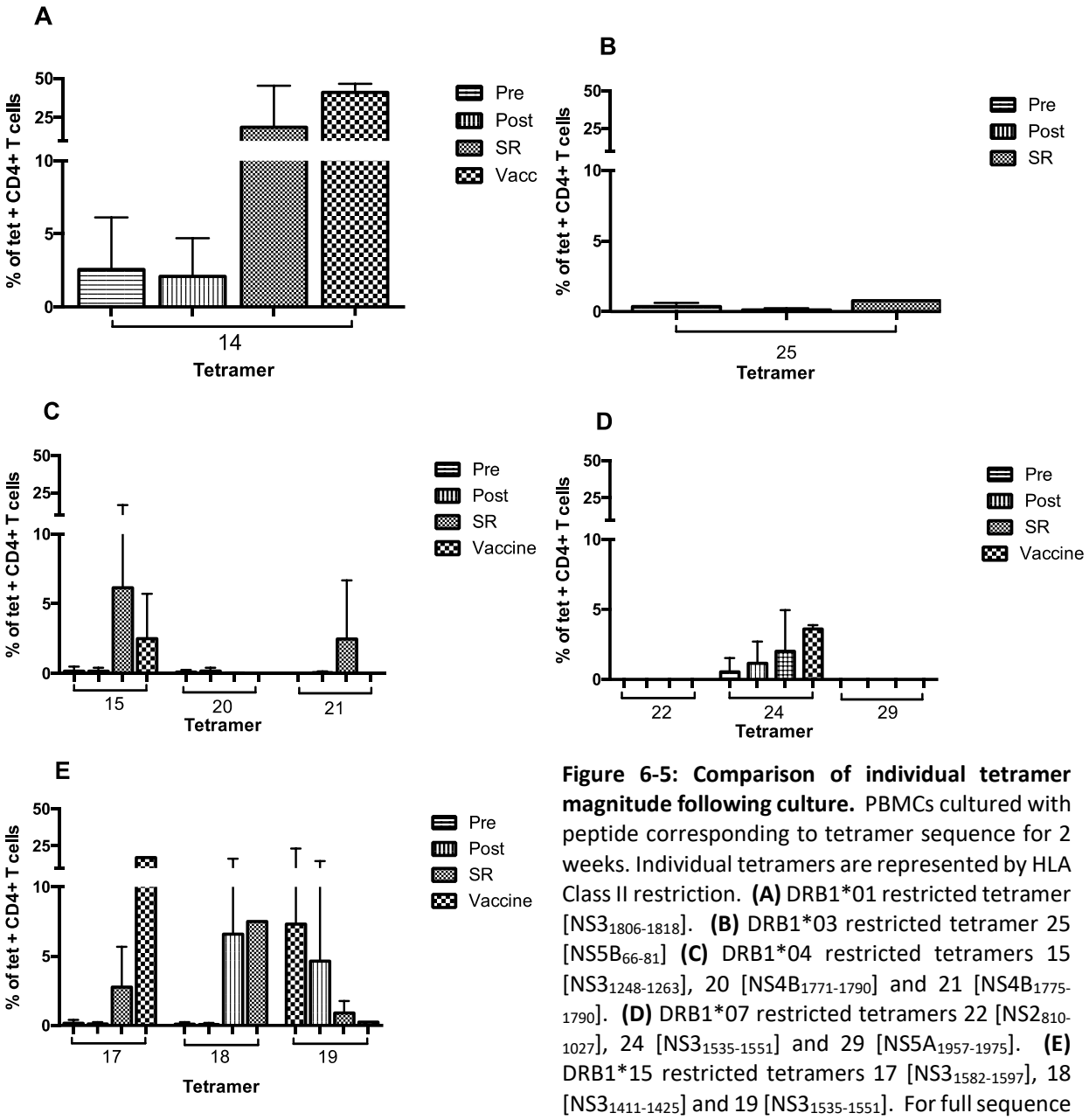


Figure 6-5: Comparison of individual tetramer magnitude following culture. PBMCs cultured with peptide corresponding to tetramer sequence for 2 weeks. Individual tetramers are represented by HLA Class II restriction. **(A)** DRB1*01 restricted tetramer [$NS3_{1806-1818}$]. **(B)** DRB1*03 restricted tetramer 25 [$NS5B_{66-81}$] **(C)** DRB1*04 restricted tetramers 15 [$NS3_{1248-1263}$], 20 [$NS4B_{1771-1790}$] and 21 [$NS4B_{1775-1790}$]. **(D)** DRB1*07 restricted tetramers 22 [$NS2_{810-1027}$], 24 [$NS3_{1535-1551}$] and 29 [$NS5A_{1957-1975}$]. **(E)** DRB1*15 restricted tetramers 17 [$NS3_{1582-1597}$], 18 [$NS3_{1411-1425}$] and 19 [$NS3_{1535-1551}$]. For full sequence description refer to **Table 3-1.** Mean percentage of tetramer positive CD4+ T cell ± SD shown. Pre = pre DAA treatment, post = post DAA treatment, SR = spontaneously resolved HCV infection, vaccine = vaccine volunteer receiving AdCh3/MVA.

6.5.4 ASSESSMENT OF CD4+ CYTOKINE PRODUCTION FOLLOWING POOLED PEPTIDE STIMULATION

ICS was used to determine the cytokine production of HCV specific T cells on peptide stimulation. The cytokines I have chosen to measure are associated with functional CD4+ and CD8+ T cells. IFNγ and TNFα are often used to assess the antiviral capacity of T cells. IL-2 stimulates clonal expansion and T cell differentiation. MIP-1β is a pro-inflammatory chemokine.

ICS was performed following *in vitro* peptide stimulation. After culturing cells with the peptide of interest for two weeks and resting in media for 24 hours, cells were stimulated with either peptide, DMSO, or PMA/I (positive control) overnight, prior to running FACS analysis on the LSRII (described in chapter 2). Analysis was made between the four groups as described above: pre-DAA (n=21), post-DAA (n=21), SR (n=10), and vaccinees (n=5).
The peptides chosen for stimulation were peptides spanning genotype 1b NS3-4 region, which corresponded to peptide pools F-H from the PEACHI-04 and HCV003 trials (**Appendix 2-1**).
These peptide pools were chosen based on their immunogenicity demonstrated in the trials, as well as published data identifying a number of immunogenic CD4+ epitopes in NS3-4 proteins (Ward et al. 2002). I was unable to stimulate with the entire HCV genome due to both lack of cell numbers (for additional culture wells) and the concern of excess DMSO concentration (by using all peptide pools in one well).

Representative ICS plots for the cytokines of interest are shown in **Figure 6-6; A-H** (for IFNγ and TNFα) and **Figure 6-7; A-H** (for MIP-1β and IL-2). Generally, the SR and vaccinees

demonstrated robust responses following stimulation with peptide pools. Patient samples on the other hand demonstrated attenuated responses, however most patients still had a detectable cloud to at least one cytokine. The DMSO (unstimulated) samples had low background (<0.5%). For each cytokine, the background cytokine level detected in unstimulated wells (DMSO) was subtracted in each individual sample.

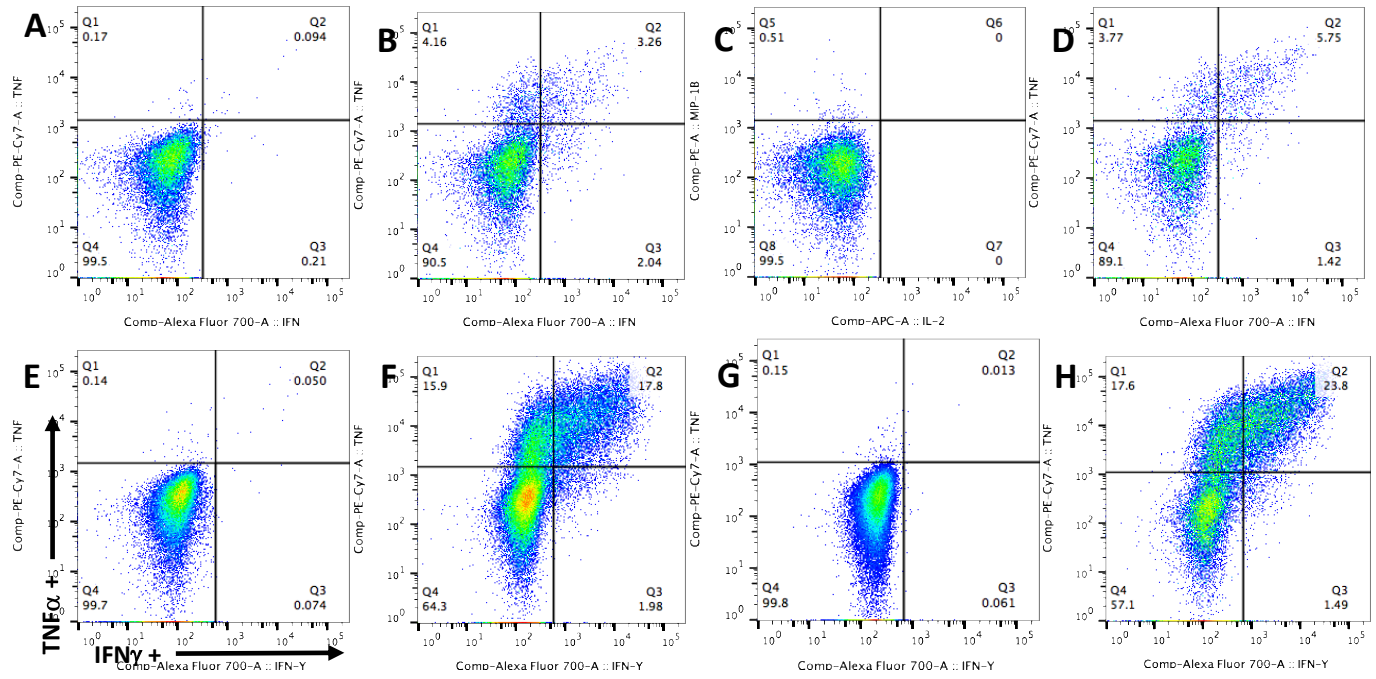


Figure 6-6: Representative ICS staining of cytokines TNFα and IFNγ on CD4+ T cells following cell culture.
(A)-(F) following overnight stimulation with either DMSO or peptide pools F-H covering NS3-4.
(A) 420 pre-DAA DMSO **(B)** 420 pre-DAA F-H. **(C)** 420 post-DAA DMSO **(D)** 420 post-DAA F-H. **(E)** SR17 DMSO **(F)** SR17 F-H **(G)** vaccinee 333 post AdCh3/MVA DMSO **(H)** vaccinee 333 post AdCh3/MVA F-H.

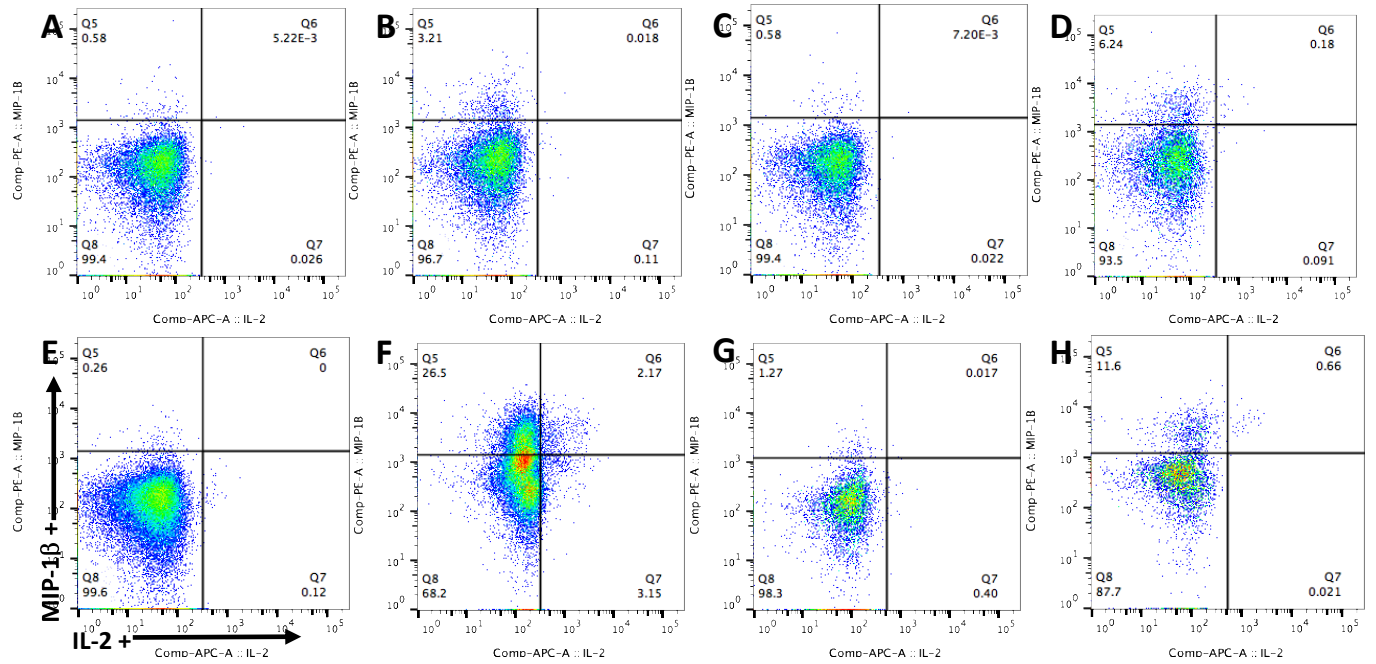


Figure 6-7: Representative ICS staining of cytokines MIP-1β and IL-2 on CD4+ T cells following cell culture.
(A)-(F) following overnight stimulation with either DMSO or peptide pools F-H covering NS3-4.
(A) 420 pre-DAA DMSO **(B)** 420 pre-DAA F-H. **(C)** 420 post-DAA DMSO **(D)** 420 post-DAA F-H. **(E)** SR16 DMSO **(F)** SR16 F-H **(G)** vaccinee 343 post AdCh3/MVA DMSO (**H)** vaccinee 343 post AdCh3/MVA F-H.

A comparison of cytokine production between the four groups was made (**Figure 6-8)**. TNFα was observed to be the most abundantly secreted cytokine in all groups except the vacinees, followed by IFNγ and MIP-β. Relatively small amounts of IL-2 production was observed in all groups.

Within the pre-DAA and post-DAA groups there was no significant change following pooled peptide stimulation in the production of any individual cytokine following treatment. Pre-DAA average individual cytokine production for HCV specific CD4+ T cells ranged from (mean; 95% CI range) 0.52%; 0.065 – 0.98% (for IL-2) to 3.6%; 2.3 – 4.8% (for TNFα). This observation varied only modestly following treatment, ranging from 0.32%; 0.076 – 0.56% (IL-2) to 3.3%; 1.8 – 4.8% (TNFα).

In comparison, vigorous cytokine production was observed in SR's and vaccinees. A significantly greater production of TNFα, IFNγ and MIP-1β was observed for both SR and vaccinees compared to both pre- and post-DAA. In the SR group, cytokine production ranged from (mean; 95% CI range) 2.4%; -0.57 – 5.4% (IL-2) to 19%; 12 – 27% (TNFα), and in the vaccinees from 0.21%; 0.045 – 0.38% (IL-2) to 16%; 5.2 – 27% (MIP-1β). No statistically significant difference was observed for any cytokine secretion between SR's and vaccinees.

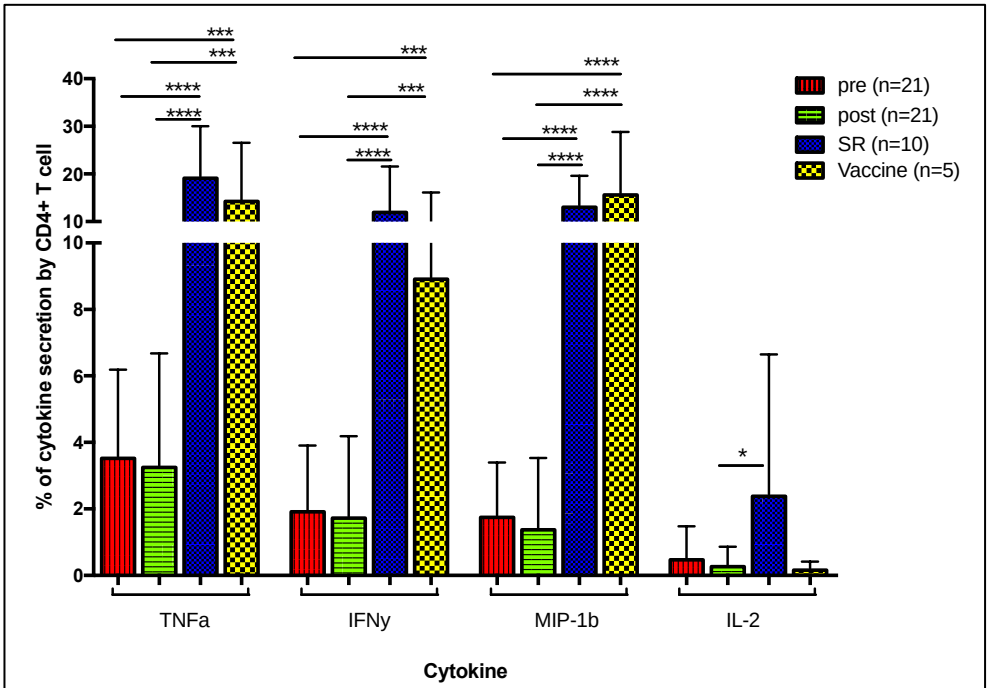


Figure 6-8: Comparison of individual cytokine secretion by HCV specific CD4+ following cell culture and overnight peptide stimulation with ICS. Shown as mean percentage of cytokine secretion with 95% CI range. Pre = pre DAA treatment (red vertical stripes), post = post DAA treatment (green horizontal stripes), SR = spontaneously resolved HCV infection (blue small squares), vaccine = AdCh3/MVA vaccine induced response (yellow large squares). Paired T tests (pre- vs post-DAA) and unpaired T tests (all other groups) used.

6.5.5 ANALYSIS OF CD4+ CYTOKINE PRODUCTION FOLLOWING INDIVIDUAL PEPTIDE STIMULATION

Next I assessed the cytokine production by T-cells after stimulation with peptides that matched the tetramer sequences. This is important for a number of reasons: Firstly, individual epitopes that are highly immunogenic can be identified. Secondly, it allows the recognition of any epitopes that may induce more functional responses following HCV cure. Thirdly, the immunogenicity of individual epitopes can be compared between

patients, SR and vaccinees. As spontaneous resolvers are a model of natural protection, it is important to identify immunogenic epitopes present in natural clearance, and if the same epitopes are immunogenic in our vaccine design. Finally, I wanted to compare the functional and proliferative capacity for the individual epitopes, by correlating cytokine production and tetramer magnitude.

Cytokine production for each individual peptide corresponding to the tetramer sequences is in **Figure 6-9; A-K.**

Similarities were observed between cytokine secretion and proliferative capacity of the HCV specific CD4+ T cells – the epitopes that induced a high proliferative capacity tended to be the same epitopes that stimulated a robust cytokine secretion. Peptide 14 [$NS4B_{1806-1818}$ TLLFNILGGWVAA] was by far the most immunogenic across the groups. Average cytokine production (measured by the average production of individual cytokines across all samples in a group) ranged from (mean % $\pm$ SD) 0.25 $\pm$ 0.3% (IL-2) to 7.5 $\pm$ 10% (MIP-1β). Peptides 22 and 29 were once again poorly immunogenic. Cytokine production for peptide 22 [$NS2_{794-810}$ WPLLLLLALPQRAYAQ] ranged from 0.09 $\pm$ 0.15% (IFNγ) to 0.18 $\pm$ 0.34% (TNFα), and for peptide 29 [$NS5A_{1957-1975}$ SLTITSLLRRLHNWITSCS] cytokine production ranged from 0.11 $\pm$ 0.19% (IFNγ) to 0.17 $\pm$ 0.3% (TNFα).

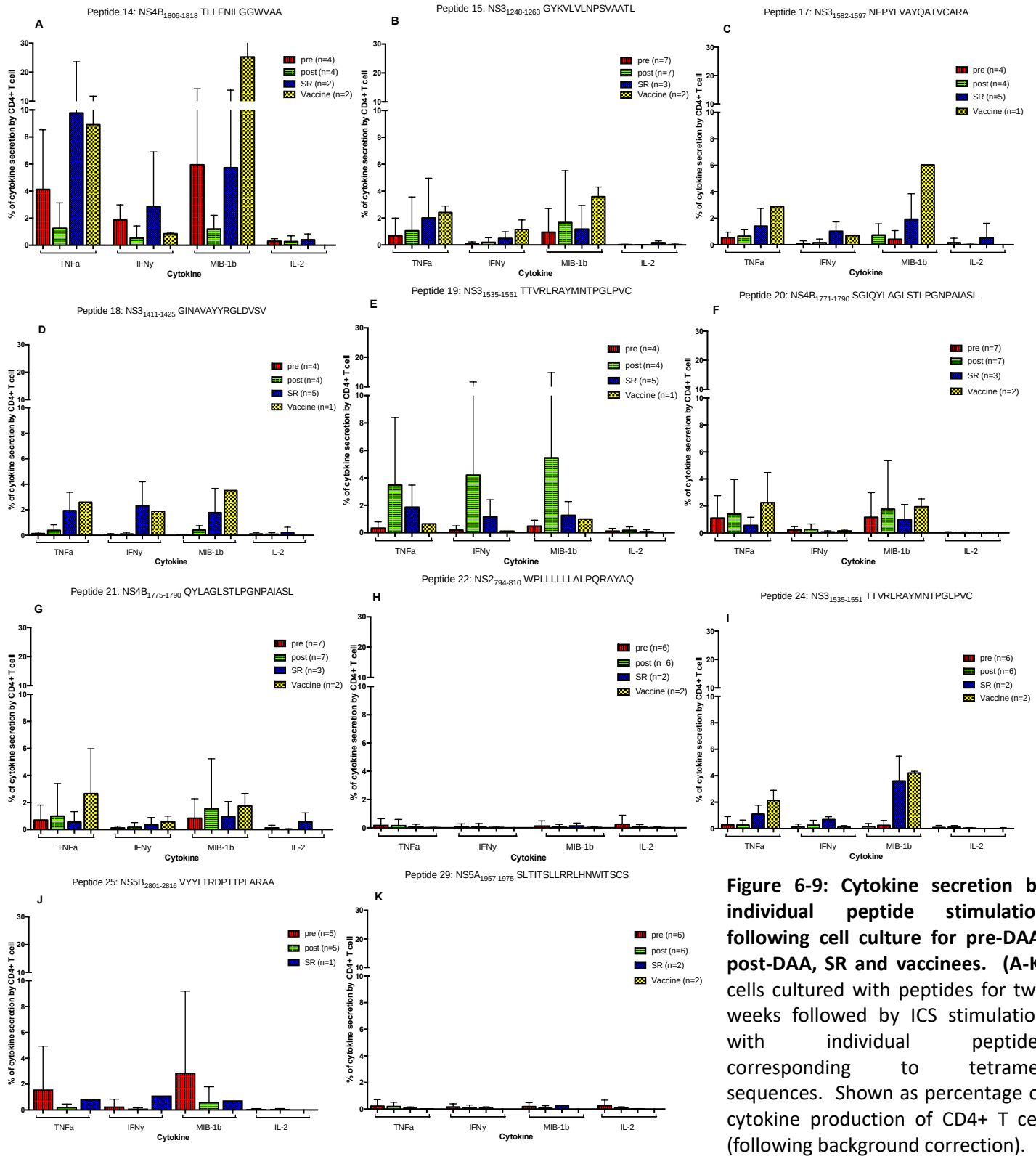


Figure 6-9: Cytokine secretion by individual peptide stimulation following cell culture for pre-DAA, post-DAA, SR and vaccinees. (A-K) cells cultured with peptides for two weeks followed by ICS stimulation with individual peptides corresponding to tetramer sequences. Shown as percentage of cytokine production of CD4+ T cell (following background correction).

6.5.6 ANALYSIS OF CD8+ CYTOKINE PRODUCTION FOLLOWING POOLED PEPTIDE STIMULATION

An analysis of cytokine production by HCV specific CD8+ T cells was performed by ICS as described previously, using peptide pools corresponding to HCV genotype 1b NS3-4 (**Figure 6-10)**. Overnight peptide stimulation was performed following two weeks cell culture (with the same peptides), and compared between pre- and post-DAA treatment (n=21), SR (n=10) and vaccinees (n=5). As described previously, background cytokine expression (negative control – DMSO) was subtracted from each value.

The vaccine sample population demonstrated the most robust cytokine production. MIP-1β was produced by on average 33% of peptide specific CD8+ T cells (95% CI range: 15-51%). This was followed by IFNγ (mean; 95% CI range = 28%; 9-47%) and TNFα (mean; 95% CI range = 27%; 9-45%). For these three cytokines, the magnitude of HCV specific CD8+ T cells making each cytokine in vaccinee samples were significantly higher than both pre-treatment patients ($p<0.0001$ for TNFα, IFNγ and MIP-1β) and post-treatment patients ($p<0.0001$ for TNFα, $p=0.0002$ for IFNγ and $p=0.0003$ for MIP-1β).

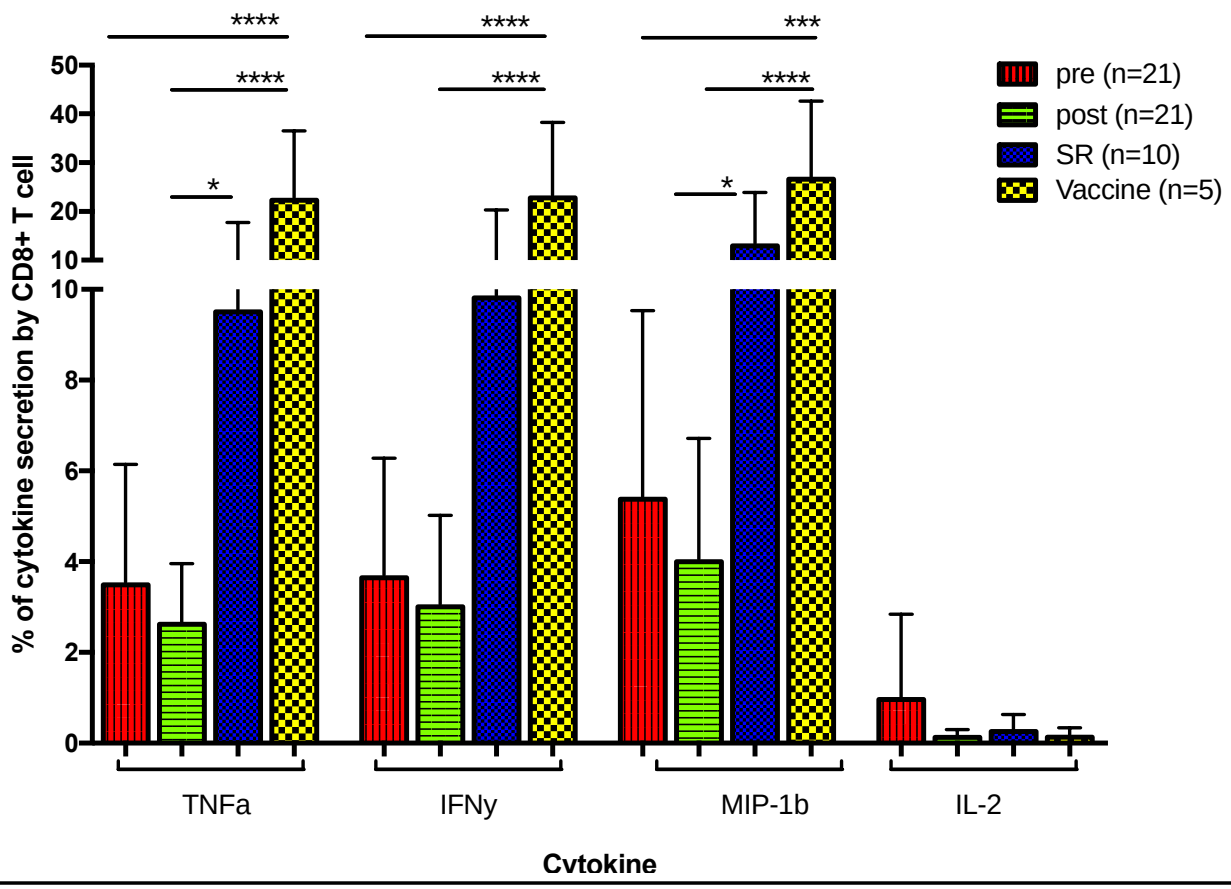


Figure 6-10: Comparison of individual cytokine secretion by HCV specific CD8+ following cell culture and overnight peptide stimulation with ICS. Shown as mean percentage of cytokine production with 95% CI range. Pre = pre DAA treatment (red vertical stripes), post = post DAA treatment (green horizontal stripes), SR = spontaneously resolved HCV infection (blue small squares), vaccine = AdCh3/MVA vaccine induced response (yellow large squares). Paired T tests (pre- vs post-DAA) and unpaired T tests (all other groups) used.

Spontaneous resolvers also demonstrated vigorous cytokine production upon stimulation with HCV peptides. Secretion of TNFα, IFNγ and MIP-1β was higher compared to both pre- and post-DAA treatment, with MIP-1β and TNFα reaching statistical significance in comparison to post treatment (p=0.01 for both).

The patient samples in comparison showed a much lower cytokine expression. The percentage of peptide specific CD8+ T cells in the pre-DAA group producing TNFα, IFNγ

and MIP-1β were (mean; 95% CI range): 3.5%; 0.95-6.1%, 3.7%; 1.1-6.3% and 1.3%; 0-2.8% respectively. Cytokine production following treatment was comparable, with average cytokine production measuring (mean; 95% CI range) 2.7%; 1.4-4%, 3.1%; 1.1-5% and 4.1%; 1.4-6.7% for TNFα, IFNγ and MIP-1β respectively.

Production of IL-2 was low in all groups (mean; 95% CI range = 0.14%; 0-0.3%, 0.31%; 0-0.63%, 1%; 0-2.8% and 0.19%; 0.068-0.3% for vaccine, SR, pre-DAA and post-DAA respectively. Of note, IL-2 expression was readily observed in positive control (PMA/I) wells, suggesting there was no technical failure in the assay.

In summary, the vaccine samples and spontaneously resolved samples were able to produce high levels of TNFα, IFNγ and MIP-1β, whereas patients demonstrated attenuated cytokine production, both prior to commencing and at the end of DAA therapy. Little IL-2 secretion by stimulated T-cells was observed in all groups.

6.5.6 ANALYSIS OF REGULATORY CD4+ T CELL SUBSET

I sought to identify if Treg populations changed in magnitude following DAA mediated HCV clearance, and if peripheral Treg numbers in chronic HCV patients (pre- and post-DAA) differed to healthy controls (defined by healthy adult HCV003 vaccine trial volunteers and individuals with spontaneously resolved HCV infection). Preferably the Treg populations would have been measured on *ex-vivo* HCV-specific (i.e. tetramer positive) samples, to give an accurate representation of the Treg populations following treatment. However as the tetramer positive populations in the patient cohorts were too small to do phenotypic

analysis, I have instead measured the number of Tregs in the bulk CD4+ and CD8+ T cell populations. Nonetheless, this may still give valuable information about the influence of chronic viral infection on T cell subsets (including bystander activation), and whether this is reversed following viral cure.

I identified Tregs both by CD4+CD25^{hi}CD127^{lo} surface expression, or CD4+CD25^{hi}CD127^{lo}foxp3+ expression. Examples plots for Treg staining are in **Figure 6-11.**

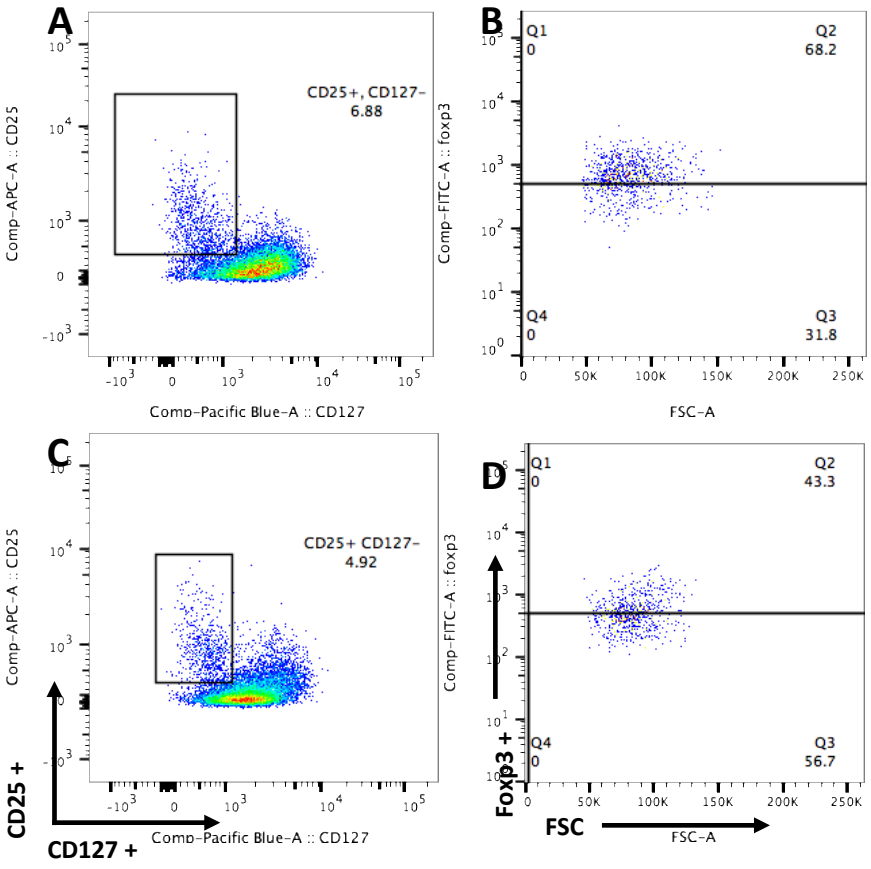


Figure 6-11: Example FACS plots of Treg staining. *Ex vivo* staining of bulk CD4+ T cells. **(A)** and **(B)** 191 pre-DAA treatment CD25^{hi}CD127^{lo} and CD25^{hi}CD127^{lo}Foxp3+ respectively **(C)** and **(D)** vaccinee 333 CD25^{hi}CD127^{lo} and CD25^{hi}CD127^{lo}Foxp3+ respectively. Full antibody panel in **Table 2-5.**

Treg populations were similar in magnitude between the 3 groups (**Figure 6-12)**. The percentage of CD4+ T cells expressing CD4+CD25^{hi}CD127^{lo} and CD4+CD25^{hi}CD127^{lo}foxp3+ in the healthy control group was (mean; 95% CI range) 7.5%; 5.7-9.2% and 5.1%; 3.4-6.8% respectively. This is in keeping with published data of peripheral Treg subsets in the general population (estimated at 5-10%) (Sakaguchi et al. 2001). Treg numbers in patients were modestly higher in patients compared with healthy controls (not reaching statistical significance), and were still within the normal population range both prior to and finishing treatment.

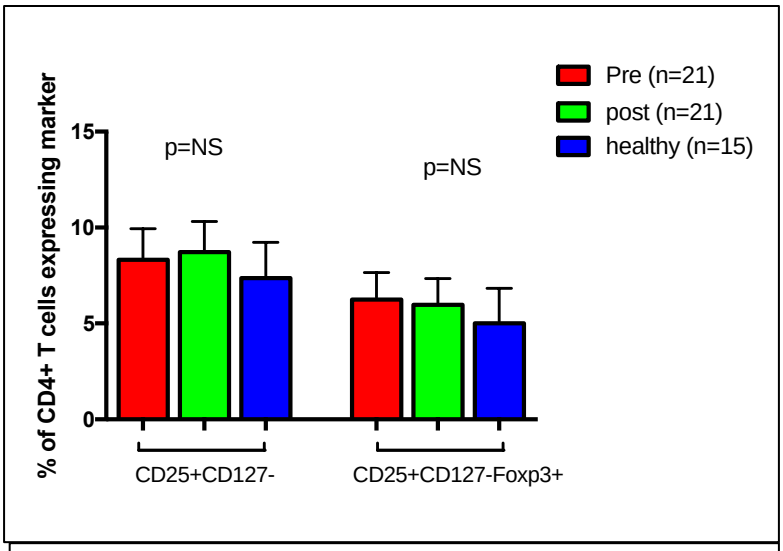


Figure 6-12: Comparison of CD4+ regulatory T cell subset (Tregs). Measured on bulk CD4+ T cells and identified by of CD25^{hi}CD127^{lo} (left) and of CD25^{hi}CD127^{lo}Foxp3+. Pre=pre-DAA treatment, post=post-DAA treatment, healthy=vaccine trial healthy adult volunteers and individuals with spontaneous HCV resolution. Paired (pre- vs post-) and non-paired (patient vs healthy) T tests used to compare groups. Pre=pre-DAA therapy (n=21), post=post-DAA therapy (n=21), healthy=spontaneous resolvers and vaccine samples from HCV003 (n=13). Mean with 95% CI range shown.

6.5.7 ANALYSIS OF PD-1 EXPRESSION

PD-1 is a member of CD28 co-stimulatory family, and its upregulation on T cells is widely regarded as a marker of exhaustion following LCMV mice studies demonstrating an upregulation on chronically exhausted CD8+ T cells, but not functional antigen-specific CD8+ T cells following viral clearance (Barber et al. 2006). In chronic infections such as HCV and HIV, increased expression of PD-1 has been correlated with disease progression (Day et al. 2006; Radziewicz et al. 2007; Trautmann et al. 2006; Urbani et al. 2006), and in particular co-expression of PD-1 with other inhibitory receptors such as 2B4, CD160, LAG-3 and KLRG1 (Bengsch et al. 2010; Blackburn et al. 2009) is associated with T cell dysfunction including impaired proliferative capacity, effector cytokine production and T cell maturation. PD-1 has been the focus of considerable research over the past decade following the discovery that blockade of the PD-1 pathway can lead to reversal of T cell exhaustion (Barber et al. 2006). This has paved the way for developing therapeutic vaccines to block PD-1 (as well as other inhibitory receptors) to treat chronic infections (Brooks et al. 2008; Ha et al. 2008).

I sought to identify changes in PD-1 expression on CD4+ and CD8+ T cells following DAA mediated viral cure. PD-1 expression was measured on bulk CD4+ and CD8+ T cells in patients (n=21) receiving DAA therapy for chronic HCV infection, and expression was compared prior to commencing treatment to expression following successful treatment. Expression of PD-1 was compared to a cohort of healthy volunteers, including HCV003 vaccine trial volunteers (n=5), and individuals that had spontaneously cleared HCV infection (n=8).

PD-1 expression was higher at both patient time points (pre- and post-DAA therapy) in comparison to healthy volunteers for CD4+ T cells. No significant difference was observed for CD8+ T cells (**Figure 6-13)**

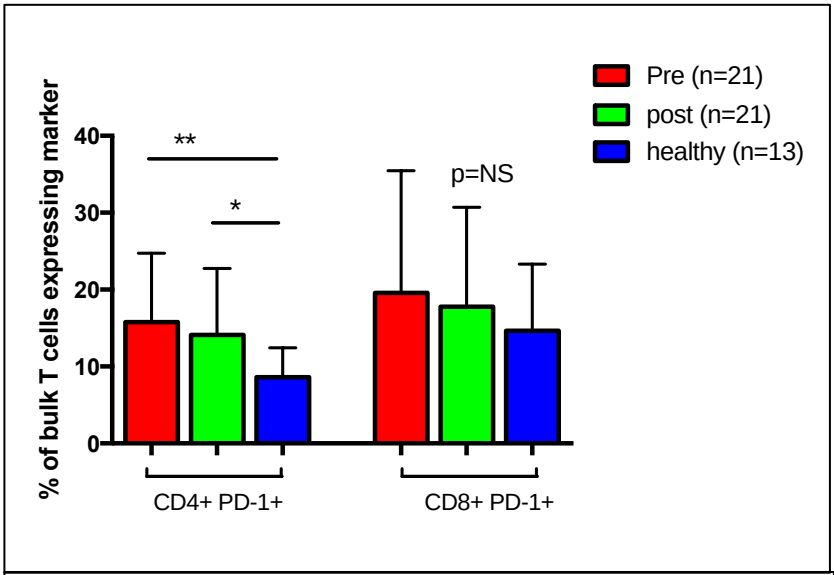


Figure 6-13: Comparison of PD-1 (programmed cell death-1) expression. Measured on bulk CD4+ and CD8+ T cells. Pre=pre-DAA treatment, post=post-DAA treatment, healthy=vaccine trial healthy adult volunteers and individuals with spontaneous HCV resolution. Mean and 95% CI range shown. Paired (pre- vs post-) and non-paired (patient vs healthy) T tests used to compare groups. Pre=pre-DAA therapy (n=21), post=post-DAA therapy (n=21), healthy=spontaneous resolvers and vaccine samples from HCV003 (n=13).

Regarding CD4+ T cells, average frequency of PD-1+ cell expression was 8.9% (95% CI range 6.8-11.1%) in healthy controls, which was significantly lower than both pre-DAA therapy (mean; 95% CI range = 16.1%; 12.1-20.0%) (p=0.008) and post-DAA therapy (mean; 95% CI range = 14.4%; 10.6-18.2%) (p=0.03).

No significant difference was observed between the pre- and post-DAA therapy groups measured with both average PD-1 expression (**Figure 6-13)** and PD-1 expression on individual samples (**Figure 6-14**).

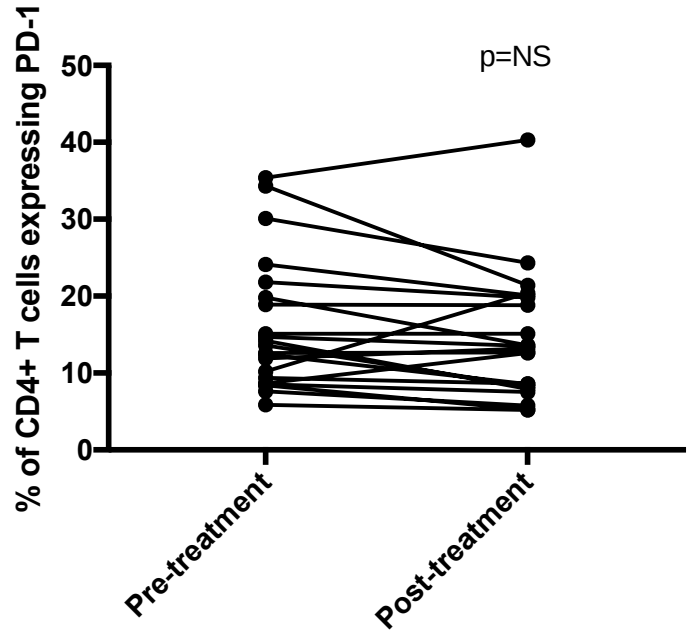


Figure 6-14: Comparison of PD-1 (programmed cell death-1) expression on individual patient samples before and after DAA therapy. Each line represents an individual patient. Measured on bulk CD4+ T cells. Pre-treatment = pre-DAA therapy (n=21), post-treatment = post-DAA therapy (n=21). Paired T tests used to compare groups.

6.5.8 ANALYSIS OF TBET AND EOMES EXPRESSION

I next sought to analyse the intranuclear expression of two transcription factors (TFs) that play pivotal roles in T-cell effector function and memory differentiation, Tbet (T-box transcription factor TBX21) and eomes (eomesodermin) in CD8+ T cells.

Healthy adults vaccinated with AdCh3/MVA-NS and spontaneous resolvers were used as healthy controls (n=13), Tbet and eomes expression in these samples was compared to both pre- and post-DAA treated patients (n=22) (example FACS plots in **Figure 6-15)**. Both the percentage of CD8+ T cells expressing a given TF, and the geometric mean fluorescence intensity (geoMFI) of each TF was measured. The proportion of Tbet^{hi} CD8+ T cells was higher in healthy individuals than in patients both pre- and post-DAA therapy **(Figure 6-16 A)**. This difference reached statistical significance when measured by MFI (p=0.005 and p=<0.0001 for pre- and post-DAA treatment respectively). Expression of eomes^{hi} CD8+ T cells was higher in patients both pre- and post-treatment than in healthy controls when measured by both percentage of CD8+ T cells expressing the marker and the MFI (**Figure 6-16 B**). Notably, eomes^{hi} in CD8+ T cells was statistically significantly higher in patients prior to commencing DAA therapy when measured by percentage of CD8+ T cells (p=0.01%). The co-expression of eomes^{hi}/Tbet^{hi} in bulk CD8+ T cells was similar between the 3 groups (**Figure 6-16 C)**

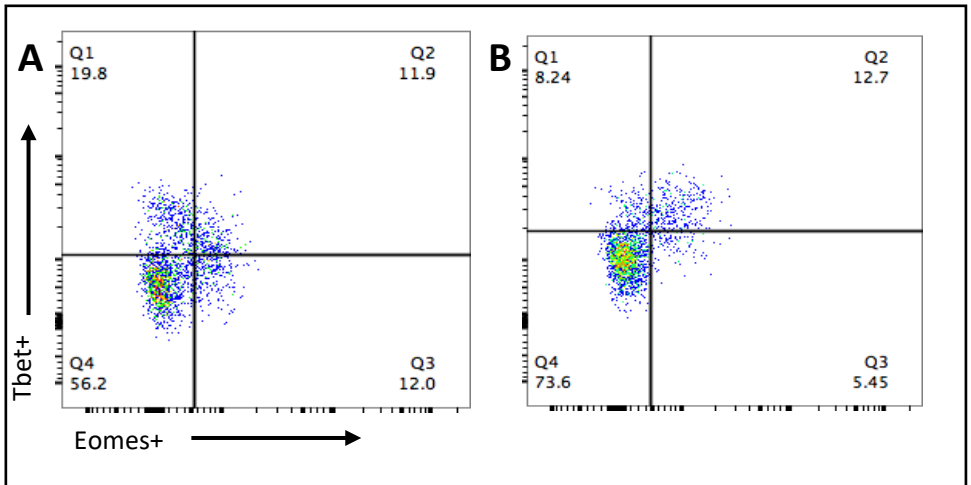


Figure 6-15: Example FACS plots of Tbet and eomes expression. Staining performed *ex vivo* on bulk CD8+ T cells **(A)** 202 (pre-treatment) **(B)** HCV003 vaccine sample 333. Refer to **Table 2-6** for full antibody panel used.

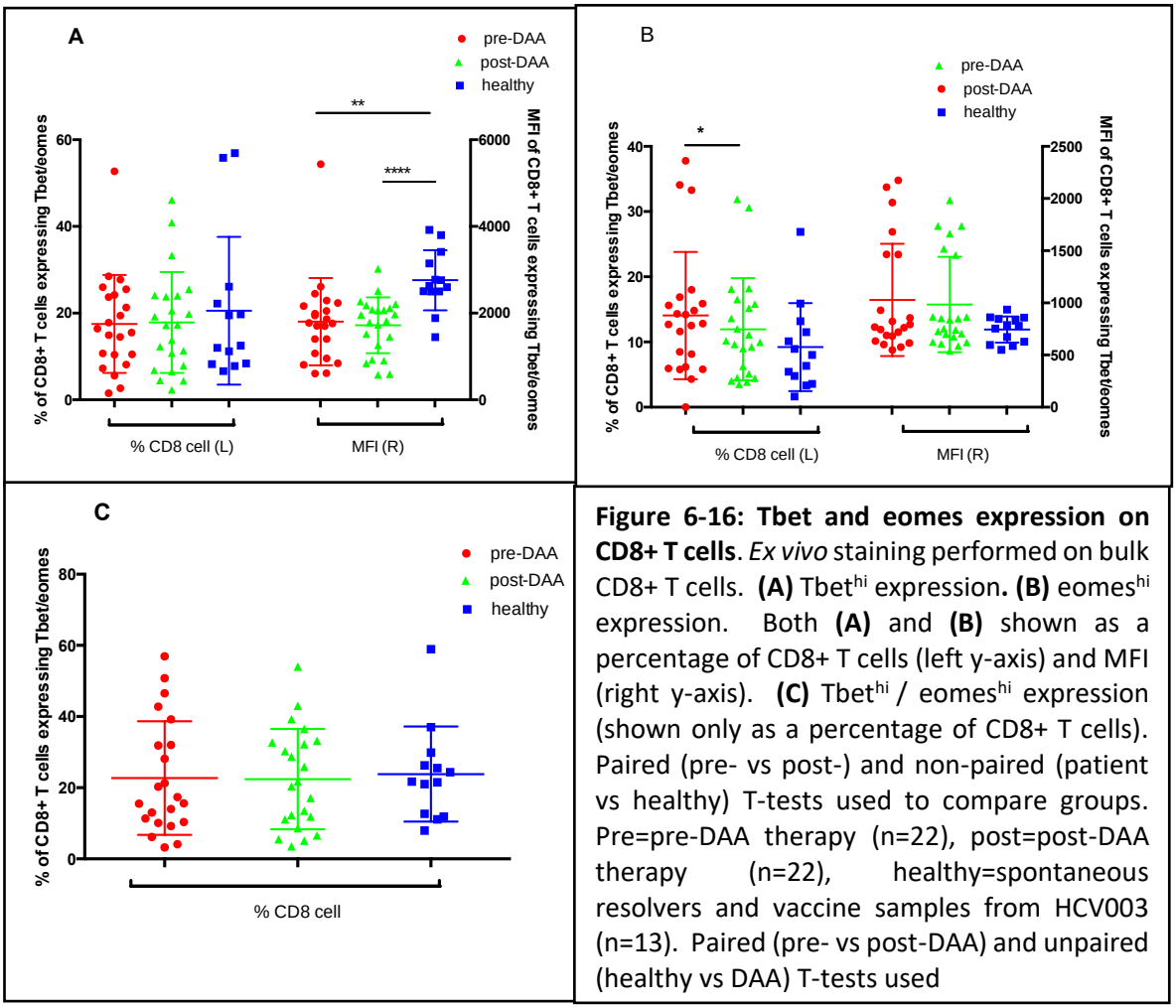


Figure 6-16: Tbet and eomes expression on CD8+ T cells. *Ex vivo* staining performed on bulk CD8+ T cells. **(A)** Tbet^{hi} expression**. (B)** eomes^{hi} expression. Both **(A)** and **(B)** shown as a percentage of CD8+ T cells (left y-axis) and MFI (right y-axis). **(C)** Tbet^{hi} / eomes^{hi} expression (shown only as a percentage of CD8+ T cells). Paired (pre- vs post-) and non-paired (patient vs healthy) T-tests used to compare groups. Pre=pre-DAA therapy (n=22), post=post-DAA therapy (n=22), healthy=spontaneous resolvers and vaccine samples from HCV003 (n=13). Paired (pre- vs post-DAA) and unpaired (healthy vs DAA) T-tests used

6.6 DISCUSSION

The primary aim of the work undertaken in this chapter was to assess the extent of T cell recovery following HCV cure with directly acting antiviral agents, and to compare the phenotype and function of T-cells before and after cure to 'gold-standard' HCV-specific T-cells from individuals who spontaneously resolved HCV or who received virally vectored T-cell vaccines.

The ability of antigen specific memory T cells to rapidly expand upon re-exposure to

antigen is a critical component of a functional and protective cell mediated response (Bowen and Walker 2005; Knolle and Thimme 2014; Protzer, Maini, and Knolle 2012). I assessed the capacity of chronic HCV-specific T cells to recover this function after HCV clearance with directly acting antiviral therapy, using a panel of 11 MHC Class II tetramers following *in vitro* cell expansion. The magnitude of antigen specific CD4+ T cells was comparable following HCV cure. This is in contrast to recent observations that HCV specific CD8+ T cells were able to recover *in vitro* proliferative capacity following IFN free DAA mediated cure in both chimpanzees and humans (Callendret et al. 2014; Martin et al. 2014). There are at least two hypotheses to explain why recovery was observed in these studies but not in my patient cohort. Firstly, both studies assessed the proliferative recovery of the CD8+ T cell population, whereas I evaluated HCV specific CD4+ T cells. Although the recovery of both T cell subsets are likely important in immune recovery, they may behave very differently in terms of their ability to recover. Secondly, the study by Martin et al. included only patients that were treatment naïve, and thus had no potentially confounding effects from previous IFN exposure. In comparison, 81% of my patient cohort were IFN experienced, and 14% had IFN in their current DAA treatment regimen. It is incompletely understood if previous IFN exposure has any impact on the recovery potential HCV specific T cells. With only 4 IFN naïve patients in my cohort, I could not adequately address this question, however it is something I am planning to assess in future experiments.

Furthermore, although a decline in proliferation was observed, this is not entirely suggestive of ongoing T cell exhaustion and dysfunctional HCV immunity. It has been established that HCV specific CD4+ T cell populations are eroded and even deleted over

the course of chronic HCV infection (Schulze Zur Wiesch et al. 2012). Therefore, it may be the case that the memory/antigen experienced T cells have been deleted from the T cell memory pool, and require 're-exposure' and re-priming (as a naïve CD4+ T cell) to elicit a functional proliferative response. An *in-vivo* HCV antigen challenge would be required to address this, either by cohort studies of patients following DAA-mediated cure at risk of re-exposure, or by vaccinating patients following HCV cure. Vaccination of HCV cured non-cirrhotic patients after DAA therapy using viral vectored vaccination as a prime-boost strategy is currently being planned in Oxford, and recruitment is likely to commence in early 2017.

Another concept that warrants exploration of the reduced proliferative capacity following antigen removal is the theory of 'antigen-addiction'. This theory states that dysfunctional T cells lose the ability of homeostatic proliferation, and become dependent on chronic antigen stimulation for survival (Shin et al. 2007). This was first described in LCMV mouse models where it was shown chronic that (and not acute) infection resulted in the down regulation of CD127 and CD122, which are receptors for the homeostatic cytokines IL-7 and IL-15 required for self-renewal of effector memory cells in the absence of antigen (Fuller et al. 2005; Lang et al. 2005; Wherry et al. 2004). Evidence in human infection comes from decreased CD127 expression observed in HIV specific CD8+ T cells compared with influenza, vaccinia and EBV specific CD8+ T cells (Wherry et al. 2006), and reduced CD127 expression in intrahepatic CD8+ T cells in chronic HCV infection (Radziewicz et al. 2007). It is plausible then, that the dysfunctional population of antigen addicted HCV specific CD4+ and CD8+ T cells in my patient cohort are eliminated following the removal of persistent antigen by HCV cure with DAAs, leaving a smaller population of more

functional HCV-specific memory T-cells. I plan to address this in future experiments by measuring *ex vivo* CD127 and CD122 expression on MHC Class I pentamer sorted HCV specific CD8+ T cells and by further assessing the phenotype and function of the T-cells persisting after HCV cure.

Antiviral CD4+ and CD8+ T cells require the ability to secrete effector cytokines as part of a successful cell-mediated response to HCV infection. As HCV specific T cells become exhausted in the context of chronic antigen stimulation, they lose the ability to produce antiviral cytokines in a step-wise manner, with IL-2 production being lost, followed by TNFα production and finally IFNγ, prior to cell deletion (Moskophidis et al. 1993; Wherry et al. 2003; Zajac et al. 1998). I wanted to assess the potential of HCV specific CD4+ and CD8+ T cells to recover these functions following cure with DAA therapy by using NS3-4 peptides in an ICS assay following *in vitro* expansion. No recovery in the ability to produce antiviral and immunomodulatory cytokines was observed following DAA treatment in either CD4+ or CD8+ T cells. With the exception of MIP-1β production in CD4+ T cells, individual cytokine production decreased for all 4 cytokines in both T cell subsets. There are a number of hypotheses to explain this observation. Firstly, it may be that the antigen specific T cells are exhausted, and although are present, are unable to produce cytokine upon stimulation.

Secondly, as discussed above, it is possible that the antigen specific T cell populations have been deleted entirely, and the naïve T cells require *in vivo* priming by HCV antigen to differentiate into cytokine producing effector T cells.

Finally, a third hypothesis is that following the removal of chronic antigen stimulation, the

dysfunctional and 'antigen addicted' T cells have been deleted from the circulation and a smaller, polyfunctional population remain. Polyfunctionality, defined as the ability of individual T cells to produce two or more cytokines, has been suggested to be more effective than groups of monofunctional T cells collectively producing the same cytokines, and therefore more effective in an antiviral response (Bogdan et al. 1990; Darrah et al. 2007). A study of long-term non-progressors (LTNPs) versus progressors in an HIV-1 cohort observed LTNPs to be enriched for HIV-1 specific CD8+ T cells co-producing IFNγ, MIP-1β, TNFα and CD107α (Betts et al. 2006). I aim to address in the future whether there is a higher population of polyfunctional CD4+ and CD8+ T cells following viral cure using Boolean gating.

T cell populations from spontaneously resolved HCV demonstrated significantly better proliferative capacity and production of effector cytokines than the patients at either time point. There are a number of observations to make about this. Firstly, spontaneous resolution of HCV infection is a model of natural protection to HCV, and therefore these observations support the use of these parameters to analyse antiviral activity. Secondly, the significant differences in functional capacity observed between SR and chronic HCV infection provide robust evidence of the substantial dysfunction and T cell exhaustion widely established to occur in chronicity (Virgin, Wherry, and Ahmed 2009; Wherry 2011). Thirdly, the responses seen in SR provides evidence of the longevity of the memory cellular response to HCV. In my cohort of spontaneous resolvers, transmission of HCV (where known) occurred many years or even decades previously. It has been observed in published studies – Including a well-defined single source outbreak cohort – that cellular immunity to HCV infection persists for decades, and often outlives the persistence of HCV

antibodies (Takaki et al. 2000), These results provide proof of principle that spontaneous viral clearance is associated with long lived HCV specific CD4+ and CD8+ memory cells, capable of effector functions upon antigen stimulation.

The 5 vaccine samples used in this study were selected from different time points following prime-boost vaccination with AdCh3/MVA-NS. All individuals demonstrated a robust proliferative and functional capacity, analogous to that seen in the SR group. This is suggestive that AdCh3/MVA-NS vaccination may induce a protective response analogous to that observed in natural HCV clearance. One criticism of this suggestion may be that the HCV specific T cells in the SR group have not encountered antigen for many years or even decades, whereas the T cells in the vaccinees were exposed to the immunogen comparatively recently (within 12 months), and thus a direct comparison of the two groups cannot be made, as the ability of the vaccine-induced memory T cell population to expand and be functional after many years cannot be assessed. It is logistically impractical to follow up the cohort of vaccinated individuals to assess the long-term memory T cell responses. However, in the individuals that could be followed for up to 2 years, a relatively stable population of HCV-specific T cells was observed to be established approximately 18 weeks after MVA boosting until the final time point (Swadling et al. 2014). The *in-vivo* potential of AdCh3/MVA-NS as a protective vaccine strategy will be addressed in a phase I/II efficacy trial currently underway in Baltimore, USA (DMID Protocol 10- 0069[1]**).** In this double-blinded randomized placebo controlled trial, a group of 472 HCV naïve individuals at high risk of infection are being administered either AdCh3/MVA or placebo as prime-

[1] https://clinicaltrials.gov/ct2/show/NCT01436357?term=NCT01436357&rank=1

boost vaccinations, with incidence of HCV infection as the primary outcome measure. The results of this trial are expected to be published in 2018.

A single epitope assessment of the proliferative and functional capacity of HCV specific CD4+ T cells in patients, SR's and vaccines was performed. Epitope 14 ($NS4B_{1806-1818}$) was by far the most immunogenic across all groups with regards to both tetramer magnitude and cytokine production. This is in keeping with published studies observing this to be an immunodominant CD4+ T cell epitope (Lucas et al. 2007; Raziorrouh et al. 2016; Schulze Zur Wiesch et al. 2012).

In contrast, peptides 22 and 29 represented epitopes that were rarely targeted by HCV-specific T-cells in all groups. These tetramers were obtained commercially through the NIH, and no information was given regarding their genotype. The peptide sequence from tetramer 29 (SLTITSLLRRLHNWITSCS) was reported on the website as being $NS5A_{1957-1975}$. However, when this peptide sequence was entered onto the EU HCV database , this exact peptide sequence was not matched with a known sequence. The closest sequence is from genotype 2A $NS4B_{1950-1969}$, with one amino acid deletion. The peptide sequence in $NS4B_{1957-1975}$ in the vaccine is vastly difference (LKRLHQWINEDCSTPCS). Both the difference genotype and different peptide sequences are two hypotheses as to why there was poor staining observed with tetramer 29.

The peptide sequence from tetramer 22 (WPLLLLLLALPQRAYAQ) was reported on the website as $NS2_{794}$. When this sequence was entered onto the same HCV database, the exact sequence was matched to genotype 1A $P7_{794-809}$ and $NS2_{810.}$ Neither P7 or NS2 are included in the vaccine immunogen, and additionally are rarely described in literature as

being an immunogenic region in spontaneously resolved infection. Therefore, it would be reasonable to expect little or no staining in my experiments.

The phenotypic assessment of HCV specific T cells in chronic disease is important, and there are a number of cell surface and intra-nuclear transcriptional changes that occur in chronicity and T cell exhaustion. Of note, magnitude of immunoregulatory T cell subsets, the expression of inhibitory receptors such as PD-1, and expression of transcription factors Tbet and eomes are suggested to be altered in chronic HCV specific T cell populations (Wherry 2011). I therefore selected these parameters to assess for any changes following DAA mediated HCV cure. I compared the expression of these markers in patients to a healthy control group, comprised of individuals with spontaneously resolved HCV infection and healthy adult vaccine trial volunteers.

Regulatory T cells (Tregs) are a subset of CD4+ T cells predominantly involved in immune tolerance and suppression of T cell effector function. They are identified by CD4+CD25^{hi}CD127^{lo} surface expression, and upregulation of the nuclear transcription factor forkhead box protein 3 (Foxp3). In persisting chronic infections such as HCV (Boettler et al. 2005; Ebinuma et al. 2008), HBV (Franzese et al. 2005), HIV (Kinter et al. 2007), they associated with ineffective cell-mediated immune response. The exact mechanisms responsible for their immunosuppressive effects are imprecisely defined, but may involve both direct (production of immunosuppressive cytokines) and indirect (depletion of essential nutrients/ cytokines) inhibition of CD8+ T cell effector function (Virgin, Wherry, and Ahmed 2009).

There was no statistically significant difference observed in the proportion of CD4+CD25^{hi}CD127^{lo}Foxp3+ CD4+ T-cells between patients and healthy controls. One explanation is that the HCV specific Treg population is too small to alter the bulk representation of the peripheral CD4+ T cell subset. Another hypothesis however is that the HCV specific Tregs are sequestered in the liver in chronic infection, and any change in Tregs will occur in this compartment and are not represented in bulk peripheral CD4+ T cell phenotyping. Tregs have been reported to comprise one third to one half of all CD4+ T cells in chronic HCV livers (Ward et al. 2007), which likely contributes to the immunosuppressive and tolerogenic liver environment in chronic HCV infection, resulting in clinically silent infection for many years or even decades (Bolacchi et al. 2006; Sturm et al. 2010).

The potential impact of regulatory T cells on exhaustion, and their role in the reversal of exhaustion, cannot be accurately assessed here. Ideally the intrahepatic Treg population prior to commencing and finishing DAA therapy would be measured, however, this would have considerable logistic and ethical constraints, as chronic HCV patients rarely have liver biopsies. Alternatively, the Treg population may be observed using MHC Class II tetramers in peripheral PBMC samples if tetramer positive CD4+ T cells are able to be isolated.

PD-1 expression was higher in patients than healthy controls in bulk CD4+ cells. These observations suggest an upregulated exhausted phenotype in bulk CD4+ T cells in chronic HCV infection. The increased PD-1 expression in patients is unlikely to be represented by HCV specific T cells, due to their small populations in chronic infection. It is therefore possible that the bulk T cell population has an elevated PD-1 expression due to the effect

of bystander activation (discussed below).

However, there are certain caveats with using PD-1 as a sole marker of T cell exhaustion. Expression of PD-1 was observed to be upregulated in acute HCV infection, and was not correlated with infection outcome (Kasprowicz et al. 2008). Furthermore, an LCMV model demonstrated that exhaustion can occur in the absence of PD-1 expression (PD-1 knock-out), suggesting this marker was not required for T cell exhaustion (Odorizzi et al. 2015). Finally, PD-1 expression exhibits significant heterogeneity among healthy adults (Duraiswamy et al. 2011).

One way to overcome the uncertainty of assessing PD-1 as a sole marker of T cell exhaustion is to measure the co-expression of PD-1 with other inhibitory markers. A number of inhibitory receptors including CTLA-4 (Brunner et al. 1999), CD160 (Cai et al. 2008) and LAG-3 (Workman et al. 2004) have been observed to inhibit cell functions such as cell cycle progression or the production of IL-2, and it has been suggested that both the pattern of co-expression and level of receptor expression on individual T cells influences the severity of exhaustion (Blackburn et al. 2009). In particular, co-expression of PD-1 with 2B4 (CD244), CD160 and KLRG1 has been observed to be upregulated in chronic HCV infection (Bengsch et al. 2010).

Using PD-1 as a marker of exhaustion on HCV-specific CD4+ T cells will be challenging using MHC Class II tetramers *ex vivo* for reasons explained previously. However, it may be worthwhile to assess the co-expression of PD-1 with other inhibitory cell surface markers *ex vivo* using MHC Class I pentamers, which is something I am planning to address in future

experiments.

As well as using PD-1 as a marker of exhaustion and reversal of exhaustion, it also may have therapeutic potential. Monoclonal antibody mediated blockade of this receptor has been widely and effectively used to restore T cell function in cancer therapy (Baitsch et al. 2011) , leading to the regression of many tumours including melanoma, non-small cell lung cancer and renal cell carcinoma. The same mechanism can potentially be applied to exhausted T cells in chronic viral infection. PD-1 blockade in chronic SIV infection resulted in expansion of SIV-specific CD8+ T cells in non-human primates (Velu et al. 2009). Additionally, PD-L1 monoclonal antibodies given to mice infected with HIV-1 resulted in a decrease in HIV-1 plasma load and increase number of antigen specific CD4+ T cells (Palmer et al. 2013). There may be a rationale to administration of PD-1 blockade (such as nivolumab) in DAA treated HCV patients prior to vaccination, in an attempt to restore T cell function. However, the potentially serious immune related adverse events that can with checkpoint inhibitors may be a barrier to trialing this in an experimental setting.

The final parameter I assessed was the expression of TFs Tbet and eomes in bulk CD8+ T cells. Tbet and eomes are nuclear transcriptional factors that have been identified to be involved in the differentiation of antigen specific CD8+ T cells upon exposure to antigen. Tbet has been postulated to drive the production of antiviral and other effector cytokines in CD8+ T cells (in particular IFNγ) (Doering et al. 2012; Kao et al. 2011; Smith et al. 2012). In chronic infection settings such as HIV, a loss of effector functions of the antigen specific cells has been observed to be associated with a depletion of Tbet expression (Hersperger et al. 2011; Ribeiro et al. 2012). Eomes is suggested to be associated with the differentiation into memory CD8+ T cells, however is also believed to be correlated with T

cell exhaustion (Doering et al. 2012; Paley et al. 2012). There is a growing body of evidence that changing levels of Tbet and eomes expression occur on the pathway to T cell exhaustion, with a $Tbet^{hi}eomes^{lo}$ population giving way to an intermediate $Tbet^{hi}eomes^{hi}$ population, and finally resulting $Tbet^{lo}eomes^{hi}$ population (Paley et al. 2012). This last group are terminally exhausted T cells unable to proliferative, and eventually die off. It is therefore plausible to expect higher expression of eomes in chronic CD8+ T cell populations, and likewise higher levels of Tbet in healthy CD8+ T cells.

I observed statistically significant higher levels of $Tbet^{hi}eomes^{lo}$ expression in healthy controls. Furthermore, higher levels of $Tbet^{lo}eomes^{hi}$ expression were seen in patients compared to healthy controls, and a significantly higher expression of this pattern was seen pre- compared with post-DAA therapy. There are a number of observations to make about these results. Firstly, the finding that a higher Tbet expression is correlated with healthy controls, and a higher eomes expression associated with chronic infection reflects observations in published studies assessing these transcription factors (Doering et al. 2012; Kao et al. 2011; Paley et al. 2012). Secondly, a declining expression of $eomes^{hi}$ was observed in patients receiving DAA therapy, with higher levels seen prior to commencing therapy, and lower expression following treatment (average 6 weeks, range 0-26 weeks following final treatment day). This may suggest a decline in the terminally exhausted subset of HCV specific CD8+ T cells following the removal of chronic antigen stimulation. However, it is established that the pool of HCV specific CD8+ T cells in chronic infection is small (Wherry 2011), and may not have an impact on bulk CD8+ T cell phenotypic analysis. MHC Class I pentamer analysis can be used as a tool to examine *ex vivo* TF expression, which is something I plan to address in future experiments.

A third hypothesis is that the systemic chronic inflammatory environment established by chronic HCV infection induces a 'bystander' effect, and causes a transcriptional change in non-HCV specific T cells. This theory of bystander activation has been explored in chronic co-infections including Schistosomiasis and HCV (Kamal et al. 2001; Whalen et al. 1995) and tuberculosis and HIV-1 (Whalen et al. 1995). It is thought that persistent chronic inflammation induced by these pathogens has a negative impact on other immune cells by decreasing their immune response to other pathogens and vaccines (Stelekati and Wherry 2012), and inducing a chronic inflammatory signature (Wherry 2011).

A significantly higher expression of markers associated with exhaustion was observed in my patient cohort in comparison to healthy controls. The higher expression of PD-1 and eomes observed in the bulk CD4+ (PD-1) and CD8+ T (both) chronic cell populations may be explained by bystander activation in the setting of chronic HCV infection. If this were the case, and this global shift in markers correlated with an exhausted phenotype does not reverse following DAA cure, this may have implications in some settings, particularly individuals co-infected with HCV and HIV-1.

The justification for the selection of my healthy control population in the phenotypic analyses was based on the absence of chronic HCV infection in the individuals. It was confirmed during the recruitment process with the spontaneous resolvers that no individual had had symptomatic chronic HCV infection in the past, and had never received treatment for HCV. The sample sizes of n=21 (patients) and n=15 (healthy controls) was of sufficient size for my study. However, to ensure the exposure of HCV antigen (in an experimental or infection setting) has no effect on the bulk T cell phenotypic response, I

would like to expand my population of healthy controls to include non-HCV antigen exposed individuals. Furthermore, I would like to compare the expression of these phenotypic markers in other chronic infection settings, such as HIV and HBV.

There are a number of limitations and potential confounding factors which need to be discussed, some of which have been alluded to already.

Firstly, the population of patients included for analysis displayed significant heterogeneity that may have had influenced the state of their immune system. These include prior exposure to, and current treatment with IFN therapy, concurrent treatment with ribavirin (discussed below), presence of cirrhosis and duration of HCV infection and level of immune 'exhaustion'. Ideally the patient population would have been treatment naïve patients with no concurrent immunomodulatory treatment and without the presence of cirrhosis. However, as these were amongst the first patients in the UK receiving DAA therapy at the time of recruitment, an 'ideal' patient population was not available.

Secondly, a potential confounding factor that needs to be addressed is the use of ribavirin, which was part of the treatment regimes in 18 of the 21 patients in my cohort.

Although it remains largely unclear what the exact mechanism of Ribavirin is and how it contributes to the clearance of HCV, it has been proposed – among other hypotheses – to drive an alteration in the cytokine production of CD4+ T cells from a Th2 to a Th1, or proinflammatory predominant response (Peavy, Powers, and Knight 1981; Tam et al. 1999). This has been supported in murine models, where ribavirin treatment has been

demonstrated to drive a Th1 response in HBV infected mice (Hultgren et al. 1998; Tian et al. 2017; Fang et al. 2000).

If this were the case, it may have been expected to observe an increase in proinflammatory cytokines (such as TNFα, IFNγ and IL-2) following treatment with DAA therapy. Additionally, it may also have been interesting to measure Th2 specfic cytokines, such as IL-10 and IL-4 both pre and post treatment, to observe if there was a decrease in production. However, if ribavirin does indeed drive CD4+ T cell immunomodulation, it would likely occur early in exposure to ribavirin at the time of active HCV clearance, and therefore alterations in T helper cell and cytokine production may not be measurable after viral clearance. Nonetheless, the potential immunomodulatory impact of ribavirin on T cell differentiaton and the immune response is a potential confounding factor in my patient cohort.

Finally, all samples in which I have done my experiments are peripheral PBMCs. It is widely understood that HCV specific lymphocytes home to the liver during both acute and chronic infection. Furthermore, there is a significant body of evidence to support the hypothesis that in both acute and chronic HCV infection the intrahepatic T cell population is different to HCV specific T cells found in the periphery. The liver is suggested to be enriched for proinflammatory Th1 cells with a paucity of Th2 cells, as well as having significantly higher numbers of activated T cells (Nuti et al. 1998). These, along with the increased number of innate lymphocytes (such as natural killer cells, natural killer T cells and gamma delta T lymphocytes), and other inflammatory cells are believed to be responsible for the inflammatory, destruction and eventual fibrosis occurring following years of chronic infection (Agrati et al. 2002). It would therefore be important to assess the phenotypic

and functional characteristics of the intrahepatic rather than peripheral CD4+ and CD8+ T cells to develop more accurate hypotheses regarding HCV specific T cell behavior in both chronic and cured infection. However, it is difficult to obtain liver biopsies for research purposes due to ethical restraints, and there are few indications to perform liver biopsies in patients with chronic HCV. Therefore the study of HCV specific T cells is largely confined to peripheral T cells.

It is important to note that paired and unpaired T-tests were used for all statistical analyses, including where there were more than two groups to compare. Performing multiple T-tests increases the chance of a 'significant' result occurring by chance. A preferred method would have been to perform ANOVA in addition to T-testing for all groups with three or more comparisons.

6.7 CONCLUSION AND FUTURE WORK

In summary, this data gives a variable picture of the extent of immune recovery following viral cure following DAA therapy. Smaller proliferative and functional T cell responses are seen which may or may not be of a higher polyfunctional quality. A change in phenotypic markers predictive of exhaustion are observed, but these may be a result of bystander activation rather than ongoing exhaustion in the HCV specific T population. This early work on HCV T cell exhaustion and recovery following cure has led to further questions rather than answers. In particular I wish to address the following in future experiments:

- Compare current and previous IFN exposure to IFN naïve patients

- Use MHC class I pentamers for further phenotypic analysis of HCV specific CD8+ T cells, including CD127, CD122, TF and inhibitory receptor co-expression
- Assess the polyfunctionality of HCV specific T cells using Boolean gating

It is unclear at present whether the patients included in my cohort would respond to HCV vaccination. Nonetheless, the next generation viral vectored vaccine, involving using the human invariant chain (CD74 or Ii) as an adjuvant to both AdCh3 and MVA-NS, are being investigated in patients with DAA mediated cure in Oxford in the near future (PEACHI-03). The early results presented here, as well as future work planned, will complement the results in PEACHI-03 to give a more comprehensive analysis of T cell recovery following HCV cure with DAA therapy.

CHAPTER 7 *EX-VIVO* PHENOTYPIC ANALYSIS OF HCV SPECIFIC CD4+ T CELLS FOLLOWING VACCINATION AND IN SPONTANEOUS VIRAL CLEARANCE

7.1 AIM

To perform a detailed phenotypic assessment on ex-vivo MHC class II tetramer specific CD4+ T cells following autologous prime boost vaccination with AdCh3/MVA-NS, and to compare these responses to the 'gold standard' of T cell induction that occurs during spontaneous viral resolution.

7.2 INTRODUCTION

The elements of a successful vaccine to control HCV infection are incompletely defined. However, they are likely to include a robust induction of HCV specific CD4+ and CD8+ T cells that target a number of different HCV antigens, produce anti-viral cytokines, and express phenotypes consistent with highly functional effector T cells which differentiate into memory T cells that endure long after exposure to HCV vaccination.

Most of these elements have been explored in chapters 4-6 of this thesis. However, the *ex-vivo* phenotypic assessment of HCV specific CD4+ T cells has not yet been explored, and, unlike CD8+ T cells, there is a paucity of knowledge in the literature regarding HCV antigen specific CD4+ T cells.

Important questions for which there are gaps in the literature, and for which I have focused on include the types of memory T cell subsets induced, the presence of activation and

costimulatory markers, and key transcription factors involved in T cell activation and differentiation following vaccination.

Memory subsets

A significant body of work has gone into defining and studying memory T cell subsets over the past decade (Newell et al. 2012; Appay et al. 2008). Two cell surface markers, CD45RA (expressed by naïve T cells and also re-expressed by a proportion of memory T cells) and CCR7 (lymph node homing receptor) are used to identify four distinct populations of T cell memory subsets (Sallusto, Geginat, and Lanzavecchia 2004; Sallusto et al. 1999). These are: central memory T-cells (Tcm; CD45RA-/CCR7+) which home to lymph nodes; effector memory T-cells (Tem; CD45RA-/CCR7-) which circulate through the periphery; multipotent T memory stem cells (Tscm; CD45RA+/CCR7+)–and 'terminally differentiated' effector memory cells which have re-expressed RA (Temra; CD45RA+/CCR7-). Tems are likely to be important for stimulating a rapid response of antigen re-exposure with immediate effector function, and have been shown to be the predominant subset involved in malaria challenge studies following viral vectored vaccination (Reyes-Sandoval et al. 2011). Tcm's are believed to have high proliferative but low effector function, and are heterogenous in their potential to differentiate (Messi et al. 2003). Tscm's are thought to be long-lived undifferentiated memory cells with stem-cell like properties that are able to self-regenerate through homeostasis and differentiate into Tcm's and Tem's (Turtle et al. 2009; Gattinoni et al. 2011). Temra's have predominantly been defined as CD8+ T cells containing increased perforin and therefore analogous with a CTL phenotype (Sallusto, Geginat, and Lanzavecchia 2004), but their role in CD4+ memory T cells is largely unknown.

Proportions of T memory subsets appear to be heterogeneous both across different disease models and at different stages of the immune response (Sallusto, Geginat, and Lanzavecchia 2004), and it is clear further work is needed to elucidate the roles and contributions of each subset. Specifically, most of the work so far has focused on CD8+ T cells, and it is important to understand the role of CD4+ memory T cells in the vaccine response to HCV peptides.

Activation markers

Co-stimulatory cell surface markers and markers of cell activation are also important components of the vaccine induced T cell response in analysing the quality of antigen specific T cells induced. Cell surface markers CD28 and CD127 were chosen for phenotypic analysis.

CD28 acts as a critical costimulatory molecule for T cell activation. Upon antigen presentation to the T cell receptor via MHC Class II, CD28 binds either B7.1/CD80 or B7.2/CD86 expressed on APCs and initiates a signalling cascade to enable T cell activation and development (Porciello and Tuosto 2016). It is expressed constitutively on almost all CD4+ T cells (Riley and June 2005).

CD127 (also known as interleukin-7 (IL-7) receptor α chain) is expressed on both naive and memory CD4+ and CD8+ T cells (Tan et al. 2001). Its expression has been associated with the development of highly effective memory T cells, with its expression early in infection believed to identify antigen specific T cells ultimately destined to become long lived memory T cells (Huster et al. 2004; Kaech et al. 2003), and its upregulation over time

correlating with the proliferation of antigen specific memory T cells (Boettler et al. 2006). Further evidence for the importance of CD127 in maintaining the memory cell population and long term viral control comes from the observation that antigen specific T cells lack CD127 expression in chronic and latent infections such as HCV (Barathan et al. 2015). It has been suggested that chronic antigen stimulation suppresses CD127 expression on activated T cells, and correlates with the development of T cell exhaustion (Lang et al. 2005).

Transcription factors

The role of transcription factors (TF) T-bet and eomes has been discussed in the previous chapter in relation to CD8+ T cells. However, they have been shown to also play an important role in CD4+ T cell activation and differentiation, including into various memory subtypes. T-bet originally was described in mouse models as the key TF involved in stimulating Th1 differentiation whilst suppressing Th2 and Th17 development (Szabo et al. 2000; Lazarevic et al. 2011; Raby et al. 2006). It has also been observed to decline as CD4+ T cells gain a more memory-like phenotype (Joshi et al. 2011). Eomes is generally not as highly expressed as T-bet (Knox et al. 2014), but is thought to be more highly expressed in effector and effector memory than central memory populations (Joshi et al. 2011; McLane et al. 2013). However, there are few studies in human *ex-vivo* CD4+ T cell populations.

The resting phenotypic behaviour of HCV specific CD4+ T cells is important in gaining insight and understanding of the roles of these cells in the HCV immune response, and also the immune response to viral vectored HCV vaccination. However, analyses of these populations have been limited in the past, most likely due to the challenges in identifying

antigen specific CD4+ T cells because of both small circulating populations and limited tools with which to identify them. Much of the prior knowledge therefore has been derived from manipulating and expanding the CD4+ T cell population making interpretation of the phenotype and function of these cells challenging.

I have attempted to overcome these previous challenges by using MHC Class II tetramers on very large cell numbers (10^6 – 10^8), with the aim of not only identifying these populations but to enable further cell surface and intranunclear phenotypic assessment whilst at the same time analysing the cells immediately *ex vivo* in the resting state.

As has been discussed in previous chapters, spontaneous resolution of HCV provides a gold standard model of a successful immune response to the virus. I have therefore chosen samples from individuals with spontaneous clearance of HCV to compare with CD4+ T cell immune response in vaccinated individuals. From this point people with spontaneous resolution of HCV infection will be referred to as 'SR'.

7.3 HYPOTHESES

1. *Ex-vivo* MHC class II tetramer HCV specific CD4+ T cell populations can be detected following viral vectored vaccination and spontaneous viral resolution
2. HCV specific CD4+ memory T cell subsets generated by viral vectored vaccination will predominantly be Tems and Tcm
3. HCV specific CD4+ T cells will express high levels of CD28 in both vaccinated individuals and spontaneous viral resolution

4. CD127 expression will be upregulated on HCV specific CD4+ T cells after vaccination, increase over time following vaccination, and will be highly expressed in spontaneous viral resolution.
5. CD4+ Tbet expression decreases and eomes expression increases after vaccination. The expression of these TF's in SR is most similar to the EOT time point.

7.4 CHAPTER SPECIFIC METHODS

Full details of the methods used in this chapter are in chapter 2. However, chapter specific methods will be briefly reviewed here.

7.4.1 RECRUITMENT

Vaccine trial samples were frozen PBMCs obtained from two separate Phase I vaccine trials: HCV003 refer to chapter 4) and PEACHI-04 (refer to chapter 5).

SR's were identified from pathology reports at the John Radcliffe Hospital, Oxford and invited to participate in the local cohort study following informed consent (see section 2.2). Samples were then selected for analysis based on HLA and tetramer matching.

7.4.2 IMMUNOASSAYS

Ex-vivo phenotypic analysis was performed on frozen PBMCs using tetramers, cell surface staining and intranuclear staining using flow cytometry. Between 10^6-10^8 PBMCs were

used for each experiment. For those samples where there were multiple available tetramers (i.e. DRB1*04, DRB1*15) tetramers were pooled in the one sample.

7.5 RESULTS

7.5.1 SAMPLE COHORT

In total 10 SR's were selected for phenotypic analysis based on having a MHC class II HLA type that matched the HLA of a tetramer available in the panel. The cohort is identical to the cohort used in chapter 6, and their characteristics can be found in **Table 6-2.**

In the vaccination cohort, 10 healthy volunteers were selected across both trials (**Table 7.1**). Sample selection was based on HLA tetramer matching between the volunteer and the available panel, and a positive ELISpot response to HCV peptide stimulation following prime vaccination, boosting vaccination and the end of trial time point. Trial identification numbers, trial week and HLA type are shown in **Table 7.1**. Despite being selected from different trials, each volunteer had the same dosing schedule: AdCh3-NSmut1 at a dose of $2.5x10^{10}$ vp at week 0 and MVA-NSmut at a dose of $2x10^{8}$ pfu at week 8 (with the exception of HCV003347 – MVA-NSmut dose of $2x10^{7}$ pfu and HCV003374 – MVA-NSmut dose of $2x10^{6}$ pfu, due to availability of stored samples). Samples were obtained between one and four weeks following vaccination were used based on availability of stored samples.

Trial number	Prime	Boost	EOT	HLA-DR	Tetramer
HCV003345	TW2	TW9	TW34	DRB1*04, *15	15, 17, 18, 19, 20, 21
HCV003347	TW4		TW34	DRB1*15	17, 18, 19
HCV003374	TW4	TW9	TW34	DRB1*01	14
HCV003333	TW2	TW9	TW32	DRB1*01	14
HCV003343	TW2	TW12	TW34	DRB1*01	14
HCV003335	TW2	TW12	TW32	DRB1*04, *15	15, 17, 18, 19, 20, 21
PEA-04009	TW2	TW9	TW34	DRB1*01	14
PEA-04011	TW4	TW12	TW34	DRB1*15	17, 18, 19
PEA-04014	TW2	TW9	TW34	DRB1*15	17, 18, 19
PEA-04028	TW2	TW9	TW34	DRB1*15	17, 18, 19

Table 7-1: Vaccine trial volunteers. TW=trial week. Prime = following priming vaccination with AdCh3. Boost = following boosting vaccination with MVA-NS. EOT = final trial time point. Full tetramer panel information can be found in **Table 3.1.**

7.5.2 FREQUENCY OF MHC CLASS II TETRAMER SPECIFIC CD4+ T CELLS IN VACCINE INDUCED AND SPONTANEOUSLY RESOLVED HCV INFECTION

I identified tetramer positive CD4+ T cells on thawed PBMCs using flow cytometry. Samples of between 6-8x10^6 cells were stained with the tetramer of interest before being stained with cell surface and intranuclear markers.

10 vaccine samples were tracked at time points during the trial: at baseline, after priming vaccination with AdCh3 (prime), post boosting with MVA-NS (boost) and at the final trial time point (EOT). Example plots are shown in (**Figure 7.1 A**). The CD4+ class II tetramer HCV+ specific T cell population was significantly higher in frequency following boosting vaccination (mean 0.062% of CD4+ T cells, 95% CI range 0.007-0.057%) compared with all other time points, including baseline (mean 0.006% of CD4+ T cells, 95% CI range: 0.001-0.017%) (p=0.044), prime (mean 0.022% of CD4+ T cells, 95% CI range: 0.001-0.045%)

(p=0.032) and EOT (mean 0.027% of CD4+ T cells, 95% CI range: 0.002-0.051%) (p=0.043) (**Figure 7.1 C**). HLA mismatched samples were used as a negative control.

HLA matched SR's also demonstrated robust tetramer specific CD4+ T cell populations. Example plots for two spontaneous resolvers are shown in **Figure 7.1 B**. The mean number of tetramer positive cells in SR samples was 0.028% (range 0.007-0.15%). This was not statistically different from either prime, boost or EOT responses from the vaccine samples, and was in fact almost identical to the EOT vales (p=0.98) (**Figure 7.1 C**).

Overall this analysis showed that HCV tetramer specific CD4+ T cells can be readily identified in their resting state following both vaccination and in SR. To date this is the first demonstration of this, and allows the opportunity for further phenotypic analysis of these cell populations.

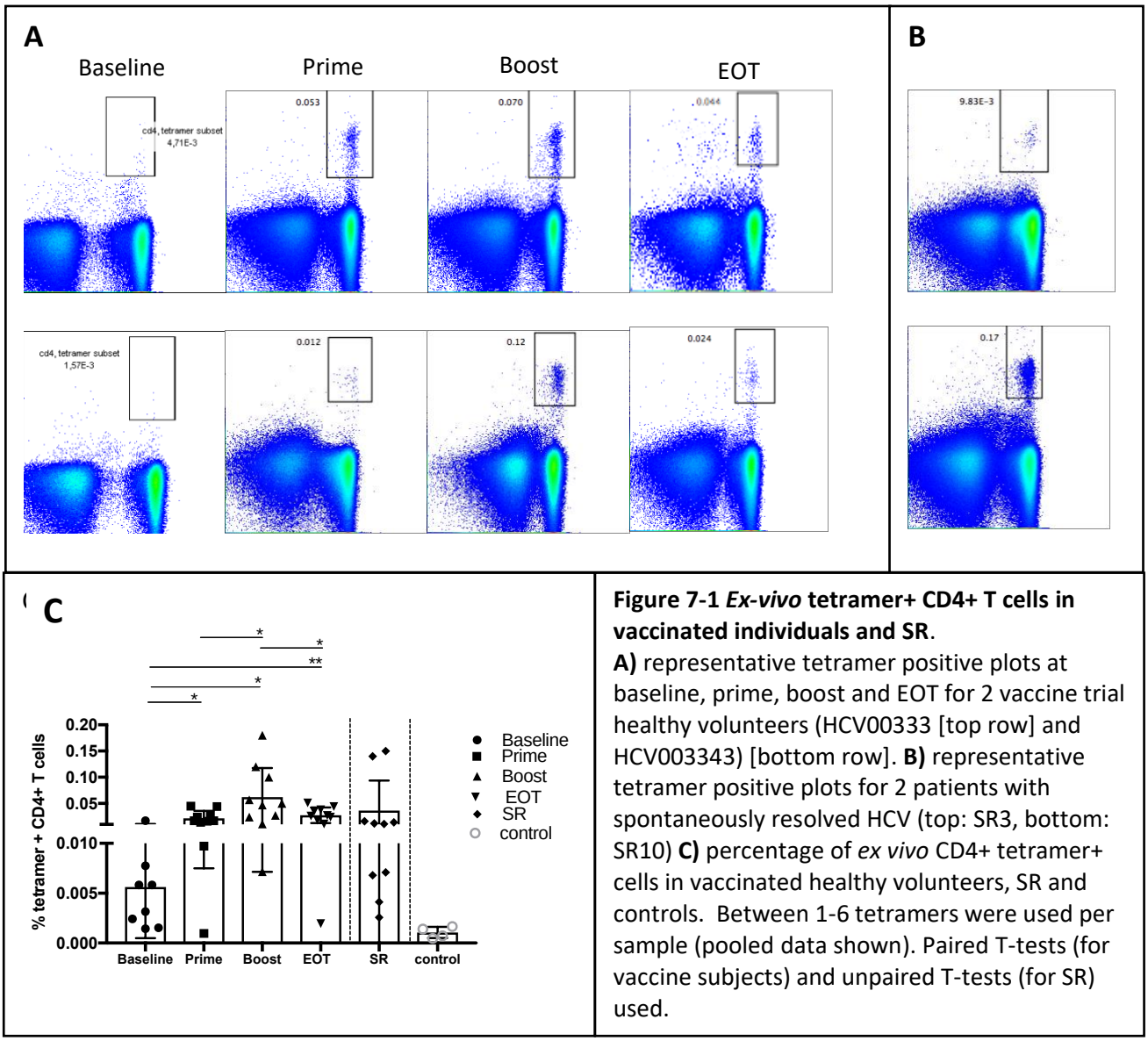


Figure 7-1 *Ex-vivo* tetramer+ CD4+ T cells in vaccinated individuals and SR.
A) representative tetramer positive plots at baseline, prime, boost and EOT for 2 vaccine trial healthy volunteers (HCV00333 [top row] and HCV003343) [bottom row]. **B)** representative tetramer positive plots for 2 patients with spontaneously resolved HCV (top: SR3, bottom: SR10) **C)** percentage of *ex vivo* CD4+ tetramer+ cells in vaccinated healthy volunteers, SR and controls. Between 1-6 tetramers were used per sample (pooled data shown). Paired T-tests (for vaccine subjects) and unpaired T-tests (for SR) used.

7.5.3 ANALYSIS OF T CELL MEMORY SUBSETS

HCV specific CD4+ memory T cell subsets were analysed using a cell surface marker panel. Following staining with the MHC class II tetramer, cells were stained with the memory markers CCR7 and CD45RA (full method described in chapter 2). Memory subsets were identified by the following: T memory stem cells (Tscm: CCR7+/CD45RA+), effector

memory T cells (Tem: CCR7-/CD45RA-); central memory T cells (Tcm: CCR7+/CD45RA-) and effector memory RA positive cells (Temra: CCR7-/CD45RA+).

Central and effector memory subsets were the most predominant at all vaccine trial time points as well as the SR population (**Figure 7.2 A**). The percentage of Tcm cells were similar between the groups, with no statistically significant differences between them (using one-way ANOVA). Mean percentage of HCV specific CD4+ T cells with 95% CI range were 32.9% (range 23.4 – 42.4%); 36.6% (range 26.0 – 47.2%); 37.2% (range 29.4 – 45.1%) and 36.4% (range 25.5 – 47.4%) for prime, boost, EOT and SR respectively. The population of Tem subsets were robust in all groups, with a higher number observed following vaccine boosting with MVA-NS. Using one-way ANOVA the groups were statistically different (p=0.011). The mean percentage of HCV specific CD4+ Tem positive tetramer specific CD4+ T cells was 37.7% (95% CI range 29.1 – 46.4%); 45.2% (95% CI range 29.4 – 61.0%); 30.0% (95% CI range 20.5 – 39.5%) and 41.6% (95% CI range 23.4 – 59.8%) for prime, boost, EOT and SR respectively.

The numbers of Tscm's and Temra's cells were lower in comparison to Tem and Tcm.

The Tscm population was similar following priming vaccination and EOT. Mean percentage of HCV specific CD4+ T cells with 95% CI range was 24.3% (13.0 – 33.6%) and 25.2% (18.3 – 32.1%) for prime and EOT respectively. These time points were also comparable to the SR group (mean percentage of HCV specific CD4+ T cells with 95% CI range = 19.7% (5.9 – 33.5%). However, the percentage of Tscm was lower following boosting vaccination (mean percentage of HCV specific CD4+ T cells with 95% CI range = 13.6% (4.4% - 22.7%). This reached statistical significance when compared with prime (p=0.0103) and EOT (p=0.0062). Groups were also significantly different using one-way ANOVA (**Figure 7.2 A).**

The Temra population was similar following prime and boost vaccination (mean percentage of HCV specific CD4+ T cells with 95% CI range = 5.08% (0.6 – 9.6%) and 4.63% (2.3 – 11.14%) for prime and boost respectively), with an increase at the EOT compared with prime

(mean percentage of HCV specific CD4+ T cells with 95% CI range = 7.6% (2.07 – 13.04%). There were fewer SR Temra cells compared with all other time points (mean percentage of HCV specific CD4+ T cells with 95% CI range = 2.64% (0.65 – 4.62%), however none significantly so. The groups were comparable using one-way ANOVA.

This data suggests that Tem's and Tcm's are the predominant memory subsets in both vaccine induced and SR HCV specific CD4+ T cells, with increasing populations of Temra's and Tscm's at later time points from vaccination. Furthermore the EOT T memory subsets were comparable to subsets in the SR population.

7.5.4 ANALYSIS OF CD28 AND CD127 IN *EX-VIVO* MHC CLASS II TETRAMER POSITIVE CD4+ T CELLS

I measured the expression of CD28 and CD127 on thawed PBMCs following staining with the MHC class II tetramer of interest (described in chapter 2). CD28 was highly expressed at every time point in the trial, as well as in SR (**Figure 7.2 B**). The populations of tetramer specific CD4+ CD28+ T cells were as follows (mean percentage of tetramer+ CD4+ T cells expressing CD28 with 95% CI range shown); prime: 94.6% (90.7 – 98.4%), boost: 96.6% (94.3 – 98.9%), EOT: 91.8% (86.3 – 97.4%) and SR 93.3% (89.2 – 97.4%). The CD28+ T cell population following boosting reached statistical significance when compared with the EOT population (p=0.046).

CD127 expression increased during the course of the trial and reached peak expression at the EOT time point, which reached statistical significance when compared with both other time points (p=0.0018 and p=0.0068 for prime and boost respectively). Expression following prime, boost and EOT was (mean percentage of tetramer+ CD4+ T cells expressing CD127 with 95% CI range shown); 49.37% (37.04 – 61.69%), 53.63% (40.74 – 66.52%) and 75.14% (66.6 – 83.7%) respectively. CD127 was also highly expressed in the SR group (mean; 95% CI range = 72.1%; 58.3-85.9%) (**Figure 7.2 C**)

In summary, the high expression of CD28 at all time points as well as SR indicates HCV specific CD4+ T cell populations that are highly active, and increasing expression of CD127 throughout the trial (culminating in comparable levels to SR) suggestive of functional memory CD4+ T cells.

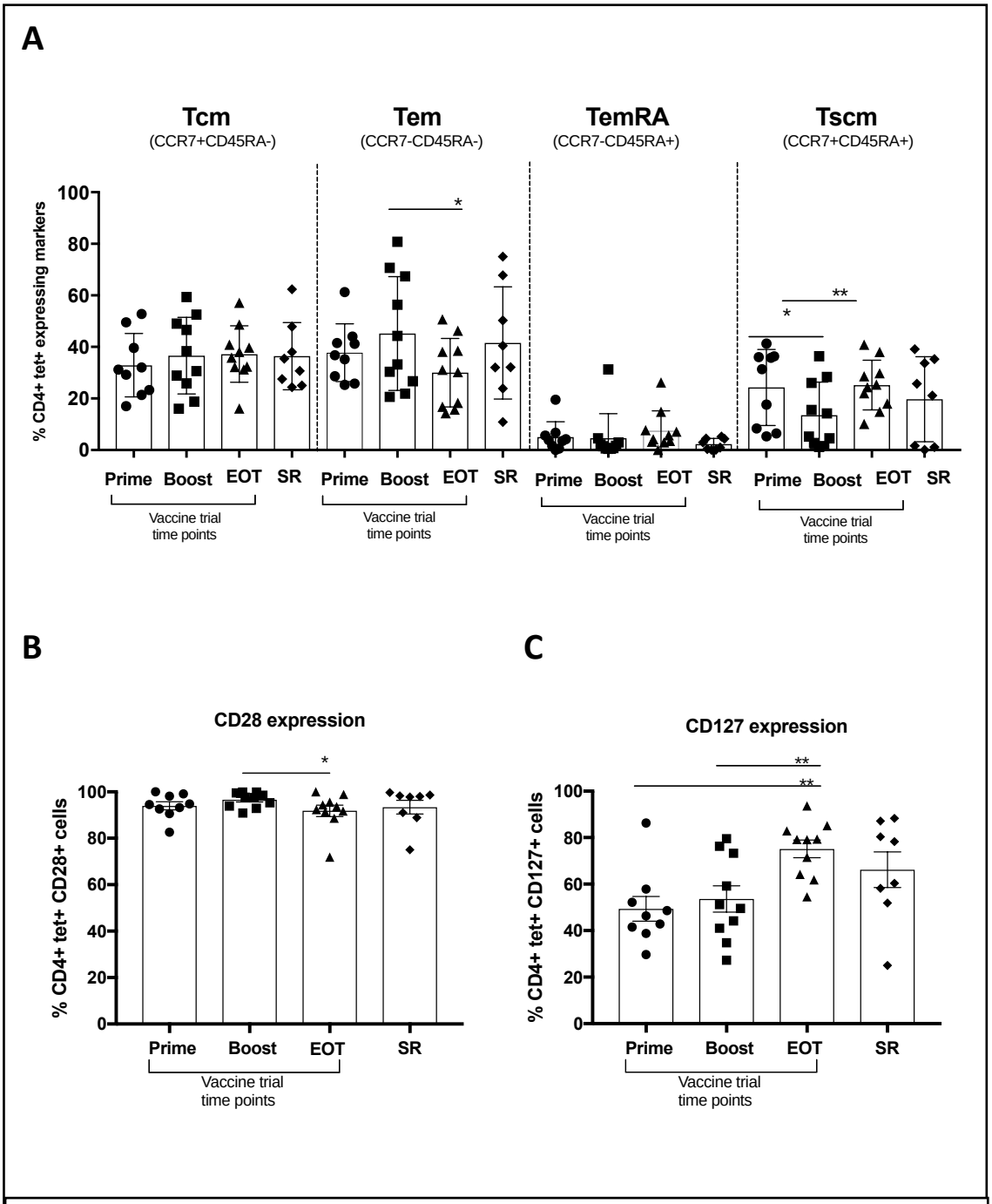


Figure 7.2: *Ex*-vivo analysis of CD4+ T cell memory subsets, co-stimulatory and activation markers in healthy vaccinated and SR volunteers.
PBMCs co-stained with tetramers (between 1 and 6 per sample) and surface antibody panel. Percentage of tetramer+ CD4+ T cells shown in each group (prime = post prime with AdCh3 [n=10), boost = post boost with MVA-NS (n=10), EOT = end of trial (n=10), SR = spontaneous viral resolution (n=8). **A)** Proportion of T memory subsets Tcm/Tem/Temra/Tscm **B)** CD28 expression **C)** CD127 expression. One-way ANOVA, paired T-tests (for vaccine subjects) and unpaired T-tests (for SR) used.

7.5.5 ANALYSIS OF TRANSCRIPTION FACTORS T-BET AND EOMES

I sought to analyse the expression of two important transcription factors (TFs) in CD4+ T cell effector function and memory differentiation, T-bet and eomes. Following staining with MHC class II tetramers and other surface markers of interest, PBMCs were fixed and permeabilsed prior to staining with intranuclear transcription factors (refer to chapter 2). Single and dual expression of the TFs were measured at the same time points (prime, boost and EOT), and compared with SR **(Figure 7.3)**.

T-bet was expressed on the majority of cells following prime and boost vaccination, with no significant difference between the two time-points (mean percentage of tetramer+ CD4+ T cells expressing T-bet with 95% CI range = 58.5% (34.4 – 92.5%) and 59.2% (39.4 – 79%) for prime and boost respectively. However, expression was reduced at EOT (mean percentage of tet+ CD4+ T-bet+ T cells with 95% CI range = 41.4% (22.1-60.6%), which was significantly lower when compared with boost (p=0.0047). Expression of T-bet following spontaneous viral resolution was lower than all other groups (mean percentage of tet+ CD4+ T-bet+ T cells with 95% CI range = 22.9% (7.5 – 38.4%)) which reached statistical significance when compared with prime and boost (p=0.047 and p=0.007 respectively).

Eomes expression was low across all groups throughout the trial as well as in SR. Expression of eomes was 16.7% (range 6.2 – 27.9%), 14.3% (range 5.8 – 22.9%) and 12.2% (range 9.0 – 15.4%) and 9.5% (range 3.8 – 15.2%) for prime, boost, EOT and SR respectively (mean percentage of tetramer+ CD4+ T cells expressing eomes with 95% CI range shown).

This data shows a reduction in both Tbet and eomes over the course of the trial, and expression of both of these TF's at the end of the trial comparable to that of SR.

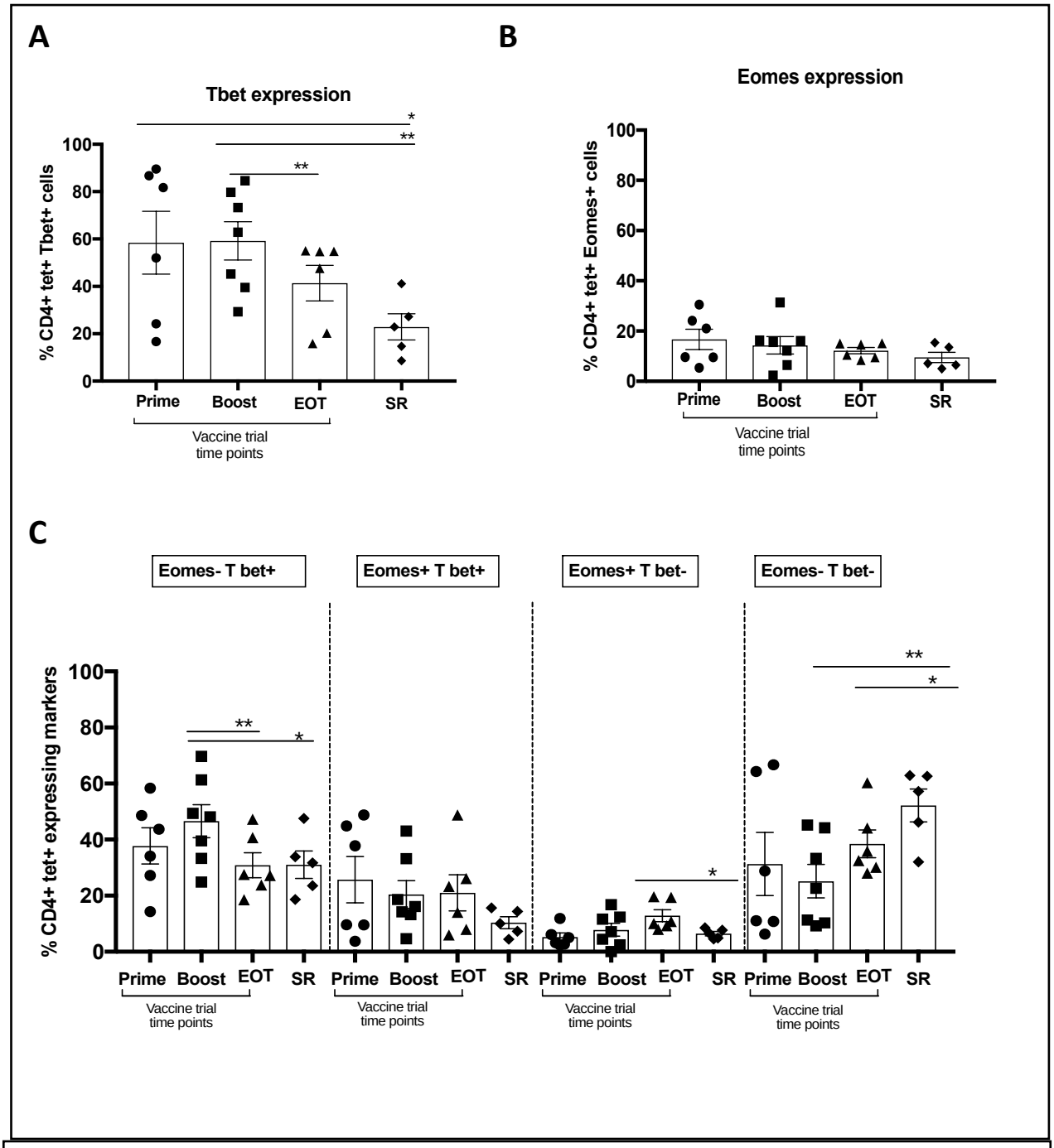


Figure 7.3: *Ex vivo* analysis of expression of Tbet and eomes on HCV tetramer+ CD4+ T cells in healthy vaccinated and SR volunteers.

PBMCs co-stained with tetramers (between 1 and 6 per sample) and TF antibody panel. Percentage of tetramer+ CD4+ T cells shown in each group (prime = post prime with AdCh3 [n=10), boost = post boost with MVA-NS (n=10), EOT = end of trial (n=10), SR = spontaneous viral resolution (n=8). **A)** expression of Tbet **B)** expression of eomes **C)** co-expression of Tbet and eomes. Paired T-tests (for vaccine subjects) and unpaired T-tests (for SR) used.

7.6 DISCUSSION

The goal of this final data chapter was to identify HCV specific CD4+ T cells in their resting state, and perform a phenotypic analysis of key surface and intranuclear markers with the aim of further understanding the behavior of antigen specific CD4+ T cells in both vaccination and spontaneous viral resolution.

The markers I chose for flow cytometry analysis were ones previously identified to be important in multiple aspects of the CD4+ T cell response, including cell activation, differentiation to effector phenotype and differentiation to various memory subsets.

The first step was to be able to identify adequate numbers of *ex-vivo* HCV specific CD4+ T cells for subsequent analysis. I overcame challenges previously identified with doing this by using very large numbers of PBMCs, and by using a panel of MHC class II tetramers (discussed in previous chapters). Tetramer positive populations were identified in all the vaccine and SR samples used, and the majority were large enough to perform further phenotypic analysis. The validity of these tetramer positive populations was supported by absent populations at the baseline of the trial, as well as absent populations in the negative controls (HLA mismatched samples). In keeping with other data looking at CD8+ and total T cell responses following AdCh3/MVA-NS vaccination (Swadling et al. 2014), the largest population of HCV specific CD4+ T cells was following boosting vaccination, with the populations reducing but still present at the final trial time point. Interestingly, the population of tetramer positive CD4+ T cells at the end of the trial was almost identical to the population size observed following spontaneous viral resolution. It could be

hypothesized therefore that only a very small population of viral specific CD4+ memory T cells is required for long term HCV control, and the population of memory T cells induced by vaccination may adequate in achieving long term HCV control following vaccination. The caveat here that needs to be noted is that exposure in spontaneous resolution in many cases occurred many years prior, whereas it was only feasible in the vaccine trial to measure responses for the duration of the trial.

However, it is almost certain that other factors, such as the phenotypic quality, production of effector cytokines and cross reactivity across genotypes and HLA types are likely to play important roles in the success of a T cell vaccine to HCV. I have addressed assessing aspects of the phenotypic quality of HCV specific CD4+ T cells, with the other factors outside the scope of this chapter.

CD4 T memory cells, and more specifically memory subsets, are likely to play an important role in a long lived successful immune response to HCV infection, whether it be from spontaneous viral clearance or following vaccination.

Since their first description by Sallusto et al (Sallusto et al. 1999) there has been an intense focus on the elucidation and roles of these subsets. Although mostly inferred from work done on CD8+ T cells in chronic infection, Tem's are likely to be important for their ability to rapidly differentiate into effector cells on antigen re-exposure, whereas Tcm's are likely to be important for their high proliferative capacity and long-half life, important for replenishing the long-lived memory cell population. Robust populations of both Tcm's and Tem's were present at all time points in the vaccine trial, as well as following spontaneous viral resolution. This is in keeping with previous studies looking at memory subsets in both

CD4 and CD8 T cells (Masopust et al. 2001; Reinhardt et al. 2001; Sallusto et al. 1999). The population of Tcm's in particular was stable across the groups, whereas the population of Tem's appeared to peak following boost and then contract by the end of the trial.

More recently, CD4+ Temra's have been described as a terminally differentiated subset of T memory cells. They have been shown to exist in much smaller populations than their CD8 counterparts, ranging from <0.3% to nearly 18% of total CD4 T cells (Burel et al. 2017). The population of Temra's in all groups were consistent with this, ranging from 2.6% (SR) to 7.5% (EOT). Although the role of Temra's in CD4 T cells is less defined, a number of studies have reported an expansion of Temra's over time in infections including CMV and dengue virus (Gordon et al. 2017; Weiskopf et al. 2015). Furthermore, this expansion of Temra's are associated with the expression of CX3CR1, which is associated with a cytotoxic phenotype (Weiskopf et al. 2015; Pachnio et al. 2016). I observed a significant increase in the population of Temra's between boosting and the EOT, which may suggest an expansion in the population with time, and potentially a switch from Tem to Temra phenotype. However, this was not reflected in the SR group, where the Temra phenotype was very low. The genetic phenotype of Temra's has been observed to vary significantly between various individuals (Tian et al. 2017), and much more work is required to further define this subset. Finally Tscm's, since their initial discovery in a mouse model of graft versus host disease (Zhang et al. 2005) are believed to be an important subset of memory T cells for their ability to self-regenerate and differentiate into other memory T cells (Turtle et al. 2009; Gattinoni et al. 2011). They exist in very small numbers (on average 2-4% of both CD4+ and CD8+ T cells) (Lugli et al. 2013; Chahroudi, Silvestri, and Lichterfeld 2015) and are differentiated from naïve T cells by the expression of other memory T cell markers such

as CD95 and CD62L (Chahroudi, Silvestri, and Lichterfeld 2015). I observed a robust population of Tscm's in all groups which – although was smaller than either Tcm's or Tem's – was larger than has been quoted in the literature. An interesting observation was the contraction of this memory subset following boosting vaccination, with a significant rise at the EOT time point. This may reflect the development and differentiation over time of vaccine induced memory cells into Tscm's, which, if were the case, would arguably lead to long lasting and successful protection against HCV reinfection.

CD28, since its discovery in the 1980's, has emerged as the most important co-stimulatory molecule in T cell activation. Upon antigen stimulation via either MHC I or II, CD28 acts in conjunction with the T cell receptor to promote T cell clonal expansion via IL-2 production, cytokine production, differentiation and survival (Boomer and Green 2010). It is expressed on approximately 80% of CD4+ and 50% of CD8+ T cells (Aruffo and Seed 1987).

Importantly, CD4+CD28- T cells are widely observed to be present in chronic viral infections such as CMV (van Leeuwen et al. 2004), HBV (Wang et al. 2009) and HIV (Haffar et al. 1995), as well as chronic inflammatory diseases (Broux et al. 2012), indicating these CD4 cell populations are dysfunctional and a marker of an attenuated adaptive immune response (Vallejo et al. 1998). In chronic HCV infection, stimulation of CD4+ T cells with anti-CD28 antibody caused an increase in production of IFN-γ and reduction in immunosuppressive cytokines (Barboza et al. 2008). In another study by Kurokohchi (Kurokohchi et al. 2003), there was a reduced population of intrahepatic CD8+CD28+ T cells compared with healthy controls. These studies suggest the upregulation of CD28 on

HCV specific CD4+ T cells to be critically important in a successful immune response to HCV.

I examined the presence of this marker following prime and boost vaccination with AdCh3 and MVA-NS respectively and observed it to be highly expressed, peaking at 96.6% following boosting vaccination, indicating an activated CD4+ phenotype. Importantly the level of expression was similar to that observed in spontaneous viral resolution.

CD127 (IL-7rα) was chosen as a marker for analysis of CD4+ T cell response to vaccination because of its strong association with the development of memory T cells. Long lived HCV specific memory T cell populations with the ability to maintain proliferation in the absence of antigen, then rapidly re-expand on antigen exposure, are critical components of a successful immune response to infection and therefore a successful vaccine (Wherry and Ahmed 2004; Klenerman and Hill 2005). I have demonstrated a progressive upregulation of CD127 in HCV specific CD4+ T cells following vaccination with adenoviral vectors, with peak expression at the final time point in the trial. Importantly, CD127 expression was comparable to that in spontaneous viral resolution, and both these groups had significantly greater CD127 expression than earlier time points in the trial.

The importance of CD127 for memory differentiation was first shown in adoptive transfer models in mice, where it was demonstrated $CD127^{hi}$ effector cells transferred into naïve mice survived and became effective memory cells, whereas $CD127^{lo}$ effector cells did not (Kaech et al. 2003). It was hypothesized the expression of CD127 was in fact required as a precursor for development of functional memory cells (Kaech et al. 2003). These observations were supported by subsequent mouse studies infected with *Listeria monocytogenes*, where it was observed CD127 upregulation was associated with the

development of functional CD8+ memory T cells in both Tem and Tcm subsets (Huster et al. 2004). There are fewer studies looking CD127 expression in CD4+ memory T cell populations. (Lohning et al. 2008) found a positive correction of IL-7 with the development of CD4+ memory cells in mouse adoptive transfer models infected with LCMV, concluding a possible role of IL-7 with homeostatic proliferation. Furthermore, a study comparing memory and naïve CD4+ T cells in humans following *in vitro* stimulation with IL-7 demonstrated proliferation of memory CD4+T cells compared with no response in naïve CD4+ T cells (Geginat, Sallusto, and Lanzavecchia 2001). In summary, the observations of CD127 expression in healthy volunteers receiving HCV vaccination and spontaneous resolution of HCV suggest that long-lived memory CD4+ T cells capable of homeostatic proliferation are induced in both of these settings, and the final time-point in the vaccine trial is analogous to the 'gold standard' of spontaneous resolution.

The final phenotypic markers selected for *ex-vivo* phenotypic analysis were the intranuclear transcription factors T-bet and eomes. T-bet, first described in 2000 as the primary TF driving Th1 differentiation (Szabo et al. 2000), has been extensively studied in both CD4 and CD8+ T cells. Eomes on the other hand was first described in 2003 to have a role in CD8+ T cell function (Pearce et al. 2003), and has been much less studied in CD4+ T cells. However, an expanding area of interest is the level of expression of these TF over time, and in various T cell subsets. There is now substantial evidence of heterogenous behaviour of these markers, albeit largely restricted to mouse models. It is therefore interesting and valuable to observe the patterns of these transcription factors *ex vivo* in human CD4+ T cells following vaccination and in spontaneous viral resolution.

I observed a robust population of CD4+ T-bet+ antigen specific cells following prime and boost vaccination, which decreased significantly by the end of the trial. The expression of T-bet in SR was comparable to EOT, but significantly reduced compared with earlier trial time points. Eomes on the other hand was found in low numbers throughout the trial, with the smallest populations at the EOT and in SR (though not significantly so).

These observations may be reflected in a study by Knox et al (Knox et al. 2014) where T-bet and eomes expression were characterized in human PBMC T cell subsets. Increased T-bet and eomes expression was associated with Tem and effector CD4+ T cell populations (albeit eomes in smaller numbers), with a lower expression found in Tcm's. In my study although it may be inferred that decreased effector CD4+ T cells (evolving to memory T cells) and T-bet levels with time are correlated, it is not possible to draw definitive parallels. Furthermore in my results Tcm levels were stable over time, and it may be expected the Tcm levels would increase in relation to decreased T-bet expression. It is interesting to note the increased populations of Tbet-/eomes- of antigen specific CD4+ T cells with time following vaccination, and particularly in the SR group (where analysis was on average many years following HCV exposure). To date there is no comparable literature assessing TF co-expression of antigen specific *ex vivo* CD4+ T cell populations. However, there are a number of different studies evaluating co-expression in CD8+ T cells with varying conclusions. Joshi et al (Joshi et al. 2011) suggested that reduced Tbet and increased eomes expression was associated with the development of a more durable memory CD8 T cell population, with low Tbet expression limiting terminal differentiation and senescence of CD8+ T cells. This is somewhat similar to Banerjee et al (Banerjee et al. 2010) who postulated an increase eomes expression was required for long term survival and proliferation following re-challenge, and that CD8+ eomes- T cells contribute

minimally to antigen specific central memory cell population. On the contrary though, CD8+ T-bet^{lo} eomes^{hi} T cells have been reported in chronic HIV infection as an exhausted and dysfunctional population of cells. Clearly there is a long way to go in the understanding of the role of T-bet and eomes in both successful and failed immune responses to viral infection as well as vaccination. Nonetheless the similarities between the final trial time point and gold standard of spontaneous clearance are promising.

7.7 CONCLUSION AND FUTURE WORK

In summary, this chapter gives a detailed phenotypic analysis of HCV specific CD4+ T cells that develop following T cell viral vectored vaccination. Importantly, these rare populations of CD4+ T cells have been analysed in their resting state, enabling evaluation of the behaviour of these cells without the need for expansion or manipulation.

The antigen specific CD4+ T cells induced by AdCh3/MVA-NS vaccination are comparable to the 'gold-standard' model of spontaneous HCV clearance. They highly express CD28, critical for cell activation, as well as increasingly express CD127 over time, known to be essential for development of effective memory cells. Furthermore, they develop into T memory subsets which are suggestive to be important for long lasting and effective memory cell populations.

Regarding Tbet and eomes, it is difficult to draw parallels between my findings and previous data due to the paucity of literature. Nonetheless the reduced expression of Tbet with time is suggestive of a long-lived memory CD4+ T cell population.

These results suggest the population of HCV specific CD4+ T cells that develop following vector vaccination is high quality and durable and may give an indication of elements of a successful vaccine, however more work is needed to be done. Future work in this area will ideally focus on doing further phenotypic work using the panel of tetramers developed, assessing the functional quality of these CD4+ T cells (expanding from chapter 6) and assessing these T cell populations following vaccination in the planned Phase II placebo-controlled trial underway currently.

Finally, *ex-vivo* phenotypic analysis of HCV specific CD4+ T cells is challenging, and as a result there is limited published data in this area. Furthermore, to my knowledge this is the first set of data analysing the resting phenotypic behaviour of antigen specific CD4+ T cells induced by viral vectors. These results will add valuable knowledge to the pool of literature in this area and pave the way for further assessment of these cell populations.

CHAPTER 8: CONCLUDING REMARKS

Despite the introduction of highly efficacious directly acting antiviral drugs, it remains an important goal to develop a prophylactic vaccination strategy to prevent HCV infection. The work presented in this thesis builds on significant groundwork in the development of an HCV vaccine, with the aim of optimising viral vectored vaccination strategies and assessing target groups that may benefit from preventative HCV vaccination.

Reboosting of HCV specific T cells is possible after prime-boost AdCh3/MVA, but is more effective following a longer interval

It has been shown that AdCh3/MVA vaccination encoding the HCV-NS immunogen given to healthy volunteers can induce a broad, high magnitude and polyfunctional HCV specific T cell response (Swadling et al. 2014). However, the memory T cell population ('set-point') was observed to decline over time. I sought to establish whether reboosting with the same vaccines after different intervals could improve either the peak T cell magnitude or memory T cell response.

It was observed that the HCV specific T cell population could be induced following revaccination with AdCh3 and MVA-NS, but this was more effective after a longer interval following priming vaccination. The exception to this was the administration of AdCh3 after a short (8 week) interval, in which no T cell induction was observed. Furthermore, MVA-NS given alone as a reboosting vaccination was able to effectively induce a T cell response. There are two hypotheses suggested for this pattern of improved T cell induction with time. Firstly, that anti-vector antibodies persisting following priming vaccination clear the AdCh3 vector if administered after a short interval, causing attenuation of the response.

This could not be adequately assessed due to the differing baseline anti-adeno antibodies titres between the groups. Published data is inconsistent regarding this hypothesis, with both a negative correlation (Kelly et al. 2016) and no correlation (Barnes et al. 2012) reported between anti-adeno titres and T cell magnitude. A second hypothesis is that the HCV specific T cells are undergoing contraction and differentiation, are more optimally boosted after a longer interval. There is evidence that antigen specific T cell responses contract with time (Appay et al. 2008; Newell et al. 2012), and furthermore, HCV specific T cells have demonstrated a shift towards a Tem and Temra phenotype with time in response to AdCh3/MVA-NS (Swadling et al. 2014). Further phenotypic assessment of these T cell populations is required to address this.

Low dose MVA can be administered without compromising immunogenicity

I next sought to address whether MVA-NS could be effectively administered at a lower dose. The justification of this came from the technical and logistic challenges associated with MVA vector production. MVA demonstrates significant potential in both prophylactic and therapeutic applications, and therefore the most efficient large-scale production of this vector is an important evaluation. When administered as a boosting vaccination following AdCh3 priming at one and two logs lower than the initial dose of $2x10^8$ pfu, MVA-NS was able to induce a T cell response. Magnitude was observed to be progressively smaller with each dose reduction. The T cell magnitude induction required to protect against HCV is unknown, and thus the application of low dose MVA-NS to future HCV vaccine development is uncertain. However, it provides an important proof of principle that antigen specific T cells can be induced by very low doses of MVA, which may be applied to other potential applications of this vector.

Co-administration of distinct adenoviral vectors is safe and immunogenic

In the next chapter of my thesis I sought to address whether distinct adenoviral vectors could be safely and effectively administered in the context of developing a vaccine strategy to prevent HIV-1 and HCV co-infection. The implications of undertaking such a study cannot be underestimated. The global burden of HCV and HIV-1 co-infection is significant. There is no licenced vaccine for either virus, and the pathogens share similar routes of transmission, risk factors and host immune responses upon viral exposure. Furthermore, viral vectors are being widely researched for both prophylactic and therapeutic applications to various pathogens and cancers, and thus the assessment of vector co-administration is likely to be useful outside the scope of this trial.

I assessed the co-administration of AdCh3 / ChAdV63 prime encoding an HCV and HIV immunogen respectively to healthy adult volunteers. This was followed by heterologous boosting 8 weeks later of dual MVA vectors encoding these same immunogens. A primary concern was that either viral inhibition or antigenic competition may be observed, causing an attenuated response in comparison to either vaccine alone. Pre-clinical studies suggest antigenic interference may be abrogated by vector administration at separate anatomical sites, thereby limiting the potential competition for antigen uptake, processing and presentation by the host antigen presenting cell (Forbes et al., 2011; Sedegah et al., 2004). Co-administration of vectors in PEACHI-04 involved replication incompetent vectors given in separate arms. As viral shedding and systemic dissemination of vectors has been established not to occur (Harro et al., 2009), we hypothesised from the outset that neither viral inhibition (with distinct adenoviruses) nor antigenic competition (with distinct immunogens in both Ad and MVA vectors) would occur.

Co-administration was observed to be safe, and did not elicit more reactogenicity compared with single administration of either vaccine. ELISpot assessment of immunogenicity analysing the breadth and magnitude of the antigen specific T cell responses was not diminished to either immunogen following co-administration. A more detailed analysis of the breadth of the antigen specific T cell response is planned, to eliminate the possibility that subdominant epitope responses have been lost with co-administration. However, for the justification given above, we hypothesise this is highly unlikely.

Limited proliferative and functional recovery is observed following DAA mediated HCV cure

In the 6th chapter of my thesis, I performed a comprehensive assessment of the potential for, and degree of, immune recovery following HCV cure with DAA therapy. Following rapid removal of chronic antigen stimulation using therapeutic agents that don't interfere with the host immune system, what type of immunity remains? Or put another way, does reversal of T cell exhaustion occur? This is a pertinent question, with implications both within the HCV setting and the wider study of chronic infections. Firstly, it may identify whether individuals who remain at risk of re-infection following viral cure would be amenable to prophylactic vaccination. Previous vaccination attempts in patients receiving IFN therapy with both Ad6/AdCh3 (Kelly et al. 2016) and AdCh3/MVA-NS (Swadling et al. 2016) have demonstrated an attenuated HCV specific T cell response to vaccination. However, it is still an open question if vaccination may be more effective following cure and in the absence of IFN therapy. Secondly, it offers an opportunity to study the cure of

chronic infection *in vivo*, of which the knowledge gained can be applied to other persisting pathogens.

I observed a limited proliferative and functional capacity of antigen specific T cells following DAA mediated cure, and a significantly lower proliferative and functional capacity of HCV specific T cells in chronic infection in comparison to spontaneous HCV resolution and vaccine induced responses. There are at least 3 potential hypotheses for these observations, each requiring further studies to address. Firstly, the HCV specific T cells may be irreversibly and terminally exhausted. An assessment of phenotypic correlates of exhaustion on antigen specific T cells following DAA treatment may help to answer this. Secondly, the largely dysfunctional and antigen addicted HCV specific T cell population may be deleted following the removal of chronic antigen stimulation, leaving a population of recent thymic emigrants that require re-priming prior to T cell induction. *In vivo* challenge using viral vectored HCV vaccination following DAA mediated cure will address whether effective antigen specific T cell priming can occur, and this is planned as part of the Oxford vaccine program in the near future. A third hypothesis is that although the functional capacity of the 'cured' T cells was diminished when measured by single cytokine production, they may be of a higher, polyfunctional quality. There is no evidence at present to suggest this is the case. Nonetheless it is a hypothesis worth exploring, and an assessment of polyfunctionality of the T cells is planned.

This analysis of the cell-mediated responses following DAA cure was commenced in the final months of my thesis. This work is by no means complete, on the contrary these initial results have led to further questions and hypotheses. Further assessment is planned,

including recruitment of IFN naïve patients, and patients post-DAA treatment at a later time point (12 months). This subgroup assessment may enable identification of factors predictive of reversible or irreversible exhaustion.

Possible bystander inflammation is observed in chronic HCV infection

As part of the above assessment I analysed phenotypic markers of exhaustion on bulk CD4+ and CD8+ T cells. Significant differences were observed between chronic infection and healthy controls in both the surface expression of PD-1 and the intra-nuclear TF expression of Tbet. Additionally, significantly lower expression of eomes was seen following HCV viral clearance. Although there are certain caveats with any conclusions drawn regarding these observations due to the heterogeneous expression of these markers, it suggests that there may be a global shift of the cell phenotype in chronic infection as a result of bystander inflammation. A more comprehensive phenotypic analysis is planned, including the co-expression of PD-1 with other markers of inhibition. Furthermore, it is important to identify if phenotypic markers of exhaustion are altered on HCV specific T cells following DAA cure. I plan to address this using MHC Class I pentamers on *ex vivo* HCV specific CD8+ T cells in the near future.

Healthy volunteers vaccinated with AdCh3/MVA demonstrate T cell induction analogous to that observed in spontaneously resolved HCV infection.

Finally, the assessment discussed above as well as the *ex-vivo* phenotypic assessment of CD4+ T cells in chapter 7 enabled the analysis of the phenotypic, proliferative and functional capacity of antigen specific T cells in spontaneous HCV resolution and vaccine induced responses. This gave an opportunity to firstly compare immune responses in

chronic, cured and naturally resolved infection, and secondly to compare the HCV specific CD4+ and CD8+ T cells induced by AdCh3/MVA-NS vaccination to the 'gold standard' of spontaneous viral resolution. The comparable phenotypic, proliferative and functional antigen specific T cell responses in spontaneous HCV clearance with those induced by vaccination suggests HCV vaccination with viral vectors induces HCV specific T cells analogous to that observed in natural protection. This hypothesis is currently being challenged in the Phase I/II efficacy study underway, the results of which are expected to be published in 2018.

Future applications of this work

The development in Oxford of a viral vectored vaccination strategy for HCV has advanced significantly over the past few years. Progression from Ad/Ad prime-boost to AdCh3/MVA prime-boost in both healthy adults and patients has occurred, and the promising results observed from these trials has enabled this strategy to progress to efficacy trials. Furthermore, this groundwork has facilitated the development of next generation AdCh3/MVA vaccines employing the invariant chain as an adjuvant, and the evaluation of these novel vaccine candidates in both healthy adults and patients with DAA-mediated HCV clearance in the near future is an exciting development, and although I will not be directly involved in PEACHI-03, I eagerly await the outcome of the trial.

Thank you for taking the time to read this work.

Sincerely
Dr Felicity Hartnell.
October 2018.

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APPENDICES

APPENDIX 2-1: PEPTIDES SPANNING HCV IMMUNOGEN

POOL F				
1 of 494	40 mg/mL	1027	APITAYSQQTRGLLG	1041
2 of 494	40 mg/mL	1031	AYSQQTRGLLGCIIT	1045
3 of 494	40 mg/mL	1035	QTRGLLGCIITSLTG	1049
4 of 494	40 mg/mL	1039	LLGCIITSLTGRDKN	1053
5 of 494	40 mg/mL	1043	IITSLTGRDKNQVEG	1057
6 of 494	40 mg/mL	1047	LTGRDKNQVEGEVQV	1061
7 of 494	10 mg/mL	1051	DKNQVEGEVQVVSTA	1065
8 of 494	10 mg/mL	1055	VEGEVQVVSTATQSF	1069
9 of 494	40 mg/mL	1059	VQVVSTATQSFLATC	1073
10 of 494	40 mg/mL	1063	STATQSFLATCVNGV	1077
11 of 494	40 mg/mL	1067	QSFLATCVNGVCWTV	1081
12 of 494	40 mg/mL	1071	ATCVNGVCWTVYHGA	1085
13 of 494	40 mg/mL	1075	NGVCWTVYHGAGSKT	1089
14 of 494	40 mg/mL	1079	WTVYHGAGSKTLAGP	1093
15 of 494	40 mg/mL	1083	HGAGSKTLAGPKGPI	1097
16 of 494	40 mg/mL	1087	SKTLAGPKGPITQMY	1101
17 of 494	40 mg/mL	1091	AGPKGPITQMYTNVD	1105
18 of 494	40 mg/mL	1095	GPITQMYTNVDQDLV	1109
19 of 494	40 mg/mL	1099	QMYTNVDQDLVGWQA	1113
20 of 494	40 mg/mL	1103	NVDQDLVGWQAPPGA	1117
21 of 494	40 mg/mL	1107	DLVGWQAPPGARSLT	1121
22 of 494	40 mg/mL	1111	WQAPPGARSLTPCTC	1125
23 of 494	40 mg/mL	1115	PGARSLTPCTCGSSD	1129
24 of 494	40 mg/mL	1119	SLTPCTCGSSDLYLV	1133
25 of 494	40 mg/mL	1123	CTCGSSDLYLVTRHA	1137
26 of 494	40 mg/mL	1127	SSDLYLVTRHADVIP	1141
27 of 494	40 mg/mL	1131	YLVTRHADVIPVRRR	1145
28 of 494	40 mg/mL	1135	RHADVIPVRRRGDSR	1149
29 of 494	40 mg/mL	1139	VIPVRRRGDSRGSLL	1153
30 of 494	40 mg/mL	1143	RRRGDSRGSLLSPRP	1157
31 of 494	40 mg/mL	1147	DSRGSLLSPRPVSYL	1161
32 of 494	40 mg/mL	1151	SLLSPRPVSYLKGSS	1165
33 of 494	40 mg/mL	1155	PRPVSYLKGSSGGPL	1169
34 of 494	40 mg/mL	1159	SYLKGSSGGPLLCPS	1173
35 of 494	40 mg/mL	1163	GSSGGPLLCPSGHAV	1177
36 of 494	40 mg/mL	1167	GPLLCPSGHAVGIFR	1181
37 of 494	40 mg/mL	1171	CPSGHAVGIFRAAVC	1185

38 of 494	40 mg/mL	1175	HAVGIFRAAVCTRGV	1189
39 of 494	40 mg/mL	1179	IFRAAVCTRGVAKAV	1193
40 of 494	40 mg/mL	1183	AVCTRGVAKAVDFVP	1197
41 of 494	40 mg/mL	1187	RGVAKAVDFVPVESM	1201
42 of 494	40 mg/mL	1191	KAVDFVPVESMETTM	1205
43 of 494	40 mg/mL	1195	FVPVESMETTMRSPV	1209
44 of 494	40 mg/mL	1199	ESMETTMRSPVFTDN	1213
45 of 494	40 mg/mL	1203	TTMRSPVFTDNSSPP	1217
46 of 494	40 mg/mL	1207	SPVFTDNSSPPAVPQ	1221
47 of 494	40 mg/mL	1211	TDNSSPPAVPQSFQV	1225
48 of 494	40 mg/mL	1215	SPPAVPQSFQVAHLH	1229
49 of 494	40 mg/mL	1219	VPQSFQVAHLHAPTG	1233
50 of 494	40 mg/mL	1223	FQVAHLHAPTGSGKS	1237
51 of 494	40 mg/mL	1227	HLHAPTGSGKSTKVP	1241
52 of 494	40 mg/mL	1231	PTGSGKSTKVPAAYA	1245
53 of 494	40 mg/mL	1235	GKSTKVPAAYAAQGY	1249
54 of 494	40 mg/mL	1239	KVPAAYAAQGYKVLV	1253
55 of 494	40 mg/mL	1243	AYAAQGYKVLVLNPS	1257
56 of 494	40 mg/mL	1247	QGYKVLVLNPSVAAT	1261
57 of 494	40 mg/mL	1251	VLVLNPSVAATLGFG	1265
58 of 494	40 mg/mL	1255	NPSVAATLGFGAYMS	1269
59 of 494	40 mg/mL	1259	AATLGFGAYMSKAHG	1273
60 of 494	40 mg/mL	1263	GFGAYMSKAHGIDPN	1277
61 of 494	40 mg/mL	1267	YMSKAHGIDPNIRTG	1281
62 of 494	40 mg/mL	1271	AHGIDPNIRTGVRTI	1285
63 of 494	40 mg/mL	1275	DPNIRTGVRTITTGA	1289
64 of 494	40 mg/mL	1279	RTGVRTITTGAPVTY	1293
65 of 494	40 mg/mL	1283	RTITTGAPVTYSTYG	1297
66 of 494	40 mg/mL	1287	TGAPVTYSTYGKFLA	1301
67 of 494	40 mg/mL	1291	VTYSTYGKFLADGGC	1305
68 of 494	40 mg/mL	1295	TYGKFLADGGCSGGA	1309
69 of 494	40 mg/mL	1299	FLADGGCSGGAYDII	1313
70 of 494	10 mg/mL	1303	GGCSGGAYDIIICDE	1317
71 of 494	40 mg/mL	1307	GGAYDIIICDECHST	1321
72 of 494	40 mg/mL	1311	DIIICDECHSTDSTT	1325
73 of 494	40 mg/mL	1315	CDECHSTDSTTILGI	1329
74 of 494	40 mg/mL	1319	HSTDSTTILGIGTVL	1333
75 of 494	10 mg/mL	1323	STTILGIGTVLDQAE	1337
76 of 494	40 mg/mL	1327	LGIGTVLDQAETAGA	1341
77 of 494	10 mg/mL	1331	TVLDQAETAGARLVV	1345
78 of 494	20 mg/mL	1335	QAETAGARLVVLATA	1349
POOL G				
79 of 494	20 mg/mL	1339	AGARLVVLATATPPG	1353

80 of 494	40 mg/mL	1343	LVVLATATPPGSVTV	1357
81 of 494	40 mg/mL	1347	ATATPPGSVTVPHPN	1361
82 of 494	40 mg/mL	1351	PPGSVTVPHPNIEEV	1365
83 of 494	40 mg/mL	1355	VTVPHPNIEEVALSN	1369
84 of 494	40 mg/mL	1359	HPNIEEVALSNTGEI	1373
85 of 494	40 mg/mL	1363	EEVALSNTGEIPFYG	1377
86 of 494	40 mg/mL	1367	LSNTGEIPFYGKAIP	1381
87 of 494	40 mg/mL	1371	GEIPFYGKAIPIEAI	1385
88 of 494	40 mg/mL	1375	FYGKAIPIEAIRGGR	1389
89 of 494	40 mg/mL	1379	AIPIEAIRGGRHLIF	1393
90 of 494	40 mg/mL	1383	EAIRGGRHLIFCHSK	1397
91 of 494	40 mg/mL	1387	GGRHLIFCHSKKKCD	1401
92 of 494	40 mg/mL	1391	LIFCHSKKKCDELAA	1405
93 of 494	40 mg/mL	1395	HSKKKCDELAAKLSG	1409
94 of 494	40 mg/mL	1399	KCDELAAKLSGLGIN	1413
95 of 494	40 mg/mL	1403	LAAKLSGLGINAVAY	1417
96 of 494	40 mg/mL	1407	LSGLGINAVAYYRGL	1421
97 of 494	20 mg/mL	1411	GINAVAYYRGLDVSV	1425
98 of 494	40 mg/mL	1415	VAYYRGLDVSVIPTI	1429
99 of 494	40 mg/mL	1419	RGLDVSVIPTIGDVV	1433
100 of 494	10 mg/mL	1423	VSVIPTIGDVVVVAT	1437
101 of 494	10 mg/mL	1427	PTIGDVVVVATDALM	1441
102 of 494	40 mg/mL	1431	DVVVVATDALMTGYT	1445
103 of 494	40 mg/mL	1435	VATDALMTGYTGDFD	1449
104 of 494	40 mg/mL	1439	ALMTGYTGDFDSVID	1453
105 of 494	10 mg/mL	1443	GYTGDFDSVIDCNTC	1457
106 of 494	40 mg/mL	1447	DFDSVIDCNTCVTQT	1461
107 of 494	40 mg/mL	1451	VIDCNTCVTQTVDFS	1465
108 of 494	40 mg/mL	1455	NTCVTQTVDFSLDPT	1469
109 of 494	40 mg/mL	1459	TQTVDFSLDPTFTIE	1473
110 of 494	40 mg/mL	1463	DFSLDPTFTIETTTV	1477
111 of 494	40 mg/mL	1467	DPTFTIETTTVPQDA	1481
112 of 494	40 mg/mL	1471	TIETTTVPQDAVSRS	1485
113 of 494	40 mg/mL	1475	TTVPQDAVSRSQRRG	1489
114 of 494	40 mg/mL	1479	QDAVSRSQRRGRTGR	1493
115 of 494	40 mg/mL	1483	SRSQRRGRTGRGRRG	1497
116 of 494	40 mg/mL	1487	RRGRTGRGRRGIYRF	1501
117 of 494	40 mg/mL	1491	TGRGRRGIYRFVTPG	1505
118 of 494	40 mg/mL	1495	RRGIYRFVTPGERPS	1509
119 of 494	40 mg/mL	1499	YRFVTPGERPSGMFD	1513
120 of 494	40 mg/mL	1503	TPGERPSGMFDSSVL	1517
121 of 494	40 mg/mL	1507	RPSGMFDSSVLCECY	1521
122 of 494	20 mg/mL	1511	MFDSSVLCECYDAGC	1525

123 of 494	40 mg/mL	1515	SVLCECYDAGCAWYE	1529
124 of 494	40 mg/mL	1519	ECYDAGCAWYELTPA	1533
125 of 494	40 mg/mL	1523	AGCAWYELTPAETSV	1537
126 of 494	40 mg/mL	1527	WYELTPAETSVRLRA	1541
127 of 494	40 mg/mL	1531	TPAETSVRLRAYLNT	1545
128 of 494	40 mg/mL	1535	TSVRLRAYLNTPGLP	1549
129 of 494	40 mg/mL	1539	LRAYLNTPGLPVCQD	1553
130 of 494	40 mg/mL	1543	LNTPGLPVCQDHLEF	1557
131 of 494	40 mg/mL	1547	GLPVCQDHLEFWESV	1561
132 of 494	40 mg/mL	1551	CQDHLEFWESVFTGL	1565
133 of 494	40 mg/mL	1555	LEFWESVFTGLTHID	1569
134 of 494	40 mg/mL	1559	ESVFTGLTHIDAHFL	1573
135 of 494	40 mg/mL	1563	TGLTHIDAHFLSQTK	1577
136 of 494	40 mg/mL	1567	HIDAHFLSQTKQAGD	1581
137 of 494	40 mg/mL	1571	HFLSQTKQAGDNFPY	1585
138 of 494	40 mg/mL	1575	QTKQAGDNFPYLVAY	1589
139 of 494	20 mg/mL	1579	AGDNFPYLVAYQATV	1593
140 of 494	40 mg/mL	1583	FPYLVAYQATVCARA	1597
141 of 494	40 mg/mL	1587	VAYQATVCARAQAPP	1601
142 of 494	40 mg/mL	1591	ATVCARAQAPPPSWD	1605
143 of 494	40 mg/mL	1595	ARAQAPPPSWDQMWK	1609
144 of 494	40 mg/mL	1599	APPPSWDQMWKCLIR	1613
145 of 494	40 mg/mL	1603	SWDQMWKCLIRLKPT	1617
146 of 494	40 mg/mL	1607	MWKCLIRLKPTLHGP	1621
147 of 494	40 mg/mL	1611	LIRLKPTLHGPTPLL	1625
148 of 494	40 mg/mL	1615	KPTLHGPTPLLYRLG	1629
149 of 494	40 mg/mL	1619	HGPTPLLYRLGAVQN	1633
150 of 494	40 mg/mL	1623	PLLYRLGAVQNEVTL	1637
151 of 494	40 mg/mL	1627	RLGAVQNEVTLTHPI	1641
152 of 494	40 mg/mL	1631	VQNEVTLTHPITKYI	1645
153 of 494	40 mg/mL	1635	VTLTHPITKYIMACM	1649
154 of 494	40 mg/mL	1639	HPITKYIMACMSADL	1653
155 of 494	40 mg/mL	1643	KYIMACMSADLEVVT	1657
156 of 494	10 mg/mL	1647	ACMSADLEVVTSTWV	1661
POOL H				
157 of 494	10 mg/mL	1651	ADLEVVTSTWVLVGG	1665
158 of 494	10 mg/mL	1655	VVTSTWVLVGGVLAA	1669
159 of 494	40 mg/mL	1659	TWVLVGGVLAALAAY	1673
160 of 494	10 mg/mL	1663	VGGVLAALAAYCLTT	1677
161 of 494	40 mg/mL	1667	LAALAAYCLTTGSVV	1681
162 of 494	20 mg/mL	1671	AAYCLTTGSVVIVGR	1685
163 of 494	40 mg/mL	1675	LTTGSVVIVGRIILS	1689
164 of 494	40 mg/mL	1679	SVVIVGRIILSGRPA	1693

165 of 494	40 mg/mL	1683	VGRIILSGRPAIVPD	1697
166 of 494	40 mg/mL	1687	ILSGRPAIVPDREFL	1701
167 of 494	40 mg/mL	1691	RPAIVPDREFLYQEF	1705
168 of 494	40 mg/mL	1695	VPDREFLYQEFDEME	1709
169 of 494	40 mg/mL	1699	EFLYQEFDEMEECAS	1713
170 of 494	40 mg/mL	1703	QEFDEMEECASHLPY	1717
171 of 494	40 mg/mL	1707	EMEECASHLPYIEQG	1721
172 of 494	20 mg/mL	1711	CASHLPYIEQGMQLA	1725
173 of 494	40 mg/mL	1715	LPYIEQGMQLAEQFK	1729
174 of 494	40 mg/mL	1719	EQGMQLAEQFKQKAL	1733
175 of 494	40 mg/mL	1723	QLAEQFKQKALGLLQ	1737
176 of 494	40 mg/mL	1727	QFKQKALGLLQTATK	1741
177 of 494	40 mg/mL	1731	KALGLLQTATKQAEA	1745
178 of 494	40 mg/mL	1735	LLQTATKQAEAAAPV	1749
179 of 494	40 mg/mL	1739	ATKQAEAAAPVVESK	1753
180 of 494	40 mg/mL	1743	AEAAAPVVESKWRAL	1757
181 of 494	40 mg/mL	1747	APVVESKWRALETFW	1761
182 of 494	40 mg/mL	1751	ESKWRALETFWAKHM	1765
183 of 494	40 mg/mL	1755	RALETFWAKHMWNFI	1769
184 of 494	40 mg/mL	1759	TFWAKHMWNFISGIQ	1773
185 of 494	40 mg/mL	1763	KHMWNFISGIQYLAG	1777
186 of 494	40 mg/mL	1767	NFISGIQYLAGLSTL	1781
187 of 494	40 mg/mL	1771	GIQYLAGLSTLPGNP	1785
188 of 494	40 mg/mL	1775	LAGLSTLPGNPAIAS	1789
189 of 494	40 mg/mL	1779	STLPGNPAIASLMAF	1793
190 of 494	40 mg/mL	1783	GNPAIASLMAFTASI	1797
191 of 494	10 mg/mL	1787	IASLMAFTASITSPL	1801
192 of 494	40 mg/mL	1791	MAFTASITSPLTTQS	1805
193 of 494	40 mg/mL	1795	ASITSPLTTQSTLLF	1809
194 of 494	40 mg/mL	1799	SPLTTQSTLLFNILG	1813
195 of 494	40 mg/mL	1803	TQSTLLFNILGGWVA	1817
196 of 494	10 mg/mL	1807	LLFNILGGWVAAQLA	1821
197 of 494	40 mg/mL	1811	ILGGWVAAQLAPPSA	1825
198 of 494	40 mg/mL	1815	WVAAQLAPPSAASAF	1829
199 of 494	40 mg/mL	1819	QLAPPSAASAFVGAG	1833
200 of 494	40 mg/mL	1823	PSAASAFVGAGIAGA	1837
201 of 494	10 mg/mL	1827	SAFVGAGIAGAAVGS	1841
202 of 494	10 mg/mL	1831	GAGIAGAAVGSIGLG	1845
203 of 494	10 mg/mL	1835	AGAAVGSIGLGKVLV	1849
204 of 494	40 mg/mL	1839	VGSIGLGKVLVDILA	1853
205 of 494	40 mg/mL	1843	GLGKVLVDILAGYGA	1857
206 of 494	40 mg/mL	1847	VLVDILAGYGAGVAG	1861
207 of 494	40 mg/mL	1851	ILAGYGAGVAGALVA	1865

208 of 494	40 mg/mL	1855	YGAGVAGALVAFKVM	1869
209 of 494	40 mg/mL	1859	VAGALVAFKVMSGEM	1873
210 of 494	40 mg/mL	1863	LVAFKVMSGEMPSTE	1877
211 of 494	40 mg/mL	1867	KVMSGEMPSTEDLVN	1881
212 of 494	40 mg/mL	1871	GEMPSTEDLVNLLPA	1885
213 of 494	40 mg/mL	1875	STEDLVNLLPAILSP	1889
214 of 494	40 mg/mL	1879	LVNLLPAILSPGALV	1893
215 of 494	40 mg/mL	1883	LPAILSPGALVVGVV	1897
216 of 494	20 mg/mL	1887	LSPGALVVGVVCAAI	1901
217 of 494	40 mg/mL	1891	ALVVGVVCAAILRRH	1905
218 of 494	40 mg/mL	1895	GVVCAAILRRHVGPG	1909
219 of 494	40 mg/mL	1899	AAILRRHVGPGEGAV	1913
220 of 494	40 mg/mL	1903	RRHVGPGEGAVQWMN	1917
221 of 494	40 mg/mL	1907	GPGEGAVQWMNRLIA	1921
222 of 494	40 mg/mL	1911	GAVQWMNRLIAFASR	1925
223 of 494	40 mg/mL	1915	WMNRLIAFASRGNHV	1929
224 of 494	40 mg/mL	1919	LIAFASRGNHVSPTH	1933
225 of 494	40 mg/mL	1923	ASRGNHVSPTHYVPE	1937
226 of 494	40 mg/mL	1927	NHVSPTHYVPESDAA	1941
227 of 494	40 mg/mL	1931	PTHYVPESDAAARVT	1945
228 of 494	40 mg/mL	1935	VPESDAAARVTQILS	1949
229 of 494	40 mg/mL	1939	DAAARVTQILSSLTI	1953
230 of 494	10 mg/mL	1943	RVTQILSSLTITQLL	1957
231 of 494	40 mg/mL	1947	ILSSLTITQLLKRLH	1961
232 of 494	40 mg/mL	1951	LTITQLLKRLHQWIN	1965
233 of 494	40 mg/mL	1955	QLLKRLHQWINEDCS	1969
234 of 494	40 mg/mL	1959	RLHQWINEDCSTPCS	1973
235 of 494	40 mg/mL	1963	WINEDCSTPCSGSWL	1977
POOL I				
236 of 494	40 mg/mL	1967	DCSTPCSGSWLRDVW	1981
237 of 494	40 mg/mL	1971	PCSGSWLRDVWDWIC	1985
238 of 494	40 mg/mL	1975	SWLRDVWDWICTVLT	1989
239 of 494	40 mg/mL	1979	DVWDWICTVLTDFKT	1993
240 of 494	40 mg/mL	1983	WICTVLTDFKTWLQS	1997
241 of 494	40 mg/mL	1987	VLTDFKTWLQSKLLP	2001
242 of 494	40 mg/mL	1991	FKTWLQSKLLPQLPG	2005
243 of 494	40 mg/mL	1995	LQSKLLPQLPGVPFF	2009
244 of 494	40 mg/mL	1999	LLPQLPGVPFFSCQR	2013
245 of 494	40 mg/mL	2003	LPGVPFFSCQRGYKG	2017
246 of 494	40 mg/mL	2007	PFFSCQRGYKGVWRG	2021
247 of 494	40 mg/mL	2011	CQRGYKGVWRGDGIM	2025
248 of 494	40 mg/mL	2015	YKGVWRGDGIMQTTC	2029
249 of 494	40 mg/mL	2019	WRGDGIMQTTCPCGA	2033

250 of 494	40 mg/mL	2023	GIMQTTCPCGAQITG	2037
251 of 494	40 mg/mL	2027	TTCPCGAQITGHVKN	2041
252 of 494	40 mg/mL	2031	CGAQITGHVKNGSMR	2045
253 of 494	40 mg/mL	2035	ITGHVKNGSMRIVGP	2049
254 of 494	40 mg/mL	2039	VKNGSMRIVGPKTCS	2053
255 of 494	40 mg/mL	2043	SMRIVGPKTCSNTWH	2057
256 of 494	40 mg/mL	2047	VGPKTCSNTWHGTFP	2061
257 of 494	40 mg/mL	2051	TCSNTWHGTFPINAY	2065
258 of 494	40 mg/mL	2055	TWHGTFPINAYTTGP	2069
259 of 494	40 mg/mL	2059	TFPINAYTTGPCTPS	2073
260 of 494	40 mg/mL	2063	NAYTTGPCTPSPAPN	2077
261 of 494	40 mg/mL	2067	TGPCTPSPAPNYSRA	2081
262 of 494	40 mg/mL	2071	TPSPAPNYSRALWRV	2085
263 of 494	40 mg/mL	2075	APNYSRALWRVAAEE	2089
264 of 494	40 mg/mL	2079	SRALWRVAAEEYVEV	2093
265 of 494	10 mg/mL	2083	WRVAAEEYVEVTRVG	2097
266 of 494	40 mg/mL	2087	AEEYVEVTRVGDFHY	2101
267 of 494	40 mg/mL	2091	VEVTRVGDFHYVTGM	2105
268 of 494	40 mg/mL	2095	RVGDFHYVTGMTTDN	2109
269 of 494	40 mg/mL	2099	FHYVTGMTTDNVKCP	2113
270 of 494	40 mg/mL	2103	TGMTTDNVKCPCQVP	2117
271 of 494	40 mg/mL	2107	TDNVKCPCQVPAPEF	2121
272 of 494	40 mg/mL	2111	KCPCQVPAPEFFTEV	2125
273 of 494	40 mg/mL	2115	QVPAPEFFTEVDGVR	2129
274 of 494	40 mg/mL	2119	PEFFTEVDGVRLHRY	2133
275 of 494	40 mg/mL	2123	TEVDGVRLHRYAPAC	2137
276 of 494	40 mg/mL	2127	GVRLHRYAPACRPLL	2141
277 of 494	40 mg/mL	2131	HRYAPACRPLLREEV	2145
278 of 494	40 mg/mL	2135	PACRPLLREEVTFQV	2149
279 of 494	40 mg/mL	2139	PLLREEVTFQVGLNQ	2153
280 of 494	40 mg/mL	2143	EEVTFQVGLNQYLVG	2157
281 of 494	40 mg/mL	2147	FQVGLNQYLVGSQLP	2161
282 of 494	40 mg/mL	2151	LNQYLVGSQLPCEPE	2165
283 of 494	40 mg/mL	2155	LVGSQLPCEPEPDVA	2169
284 of 494	40 mg/mL	2159	QLPCEPEPDVAVLTS	2173
285 of 494	40 mg/mL	2163	EPEPDVAVLTSMLTD	2177
286 of 494	40 mg/mL	2167	DVAVLTSMLTDPSHI	2181
287 of 494	40 mg/mL	2171	LTSMLTDPSHITAET	2185
288 of 494	40 mg/mL	2175	LTDPSHITAETAKRR	2189
289 of 494	40 mg/mL	2179	SHITAETAKRRLARG	2193
290 of 494	40 mg/mL	2183	AETAKRRLARGSPPS	2197
291 of 494	40 mg/mL	2187	KRRLARGSPPSLASS	2201
292 of 494	40 mg/mL	2191	ARGSPPSLASSSASQ	2205

293 of 494	40 mg/mL	2195	PPSLASSSASQLSAP	2209
294 of 494	40 mg/mL	2199	ASSSASQLSAPSLKA	2213
295 of 494	40 mg/mL	2203	ASQLSAPSLKATCTT	2217
296 of 494	40 mg/mL	2207	SAPSLKATCTTHHVS	2221
297 of 494	40 mg/mL	2211	LKATCTTHHVSPDAD	2225
298 of 494	40 mg/mL	2215	CTTHHVSPDADLIEA	2229
299 of 494	40 mg/mL	2219	HVSPDADLIEANLLW	2233
300 of 494	40 mg/mL	2223	DADLIEANLLWRQEM	2237
301 of 494	40 mg/mL	2227	IEANLLWRQEMGGNI	2241
302 of 494	40 mg/mL	2231	LLWRQEMGGNITRVE	2245
303 of 494	40 mg/mL	2235	QEMGGNITRVESENK	2249
304 of 494	20 mg/mL	2239	GNITRVESENKVVVL	2253
305 of 494	40 mg/mL	2243	RVESENKVVVLDSFD	2257
306 of 494	40 mg/mL	2247	ENKVVVLDSFDPLRA	2261
307 of 494	40 mg/mL	2251	VVLDSFDPLRAEEDE	2265
308 of 494	40 mg/mL	2255	SFDPLRAEEDEREVS	2269
309 of 494	40 mg/mL	2259	LRAEEDEREVSVPAE	2273
310 of 494	40 mg/mL	2263	EDEREVSVPAEILRK	2277
311 of 494	40 mg/mL	2267	EVSVPAEILRKSKKF	2281
312 of 494	40 mg/mL	2271	PAEILRKSKKFPAAM	2285
313 of 494	40 mg/mL	2275	LRKSKKFPAAMPIWA	2289
314 of 494	40 mg/mL	2279	KKFPAAMPIWARPDY	2293
315 of 494	40 mg/mL	2283	AAMPIWARPDYNPPL	2297
316 of 494	40 mg/mL	2287	IWARPDYNPPLLESW	2301
317 of 494	40 mg/mL	2291	PDYNPPLLESWKDPD	2305
318 of 494	40 mg/mL	2295	PPLLESWKDPDYVPP	2309
319 of 494	40 mg/mL	2299	ESWKDPDYVPPVVHG	2313
320 of 494	40 mg/mL	2303	DPDYVPPVVHGCPLP	2317
321 of 494	40 mg/mL	2307	VPPVVHGCPLPPIKA	2321
322 of 494	40 mg/mL	2311	VHGCPLPPIKAPPIP	2325
323 of 494	40 mg/mL	2315	PLPPIKAPPIPPPRR	2329
324 of 494	40 mg/mL	2319	IKAPPIPPPRRKRTV	2333
325 of 494	40 mg/mL	2323	PIPPPRRKRTVVLTE	2337
326 of 494	40 mg/mL	2327	PRRKRTVVLTESSVS	2341
327 of 494	40 mg/mL	2331	RTVVLTESSVSSALA	2345
328 of 494	10 mg/mL	2335	LTESSVSSALAELAT	2349
329 of 494	40 mg/mL	2339	SVSSALAELATKTFG	2353
330 of 494	40 mg/mL	2343	ALAELATKTFGSSES	2357
331 of 494	40 mg/mL	2347	LATKTFGSSESSAVD	2361
332 of 494	40 mg/mL	2351	TFGSSESSAVDSGTA	2365
333 of 494	40 mg/mL	2355	SESSAVDSGTATALP	2369
334 of 494	40 mg/mL	2359	AVDSGTATALPDQAS	2373
335 of 494	40 mg/mL	2363	GTATALPDQASDDGD	2377

336 of 494	40 mg/mL	2367	ALPDQASDDGDKGSD	2381
337 of 494	40 mg/mL	2371	QASDDGDKGSDVESY	2385
338 of 494	40 mg/mL	2375	DGDKGSDVESYSSMP	2389
339 of 494	40 mg/mL	2379	GSDVESYSSMPPLEG	2393
340 of 494	40 mg/mL	2383	ESYSSMPPLEGEPGD	2397
341 of 494	40 mg/mL	2387	SMPPLEGEPGDPDLS	2401
342 of 494	40 mg/mL	2391	LEGEPGDPDLSDGSW	2405
343 of 494	40 mg/mL	2395	PGDPDLSDGSWSTVS	2409
344 of 494	40 mg/mL	2399	DLSDGSWSTVSEEAS	2413
345 of 494	10 mg/mL	2403	GSWSTVSEEASEDVV	2417
346 of 494	10 mg/mL	2407	TVSEEASEDVVCCSM	2421
347 of 494	40 mg/mL	2411	EASEDVVCCSMSYTW	2425
POOL L				
348 of 494	40 mg/mL	2415	DVVCCSMSYTWTGAL	2429
349 of 494	40 mg/mL	2419	CSMSYTWTGALITPC	2433
350 of 494	40 mg/mL	2423	YTWTGALITPCAAEE	2437
351 of 494	40 mg/mL	2427	GALITPCAAEESKLP	2441
352 of 494	40 mg/mL	2431	TPCAAEESKLPINAL	2445
353 of 494	40 mg/mL	2435	AEESKLPINALSNSL	2449
354 of 494	40 mg/mL	2439	KLPINALSNSLLRHH	2453
355 of 494	40 mg/mL	2443	NALSNSLLRHHNMVY	2457
356 of 494	40 mg/mL	2447	NSLLRHHNMVYATTS	2461
357 of 494	40 mg/mL	2451	RHHNMVYATTSRSAG	2465
358 of 494	40 mg/mL	2455	MVYATTSRSAGLRQK	2469
359 of 494	40 mg/mL	2459	TTSRSAGLRQKKVTF	2473
360 of 494	40 mg/mL	2463	SAGLRQKKVTFDRLQ	2477
361 of 494	40 mg/mL	2467	RQKKVTFDRLQVLDD	2481
362 of 494	40 mg/mL	2471	VTFDRLQVLDDHYRD	2485
363 of 494	40 mg/mL	2475	RLQVLDDHYRDVLKE	2489
364 of 494	40 mg/mL	2479	LDDHYRDVLKEMKAK	2493
365 of 494	40 mg/mL	2483	YRDVLKEMKAKASTV	2497
366 of 494	40 mg/mL	2487	LKEMKAKASTVKAKL	2501
367 of 494	40 mg/mL	2491	KAKASTVKAKLLSVE	2505
368 of 494	40 mg/mL	2495	STVKAKLLSVEEACK	2509
369 of 494	40 mg/mL	2499	AKLLSVEEACKLTPP	2513
370 of 494	40 mg/mL	2503	SVEEACKLTPPHSAK	2517
371 of 494	40 mg/mL	2507	ACKLTPPHSAKSKFG	2521
372 of 494	40 mg/mL	2511	TPPHSAKSKFGYGAK	2525
373 of 494	40 mg/mL	2515	SAKSKFGYGAKDVRN	2529
374 of 494	40 mg/mL	2519	KFGYGAKDVRNLSSK	2533
375 of 494	40 mg/mL	2523	GAKDVRNLSSKAVNH	2537
376 of 494	40 mg/mL	2527	VRNLSSKAVNHIHSV	2541
377 of 494	40 mg/mL	2531	SSKAVNHIHSVWKDL	2545

378 of 494	40 mg/mL	2535	VNHIHSVWKDLLEDT	2549
379 of 494	40 mg/mL	2539	HSVWKDLLEDTVTPI	2553
380 of 494	40 mg/mL	2543	KDLLEDTVTPIDTTI	2557
381 of 494	40 mg/mL	2547	EDTVTPIDTTIMAKN	2561
382 of 494	40 mg/mL	2551	TPIDTTIMAKNEVFC	2565
383 of 494	40 mg/mL	2555	TTIMAKNEVFCVQPE	2569
384 of 494	40 mg/mL	2559	AKNEVFCVQPEKGGR	2573
385 of 494	40 mg/mL	2563	VFCVQPEKGGRKPAR	2577
386 of 494	40 mg/mL	2567	QPEKGGRKPARLIVF	2581
387 of 494	40 mg/mL	2571	GGRKPARLIVFPDLG	2585
388 of 494	40 mg/mL	2575	PARLIVFPDLGVRVC	2589
389 of 494	40 mg/mL	2579	IVFPDLGVRVCEKMA	2593
390 of 494	40 mg/mL	2583	DLGVRVCEKMALYDV	2597
391 of 494	40 mg/mL	2587	RVCEKMALYDVVSTL	2601
392 of 494	40 mg/mL	2591	KMALYDVVSTLPQVV	2605
393 of 494	40 mg/mL	2595	YDVVSTLPQVVMGSS	2609
394 of 494	40 mg/mL	2599	STLPQVVMGSSYGFQ	2613
395 of 494	40 mg/mL	2603	QVVMGSSYGFQYSPG	2617
396 of 494	40 mg/mL	2607	GSSYGFQYSPGQRVE	2621
397 of 494	40 mg/mL	2611	GFQYSPGQRVEFLVN	2625
398 of 494	40 mg/mL	2615	SPGQRVEFLVNTWKS	2629
399 of 494	40 mg/mL	2619	RVEFLVNTWKSKKNP	2633
400 of 494	40 mg/mL	2623	LVNTWKSKKNPMGFS	2637
401 of 494	40 mg/mL	2627	WKSKKNPMGFSYDTR	2641
402 of 494	40 mg/mL	2631	KNPMGFSYDTRCFDS	2645
403 of 494	40 mg/mL	2635	GFSYDTRCFDSTVTE	2649
404 of 494	40 mg/mL	2639	DTRCFDSTVTENDIR	2653
405 of 494	40 mg/mL	2643	FDSTVTENDIRVEES	2657
406 of 494	40 mg/mL	2647	VTENDIRVEESIYQC	2661
407 of 494	20 mg/mL	2651	DIRVEESIYQCCDLA	2665
408 of 494	40 mg/mL	2655	EESIYQCCDLAPEAR	2669
409 of 494	40 mg/mL	2659	YQCCDLAPEARQAIK	2673
410 of 494	40 mg/mL	2663	DLAPEARQAIKSLTE	2677
411 of 494	40 mg/mL	2667	EARQAIKSLTERLYI	2681
412 of 494	40 mg/mL	2671	AIKSLTERLYIGGPL	2685
413 of 494	40 mg/mL	2675	LTERLYIGGPLTNSK	2689
414 of 494	40 mg/mL	2679	LYIGGPLTNSKGQNC	2693
415 of 494	40 mg/mL	2683	GPLTNSKGQNCGYRR	2697
416 of 494	40 mg/mL	2687	NSKGQNCGYRRCRAS	2701
417 of 494	40 mg/mL	2691	QNCGYRRCRASGVLT	2705
418 of 494	40 mg/mL	2695	YRRCRASGVLTTSCG	2709
419 of 494	40 mg/mL	2699	RASGVLTTSCGNTLT	2713
420 of 494	40 mg/mL	2703	VLTTSCGNTLTCYLK	2717

POOL M				
421 of 494	40 mg/mL	2707	SCGNTLTCYLKASAA	2721
422 of 494	40 mg/mL	2711	TLTCYLKASAACRAA	2725
423 of 494	40 mg/mL	2715	YLKASAACRAAKLQD	2729
424 of 494	40 mg/mL	2719	SAACRAAKLQDCTML	2733
425 of 494	40 mg/mL	2723	RAAKLQDCTMLVNAA	2737
426 of 494	10 mg/mL	2727	LQDCTMLVNAAGLVV	2741
427 of 494	20 mg/mL	2731	TMLVNAAGLVVICES	2745
428 of 494	10 mg/mL	2735	NAAGLVVICESAGTQ	2749
429 of 494	40 mg/mL	2739	LVVICESAGTQEDAA	2753
430 of 494	40 mg/mL	2743	CESAGTQEDAASLRV	2757
431 of 494	40 mg/mL	2747	GTQEDAASLRVFTEA	2761
432 of 494	40 mg/mL	2751	DAASLRVFTEAMTRY	2765
433 of 494	40 mg/mL	2755	LRVFTEAMTRYSAPP	2769
434 of 494	40 mg/mL	2759	TEAMTRYSAPPGDPP	2773
435 of 494	40 mg/mL	2763	TRYSAPPGDPPQPEY	2777
436 of 494	40 mg/mL	2767	APPGDPPQPEYDLEL	2781
437 of 494	40 mg/mL	2771	DPPQPEYDLELITSC	2785
438 of 494	40 mg/mL	2775	PEYDLELITSCSSNV	2789
439 of 494	40 mg/mL	2779	LELITSCSSNVSVAH	2793
440 of 494	40 mg/mL	2783	TSCSSNVSVAHDASG	2797
441 of 494	40 mg/mL	2787	SNVSVAHDASGKRVY	2801
442 of 494	40 mg/mL	2791	VAHDASGKRVYYLTR	2805
443 of 494	40 mg/mL	2795	ASGKRVYYLTRDPTT	2809
444 of 494	40 mg/mL	2799	RVYYLTRDPTTPLAR	2813
445 of 494	40 mg/mL	2803	LTRDPTTPLARAAWE	2817
446 of 494	40 mg/mL	2807	PTTPLARAAWETARH	2821
447 of 494	40 mg/mL	2811	LARAAWETARHTPVN	2825
448 of 494	40 mg/mL	2815	AWETARHTPVNSWLG	2829
449 of 494	40 mg/mL	2819	ARHTPVNSWLGNIIM	2833
450 of 494	40 mg/mL	2823	PVNSWLGNIIMYAPT	2837
451 of 494	40 mg/mL	2827	WLGNIIMYAPTLWAR	2841
452 of 494	40 mg/mL	2831	IIMYAPTLWARMILM	2845
453 of 494	40 mg/mL	2835	APTLWARMILMTHFF	2849
454 of 494	40 mg/mL	2839	WARMILMTHFFSILL	2853
455 of 494	40 mg/mL	2843	ILMTHFFSILLAQEQ	2857
456 of 494	40 mg/mL	2847	HFFSILLAQEQLEKA	2861
457 of 494	40 mg/mL	2851	ILLAQEQLEKALDCQ	2865
458 of 494	40 mg/mL	2855	QEQLEKALDCQIYGA	2869
459 of 494	40 mg/mL	2859	EKALDCQIYGACYSI	2873
460 of 494	40 mg/mL	2863	DCQIYGACYSIEPLD	2877
461 of 494	40 mg/mL	2867	YGACYSIEPLDLPQI	2881
462 of 494	40 mg/mL	2871	YSIEPLDLPQIIERL	2885

463 of 494	40 mg/mL	2875	PLDLPQIIERLHGLS	2889
464 of 494	40 mg/mL	2879	PQIIERLHGLSAFSL	2893
465 of 494	40 mg/mL	2883	ERLHGLSAFSLHSYS	2897
466 of 494	40 mg/mL	2887	GLSAFSLHSYSPGEI	2901
467 of 494	40 mg/mL	2891	FSLHSYSPGEINRVA	2905
468 of 494	40 mg/mL	2895	SYSPGEINRVASCLR	2909
469 of 494	40 mg/mL	2899	GEINRVASCLRKLGV	2913
470 of 494	40 mg/mL	2903	RVASCLRKLGVPPLR	2917
471 of 494	40 mg/mL	2907	CLRKLGVPPLRVWRH	2921
472 of 494	40 mg/mL	2911	LGVPPLRVWRHRARS	2925
473 of 494	40 mg/mL	2915	PLRVWRHRARSVRAR	2929
474 of 494	40 mg/mL	2919	WRHRARSVRARLLSQ	2933
475 of 494	40 mg/mL	2923	ARSVRARLLSQGGRA	2937
476 of 494	40 mg/mL	2927	RARLLSQGGRAATCG	2941
477 of 494	40 mg/mL	2931	LSQGGRAATCGKYLF	2945
478 of 494	40 mg/mL	2935	GRAATCGKYLFNWAV	2949
479 of 494	40 mg/mL	2939	TCGKYLFNWAVKTKL	2953
480 of 494	40 mg/mL	2943	YLFNWAVKTKLKLTP	2957
481 of 494	40 mg/mL	2947	WAVKTKLKLTPIPAA	2961
482 of 494	40 mg/mL	2951	TKLKLTPIPAASQLD	2965
483 of 494	40 mg/mL	2955	LTPIPAASQLDLSGW	2969
484 of 494	10 mg/mL	2959	PAASQLDLSGWFVAG	2973
485 of 494	40 mg/mL	2963	QLDLSGWFVAGYSGG	2977
486 of 494	40 mg/mL	2967	SGWFVAGYSGGDIYH	2981
487 of 494	40 mg/mL	2971	VAGYSGGDIYHSLSR	2985
488 of 494	40 mg/mL	2975	SGGDIYHSLSRARPR	2989
489 of 494	40 mg/mL	2979	IYHSLSRARPRWFML	2993
490 of 494	40 mg/mL	2983	LSRARPRWFMLCLLL	2997
491 of 494	40 mg/mL	2987	RPRWFMLCLLLLSVG	3001
492 of 494	10 mg/mL	2991	FMLCLLLLSVGVGIY	3005
493 of 494	10 mg/mL	2995	LLLLSVGVGIYLLPN	3009
494 of 494	10 mg/mL	2999	LLLSVGVGIYLLPNR	3013

APPENDIX 2-2: PEPTIDES SPANNING THE HIV IMMUNOGEN (HIV.CONSV)

Pool 1	
HIVCON 1	MEEKAFSPEVIPMFT
HIVCON 2	AFSPEVIPMFTALSE
HIVCON 3	EVIPMFTALSEGATP
HIVCON 4	MFTALSEGATPQDLN
HIVCON 5	LSEGATPQDLNTMLN
HIVCON 6	ATPQDLNTMLNTVGG
HIVCON 7	DLNTMLNTVGGHQAA
HIVCON 8	MLNTVGGHQAAMQML
HIVCON 9	VGGHQAAMQMLKDTI
HIVCON 10	KQAAMQMLKDTINEEA
HIVCON 11	KQMLKDTINEEAAEWD
HIVCON 12M	DTINEEAAEWDR
HIVCON 15	IYKRWIILGLNKIVR
HIVCON 16	WIILGLNKIVRMYSP
HIVCON 17	GLNKIVRMYSPVSIL
HIVCON 18	IVRMYSPVSILDIRQ
HIVCON 19	YSPVSILDIRQGPKE
HIVCON 20	SILDIRQGPKEPFRD
HIVCON 21	IRQGPKEPFRDYVDR
HIVCON 22M	PKEPFRDYVDRF
HIVCON 25	ARNCRAPRKKGCWKC
HIVCON 26	RAPRKKGCWKCGKEG
HIVCON 27	KKGCWKCGKEGHQMK
HIVCON 28	WKCGKEGHQMKDCTE
HIVCON 29	KEGHQMKDCTERQAN
HIVCON 30	KQMKDCTERQANFLGK
HIVCON 31	CTERQANFLGKIWPS
HIVCON 32M	KQANFLGKIWPS
Pool 2	
HIVCON 35	RWKPKMIGGIGGFIKV
HIVCON 36	MIGGIGGFIKVRQYD
HIVCON 37	IGGFIKVRQYDQILI
HIVCON 38R	IKVRQYDQILIEICG
HIVCON 39	KQYDQILIEICGHKAI
HIVCON 40	ILIEICGHKAIGTVL
HIVCON 41	ICGHKAIGTVLVGPT
HIVCON 42	KAIGTVLVGPTPVNI
HIVCON 43	TVLVGPTPVNIIGRN
HIVCON 44	GPTPVNIIGRNLLTQ
HIVCON 45	VNIIGRNLLTQIGCT
HIVCON 46	GRNLLTQIGCTLNFP
HIVCON 47	LTQIGCTLNFPISPI
HIVCON 48	GCTLNFPISPIETVP
HIVCON 49	KNFPISPIETVPVKLK
HIVCON 50	SPIETVPVKLKPGMD
HIVCON 51	TVPVKLKPGMDGPKV

HIVCON 52	KLKPGMDGPKVKQWP
HIVCON 53	GMDGPKVKQWPLTEE
HIVCON 54	PKVKQWPLTEEKIKA
HIVCON 55	KQWPLTEEKIKALVEI
HIVCON 56	TEEKIKALVEICTEM
HIVCON 57	IKALVEICTEMEKEG
HIVCON 58	VEICTEMEKEGKISK
HIVCON 59	TEMEKEGKISKIGPE
HIVCON 60	KEGKISKIGPENPYN
HIVCON 61	ISKIGPENPYNTPVF
HIVCON 62	GPENPYNTPVFAIKK
HIVCON 63	PYNTPVFAIKKKDST
HIVCON 64M	PVFAIKKKDSTKW
Pool 3	
HIVCON 67M	RKLVDFRELNKRTQ
HIVCON 68	VDFRELNKRTQDFWE
HIVCON 69	ELNKRTQDFWEVQLG
HIVCON 70	RTQDFWEVQLGIPHP
HIVCON 71	FWEVQLGIPHPAGLK
HIVCON 72	KQLGIPHPAGLKKKKS
HIVCON 73	PHPAGLKKKKSVTVL
HIVCON 74	GLKKKKSVTVLDVGD
HIVCON 75	KKSVTVLDVGDAYFS
HIVCON 76	TVLDVGDAYFSVPLD
HIVCON 77	VGDAYFSVPLDEGFR
HIVCON 78	YFSVPLDEGFRKYTA
HIVCON 79	PLDEGFRKYTAFTIP
HIVCON 80	GFRKYTAFTIPSINN
HIVCON 81	YTAFTIPSINNETPG
HIVCON 82	TIPSINNETPGIRYQ
HIVCON 83	INNETPGIRYQYNVL
HIVCON 84	TPGIRYQYNVLPQGW
HIVCON 85	RYQYNVLPQGWKGSP
HIVCON 86	KNVLPQGWKGSPAIFQ
HIVCON 87	KQGWKGSPAIFQSSMT
HIVCON 88	GSPAIFQSSMTKILE
HIVCON 89	IFQSSMTKILEPFRA
HIVCON 90	SMTKILEPFRAQNPE
HIVCON 91	ILEPFRAQNPEIVIY
HIVCON 92	FRAQNPEIVIYQYMD
HIVCON 93	NPEIVIYQYMDDLYV
HIVCON 94	VIYQYMDDLYVGSDL
HIVCON 95	YMDDLYVGSDLEIGQ
HIVCON 96M	LYVGSDLEIGQHR
Pool 4	
HIVCON 99M	MENRWQVMIVWQVD
HIVCON 100	RWQVMIVWQVDRMRI
HIVCON 101	MIVWQVDRMRIRTWK
HIVCON 102	QVDRMRIRTWKSLVK

HIVCON 103M	MRIRTWKSLVKHH
HIVCON 106M	LTEEAELELAENRE
HIVCON 107	EAELELAENREILKD
HIVCON 108	ELAENREILKDPVHG
HIVCON 109	KNREILKDPVHGVYYD
HIVCON 110	LKDPVHGVYYDPSKD
HIVCON 111	VHGVYYDPSKDLIAE
HIVCON 112M	YYDPSKDLIAEIQ
HIVCON 115M	YWQATWIPEWEFVN
HIVCON 116	ATWIPEWEFVNTPPL
HIVCON 117	PEWEFVNTPPLVKLW
HIVCON 118	FVNTPPLVKLWYQLE
HIVCON 119M	PPLVKLWYQLEK
HIVCON 122M	NVTENFNMWKNDMVD
HIVCON 123	KNFNMWKNDMVDQMHE
HIVCON 124	WKNDMVDQMHEDIIS
HIVCON 125	MVDQMHEDIISLWDQ
HIVCON 126	MHEDIISLWDQSLKP
HIVCON 127	IISLWDQSLKPCVKL
HIVCON 128M	WDQSLKPCVKLTP
Pool 5	
HIVCON 131M	WVPAHKGIGGNEQV
HIVCON 132	AHKGIGGNEQVDKLV
HIVCON 133	IGGNEQVDKLVSQGI
HIVCON 134	EQVDKLVSQGIRKVL
HIVCON 135	KLVSQGIRKVLFLDG
HIVCON 136	SQGIRKVLFLDGIDKA
HIVCON 137M	KVLFLDGIDKAQ
HIVCON 140	AKEIVASCDKCQLKG
HIVCON 141	VASCDKCQLKGEAMH
HIVCON 142	DKCQLKGEAMHGQVD
HIVCON 143	LKGEAMHGQVDCSPG
HIVCON 144	AMHGQVDCSPGIWQL
HIVCON 145	GQVDCSPGIWQLDCTH
HIVCON 146	SPGIWQLDCTHLEGK
HIVCON 147	WQLDCTHLEGKVILV
HIVCON 148	CTHLEGKVILVAVHV
HIVCON 149	EGKVILVAVHVASGY
HIVCON 150	ILVAVHVASGYIEAE
HIVCON 151	VHVASGYIEAEVIPA
HIVCON 152	SGYIEAEVIPAETGQ
HIVCON 153	EAEVIPAETGQETAY
HIVCON 154	IPAETGQETAYFLLK
HIVCON 155M	TGQETAYFLLKLA
HIVCON 158M	MNKELKKIIGQVRD
HIVCON 159	ELKKIIGQVRDQAEH
HIVCON 160	IIGQVRDQAEHLKTA
HIVCON 161	VRDQAEHLKTAVQMA
HIVCON 162	AEHLKTAVQMAVFIH

HIVCON 163	KTAVQMAVFIHNFKR
HIVCON 164	VQMAVFIHNFKRKGGI
HIVCON 165	FIHNFKRKGGIGGYS
Pool 6	
HIVCON 166	FKRKGGIGGYSAGER
HIVCON 167M	GGIGGYSAGERI
HIVCON 170	WKGPAKLLWKGEGAV
HIVCON 171	AKLLWKGEGAVVIQD
HIVCON 172	WKGEGAVVIQDNSDI
HIVCON 173	GAVVIQDNSDIKVVP
HIVCON 174	IQDNSDIKVVPRRKA
HIVCON 175	SDIKVVPRRKAKIIR
HIVCON 176	VVPRRKAKIIRDYGK
HIVCON 177	RKAKIIRDYGKQMAG
HIVCON 178	IIRDYGKQMAGADCV
HIVCON 181M	FLGAAGSTMGAA
HIVCON 182	LGAAGSTMGAASMTL
HIVCON 183	GSTMGAASMTLTVQA
HIVCON 184	GAASMTLTVQARQLL
HIVCON 185	MTLTVQARQLLSGIV
HIVCON 186	VQARQLLSGIVQQQN
HIVCON 187	RQLLSGIVQQQNNLLR
HIVCON 188	GIVQQQNNLLRAIEA
HIVCON 189	VQQQNNLLRAIEAQQHL
HIVCON 190	LLRAIEAQQHLLQLT
HIVCON 191	IEAQQHLLQLTVWGI
HIVCON 192M	QHLLQLTVWGIKQ