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Novel adenoviral vectored vaccines and the implications of viral diversity in therapeutic strategies against Hepatitis C Virus infection

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Dedication

At the beginning of my DPhil, a wise friend took me aside and said,

"This will be totally different to studying for your Medical Degree, or even your Physician's exams. Those are like being put in the starting blocks of an Olympic swimming pool and told you will have to swim the length of a marathon as fast as you can before you can get out. A DPhil is like being dropped in the middle of the ocean with no idea of where the shore is and being told to find it."

Fortunately I have had several guiding lights to help me navigate these years. My supervisor Ellie Barnes is inspirational as a woman, a clinician scientist and energetic cyclist and mountaineer. She has become a friend I will value for life, who has generously shared her time, her intellect and her irreverent wit with me. One day I hope to convince her that adverbs have a place in scientific literature! Paul Klenerman has been awe-inspiring as my co-supervisor. His capacity to conceptualise incredibly complex ideas and reduce them to one simple sentence or diagram is amazing. I will never forget Paul's rock solid support in face of everything from visa problems to freezer melt downs. He also has the unusual gift of managing to be everywhere at once, whilst appearing to have all the time in the world. I am yet to hear him say he is too busy or raise his voice!

I thank my colleagues at the John Radcliffe Hospital; Dr John Halliday, Research nurses Lizzie Stafford and Denise O'Donnell, as well as the Klenerman and Barnes group, especially Tony Brown, Leo Swadling and Abby Harrison. At the WIMM, Mao Salio kindly shared samples and her expertise on dendritic cells with me. I also thank the patients and volunteers who have taken part in these studies; none of this work would have been possible without their good will.

The people I have met and close friends I have made while in Oxford have made my DPhil experience a joy. I have been especially lucky to have met and married the most warm, generous hearted and multi-talented man- Jay. His enthusiasm, optimism and willingness to help in whatever way possible have been invaluable, especially during the seemingly endless write up period. These traits are clearly shared by my mother-in-law Jo who did not hesitate to give up her holiday time this Summer, to offer her assistance.

Making the transition from clinician to clinician-scientist has been challenging, but ultimately exciting and rewarding. I look forward to continuing my quest in search of the shore in the years ahead.

Abstract

Novel adenoviral vectored vaccines and the implications of viral diversity in therapeutic strategies against Hepatitis C Virus infection.

Dr Christabel Kelly, Keble College
Submitted for DPhil. in Biomedical and Clinical Sciences, Trinity Term 2013

Hepatitis C virus (HCV) is a major global pathogen estimated to infect over 170 million people worldwide. A recent study has shown that vaccination with adenoviral vectors, based on rare human and simian serotypes encoding the non-structural (NS) proteins of HCV, induces highly potent, multi-specific and durable T cell responses in healthy human volunteers. In this thesis I assess the safety and immunogenicity of these vaccines (ChAd3–NSmut and Ad6-NSmut), for the first time in HCV infected patients. This work also explores whether vaccine-induced T cell responses target *in vivo* circulating HCV antigens and common naturally occurring epitope variants.

Patients with treatment naive chronic genotype 1 HCV infection were vaccinated (i.m.) with ChAd3-NSmut and Ad6-NSmut in a heterologous prime boost schedule, either with or without current IFN and ribavirin (IFN/RBV). Epitope-specific T cell responses were defined by fine mapping using HCV peptides. Circulating viral genomic sequence was determined in vaccinated patients at baseline and at any point of viral relapse. Cross-reactivity of vaccine-induced T cell responses was determined in T cell assays, using peptides corresponding to both circulating host virus and common population HCV epitope variants. An in vitro dendritic cell /T cell priming model was used to identify possible candidates for a cross-reactive vaccine immunogen at the most immunodominant epitope, $NS3_{1406}$.

33 patients were vaccinated. Vaccination was well tolerated. At the highest vaccine dose (2.5×10^{10}vp) vaccine-induced T cell responses were detectable in 11/20 patients receiving concurrent IFN/RBV and 2/4 patients receiving vaccination alone. In total 14 antigenic targets were identified, 2 of which have not previously been described. However, T cell responses were of lower magnitude and more narrowly focused than those observed in healthy volunteers vaccinated with the same regimen.

Analysis of viral sequence showed that in many cases vaccine-induced T cells did not target the circulating virus. At the most immunodominant epitope ($NS3_{1406}$), T cells induced by vaccination failed to target common circulating genotype 1 HCV variants. An *in vitro* model suggested that in order to target all genotype 1 sequences at this epitope, it would be necessary to insert both a genotype 1a and 1b version of this epitope into a vaccine immunogen.

Vaccination with adenoviral vectors induces T cell responses in patients with chronic HCV infection, however immune responses are attenuated compared with healthy volunteers. Ultimately a successful therapeutic or prophylactic vaccine strategy will rely on inducing responses that target conserved or cross-reactive epitopes.

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Abbreviations

Ab	Antibody
Ad6	Human Adenovirus 6
Ad6-NSmut	Replication defective human adenovirus encoding an immunogen representing the non-structural region of the HCV genome, with a mutation in the RNA polymerase.
AE	Adverse event
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
ChAd3	Chimpanzee Adenovirus 3
ChAd3-NSmut	Replication defective Chimpanzee Adenovirus encoding an immunogen representing the non-structural region of the HCV genome, with a mutation in the RNA polymerase.
ConA	Concavalin A
DC	Dendritic Cell
DMSO	Dimethyl sulfoxide
ELISpot	Enzyme Linked ImmunoSpot
FACS	Fluorescence-activated cell sorter
FBC	Full Blood Count
FCS	Foetal calf serum
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICS	Intracellular cytokine staining
IFN	Interferon
IFN-α	Interferon alpha
IFN/RBV	Pegylated Interferon and ribavirin
IFNλ3	Interferon Lambda 3
IM	Intramuscular
IVDU	Intravenous drug user
LPS	Lipopolysaccharide
MSM	Men who have sex with men
MVA	Modified Vaccinia Ankara
nAb	Neutralising antibody
NK	Natural killer cell
PBS	Phosphatase buffer saline
PCR	Polymerase chain reaction
PEG-IFNa	Pegylated Interferon Alpha
pfu	plaque forming units
PMA	Phorbal 12-myristate 13-acetate
Pt	patient
RBV	Ribavirin
SAE	Serious Adverse Event
SFC	Spot forming colonies
SVR	Sustained Virological Response
vp	Virus Particles

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1 Introduction: Hepatitis C Virus and the rationale for vaccination

1.1 Hepatitis C Virus Infection is a significant global problem

Hepatitis C virus is a major global pathogen, infecting approximately 180 million people world wide, with 3-4 million people newly infected every year (Muhlberger et al. 2009) In the UK, prevalence is approximately 0.5% of the population, similar to Australia, Northern Europe and Canada, although in some immigrant communities in the UK it is considerably higher. For example, in a recent study of immigrants born in Pakistan, approximately 2% of people were infected with chronic HCV (Costella, 2008). This reflects a higher prevalence in developing countries across the globe, with figures between 5% and 10% reported in Africa, Latin America and Central and South-East Asia (Shepard, Finelli and Alter 2005). Many in these areas have limited access to expensive antiviral therapies and complex treatments for chronic liver disease such as transplantation. Furthermore, it is likely that the true prevalence of infection is underestimated, given that primary infection is usually asymptomatic and often occurs in socioeconomic groups less likely to seek health care.

The genotype of HCV infection also differs across the globe. HCV is divided into related, but genetically distinct genotypes (discussed further in Section 1.4.2 Variability of the HCV genome). Genotypes vary in distribution between geographical areas and amongst risk groups and are further divided into over 100 subtypes (1a, b, c etc) (Simmonds et al. 2005). In Europe genotype 1a and 1b are the most commonly identified types, while those infected via IVDU more frequently carry genotype 3a. The UK is unusual in that there is a also a large

group of genotype 3 infected patients, representing approximately half of all HCV infections, making this an important genotype to consider for the local effectiveness of any vaccination or treatment strategy (Costella, 2008). In the USA and UK, genotype 1a accounts for over 50% infections, whereas in Japan, genotype 1b is the most common. Central Africa and Egypt also have high levels of genotype 1, but also have extremely high levels of genotype 4 infection. In Asia, genotypes 3 and 6 are common. Clearly, such global diversity poses a challenge for any prophylactic vaccine or treatment strategy.

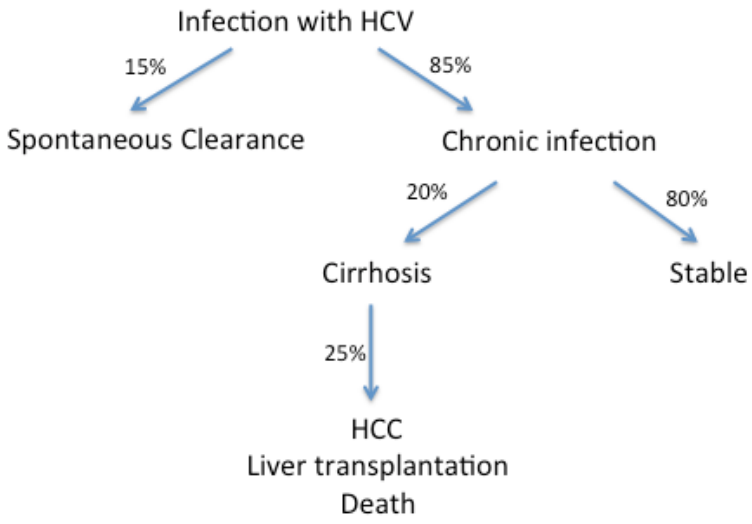
1.2 What is the natural history of HCV infection?

HCV is a blood borne virus, most commonly spread in developed countries through the sharing of needles and associated equipment such as during IV drug use (IVDU) and tattooing. Approximately 40% of IV drug users (IVDUs) in the UK are infected with HCV, with approximately half of these becoming infected with HCV within 3 years of initiating IVDU (Hope et al. 2012). Unlike HIV, sexual transmission is not thought to be a means of HCV transmission, apart from in a subset of HIV positive men who have sex with men. Iatrogenic transmission during blood product administration was also responsible for infections prior to the discovery of the virus in 1989. This has caused notable outbreaks following Anti-D immunoglobulin administration to women in Ireland and the former East Germany (Kenny-Walsh 1999; Takaki et al. 2000).

Following acute infection, approximately 20% of patients spontaneously clear the virus, while the remainder go on to develop chronic infection (Figure 1-1). Approximately 20% of those with chronic infection will progress to liver cirrhosis, which brings with it serious complications, including a 1-4% annual

risk of liver carcinoma (Seeff 2002). Across the developed world, HCV is estimated to be responsible for between 30-60% of liver cirrhosis and primary liver malignancies (Lauer and Walker 2001). In the UK, end stage liver disease and HCV related HCC carry an annual mortality of 25% each and are a leading cause of end stage liver disease and liver transplantation, despite the provision of universal free healthcare.

Figure 1-1 Natural History of HCV Infection



Statistics from National Institute of Clinical Excellence (NICE) UK (http://www.nice.org.uk/newsroom/pressreleases/BoceprevirAndTelaprevirForCHCGuidance.jsp accessed 20/5/13)

The significant morbidity and mortality associated with chronic HCV infection are predicted to rise considerably over the coming decades in developed countries, when individuals infected during the high incidence era of the 1980s and 1990s, develop progressive liver disease (Costella 2008). In the UK, hospital admissions and deaths from HCV related liver disease and HCC have more than tripled between 1998 and 2012, despite the availability of antiviral therapy during that time (Health Protection Agency report 2012 http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317135237219 accessed 23/5/13).

Furthermore, costs of treatment and care are predicted to escalate over the coming year making a prophylactic vaccine and readily available affordable treatments a pressing necessity for this disease.

1.3 What is the current treatment for HCV infection?

The goals of treatment for HCV are

(i) Clearance of the virus from infected patients, to prevent the progression of liver disease and its complications. This is determined by measuring an undetectable level of virus (viral load) in the patient's plasma 6 months after the end of treatment.

(ii) Prevention of transmission of infection to others

Until recently, gold standard treatment for HCV was weekly pegylated interferon alpha injected subcutaneously, with daily oral ribavirin (IFN/RBV) (1000mg daily for patients <75kg or 1200mg daily for those >75kg). Unfortunately, this treatment is prolonged (24-48 weeks), expensive (approximately £11,000) and fraught with side effects, which often result in early cessation of treatment (NICE http://guidance.nice.org.uk/TA200, accessed 20/5/2013). Furthermore, response rates to this combination are poor: cure rates or Sustained Virological Response (SVR) (see Table 1-1) are only 40-50% in genotype 1 infection, although are higher in genotypes 2 and 3 (75-85%).

Table 1-1 Terminology used to define virological response to therapy for HCV

Term	Definition
Rapid Viroloical Response (RVR)	Undetectable HCV RNA by PCR after **4 weeks** of treatment
Early Virological Response (EVR)	Undetectable viral load after **12 weeks** of treatment
Sustained Virological Response (SVR)	Undetectable viral load **6 months** after the end of treatment

Direct Acting Antivirals (DAAs) are now changing the landscape of therapy for HCV. The two DAAs currently in clinical use are the protease inhibitors (PIs) boceprevir and telaprevir, administered for between 12 and 44 weeks in conjunction with IFN/RBV. These PIs inhibit the NS3/4A serine protease, essential for viral replication and increase SVR rates both in treatment naïve individuals and in those who have been previously unsuccessfully treated with IFN/RBV (Table 1-2). However, a significant proportion of patients remain untreatable with these drugs. They are not suitable for patients who are intolerant of IFN/RBV or for those with non-genotype 1 infection. Their effectiveness in patients with cirrhosis is still under evaluation. Furthermore these PIS add a significant cost and side effect burden to standard therapy; the estimated additional cost of 12 weeks of boceprevir or telaprevir is £8400 and £22,398 respectively (http://www.nice.org.uk/newsroom/pressreleases/BoceprevirAndTelaprevirForCHCGuidance.jsp accessed 20/5/13).

Table 1-2 Response rates to Protease Inhbitors boceptrivir and telaprevir in different patient groups infected with HCV genotype 1

Patient response to previous treatment	Definition	Response Rate to PIs (%)
Treatment Naïve	Never treated for HCV	67-75[1]
Prior relapsers	Undetectable viral load at end of treatment, but positive viral load by 24 weeks after end of treatment (non-SVR)	69-88[2]
Prior partial responders	$2\log_{10}$ drop in viral load by week 12 of treatment, but positive viral load by end of treatment	40-59[2]
Prior null responders	Did not achieve a $2\log_{10}$ drop in viral load by week 12 of treatment	23-38[3]

1. Poordad et al. 2011, Jacobson et al. 2011
2. McHutichson et al. 2010, Zeuzem et al. 2011, Bacon et al. 2011
3. McHutichson 2010 et al., Zeuzem et al. 2011. Boceprivir has not been studied in this group.

Other DAAs are under development. This year saw the release of the first Phase III data on polymerase inhibitiors and interferon free regimes. Licensing of some of these drugs is expected in the coming year. DAAs in development include:

(i) NS5B polymerase inhibitors such as sofosbuvir (Lawitz et al. 2013)

(ii) NS5A inhibitors such as ledipasvir or daclatsavir (Pol et al. 2012) and

(iii) Cyclophilin inhibitors (Flisiak et al. 2011).

Preliminary trials suggest that interferon free or at least interferon sparing regimens are realistic goals in the near future. A recent non-inferiority trial using the NS5B inhibitor sofosbuvir plus ribavirin without interferon in genotype 2/3 infected patients, demonstrated a 97% and 56% SVR rate in genotype 2 and 3 patients respectively. This was compared with 78% and 63% SVR rates with conventional IFN/RBV alone (Lawitz et al. 2013). Whilst these results for genotype 2 are encouraging, DAAs have had limited success against genotype 3 infection and these drugs are yet to be trialed against many of the non-genotype 1 infections common in developing countries. Therefore, although

more DAAs are entering the clinical arena, there will still remain a substantial group of difficult to treat patients with advanced fibrosis or cirrhosis and difficult to treat HCV genotypes or subtypes.

DAAs will also have to contend with the rapid emergence of escape mutations, which limits their efficacy when they are used as monotherapy (McHutchison et al. 2010, Hezode et al. 2009). DAAs will most likely be used in combination with each other in the future, similar to the current approach to HIV treatment, or combined with PEG-IFNα /RBV to limit this possibility. This of course adds to the potential risks of drug interactions and reintroduces the problem of the significant side effect profile of IFN. There is not yet a successful vaccine for the prevention or treatment of HCV.

1.4 Structure and function of HCV

HCV is an enveloped single-stranded positive sense RNA virus, from the family Flaviviridae, and genus Hepacivirus. HCV virions exist in the infected host as lipoviral particles in association with low and very low density lipoproteins (LDL and VLDL) (Andre et al. 2002). They gain entry into host cells by both direct viral infection of target cells and by cell to cell transmission (Timpe et al. 2008). Cell entry receptors include the LDL receptor, SRB1, CD81, claudin and occludin (Scheel et al. 2013). Viral particles are then taken up by endocytosis into the host cells where virus uncoats and uses the host machinery and its own enzymes to replicate. RNA replication takes place under the influence of the HCV RNA polymerase in association with the endoplasmic reticulum.

This process lends itself to intervention by therapeutic agents at multiple levels. Firstly cell entry can be blocked using antibodies, such as CD81 or SRB1

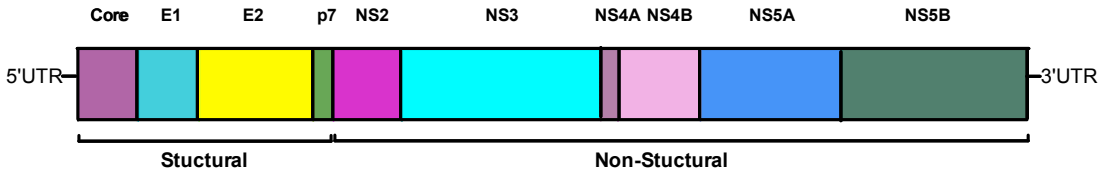
antibodies (Meuleman et al. 2008). DAAs can also interfere with the enzymes used by HCV such as polymerase inhibitors and protease inhibitors (see section 1.3 p14). It is also possible to disrupt the proteins which are necessary for viral replication such as cyclophilin inhibitors, however trials with these have been discontinued due to safety concerns including a fatal case of pancreatitis (Hopkins and Gallay, 2012). Finally T cell therapies such as a T cell vaccine can be used to target virally infected cells.

1.4.1 The HCV genome

HCV has a 9.6kb long genome is contained within an icosahedral capsid, and consists of an open reading frame, flanked by two un-translated regions (UTR) (Figure 1-2). It encodes a 3011 amino acid (a.a.) polyprotein containing structural and non-structural regulatory proteins, which is cleaved into 10 viral proteins using proteases. These are the structural proteins (core, E1, E2, p7) and the non-structural proteins (NS2, NS3 NS4A/B, NS5A/B) (Reed and Rice 2000).

The structural region comprises core proteins encoding the viral capsid, and two glycoproteins E1 and E2, which form heterodimers at the surface of the viral envelope. They are important in facilitating cell entry by binding cell receptors. The HCV envelope is the target of the host antibody response, and it may be in response to this that E2 is the most variable region of the HCV genome (Prentoe et al. 2011). The last structural protein is a small ion chanel designated p7, thought to be essential for viral particle assembly and release formation, although its precise function remains a subject of ongoing research.

Figure 1-2 Schematic representation of the HCV genome



The large black rectangle represents the 3011 amino acid long HCV polyprotein. Each section of the genome is indicated by a different colour, with the corresponding label above each protein. Proteins are drawn to scale. Structural proteins (core, E1, E2 and p7) and non-structural proteins (NS2, NS3, NS4A/B, NS5A/B) are shown. UTR=untranslated region.

The non-structural proteins NS3-NS5B contain elements important for viral replication and auto-cleavage of proteins. There are two proteases; a cystine protease (NS2-NS3) and a serine protease formed by the N-terminal of NS3 and a cofactor (NS4A). These are responsible for cleaving the remainder of the non-structural proteins. NS3 also contains a viral helicase which facilitates RNA processing. HCV also requires an RNA polymerase for replication, which is encoded by NS5B. There are two segments which have an undetermined role in viral replication (NS4B, NS5A). It is possible that NS5A is important for interferon sensitivity, containing a "interferon determining region", mutations in which has been associated with response to IFN/RBV therapy in many Japanese studies (Kohashi et al. 2006; Yen et al. 2008).

1.4.2 Variability of the HCV genome

The HCV genome pictured in Figure 1-2 is hugely variable. It is divided into genotypes, which have an identical complement of genes of similar or identical size contained within the open reading frame. However there is considerable divergence in genomic sequences both within and between the genotypes.

Genotypes differ by 31 -33% at the nucleotide level, and can be further divided into subtypes which differ by 20-25% (Simmonds et al. 2005). Within the HCV genome itself, different segments have different levels of sequence homology. The 5' UTR end and the terminal 99 bases for the 3' UTR end are the most invariant sections, and are frequently used in clinical assays for HCV genotyping, while the core protein is the next most conserved (Simmonds et al. 2005). The envelope proteins and NS2 protease are the most variable regions, with the NS3-NS5 proteins being relatively conserved.

The high variability of HCV is in part due to the NS5B RNA polymerase, which lacks proof reading capacity. This, combined with a high replication rate of up to 10^{12} virions per day (Neumann et al. 1998) results in a heterogenous group of related but distinct viral strains within the same patient, known as quasi-species. Furthermore, in countries where IVDU is the predominant mode of transmission, ongoing risk behaviour can result in superinfection from different viral sources, producing a complex viral milieu in each individual. Any vaccination strategy, whether that be prophylactic or therapeutic, must either be able to combat multiple divergent viral strains simultaneously or allow for the provision of different vaccines for different populations.

1.5 What is the innate immune response to HCV infection?

Once HCV has breached the epithelial barrier, it is immediately confronted with a host of innate immune defenses including phagocytes (macrophages and neutrophils), dendritic cells, natural killer (NK) cells and circulating proteins such as complement and defensins. These aim to control viraemia in the first hours to days of infection until the adaptive immune response is induced.

The interferon (IFN) family is the key anti-viral cytokine system employed by the infected host and is also used, in the form of exogenous IFNα, for HCV treatment (see page 14, What is the current treatment for HCV infection?). This family of cytokines forms part of the Class II cytokine group and consists of three types of interferons: Type I, II and III. Type I IFNs include IFNα and IFNß, Type II IFN is IFNγ and Type III IFNs consist of IFNλ1, IFNλ2, and IFNλ3, which are also called IL29, IL28A and IL28B respectively. A fourth Type III IFN has also just been described this year, known as IFNλ4 (Prokunina-Olsson et al. 2013).

1.5.1 Production of Interferon

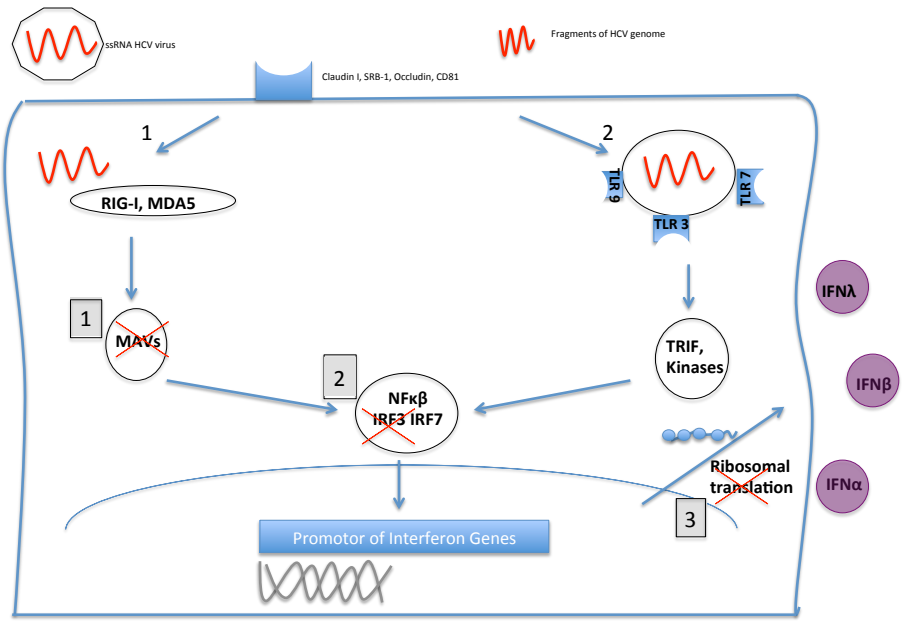
Knowledge of the precise mechanisms of IFN induction and Interferon stimulated gene (ISG) activation continues to evolve. Current understanding is that host cells detect viral RNA through two main pathways, outlined in Figure 1-3 (Iwasaki and Medzhitov 2004; Yoneyama and Fujita 2007). This results in the production of Type I and probably Type III interferons, although much less is known about the latter.

(i) **Cytosolic Pathway**: Cystosolic caspase recruitement domain (CARD) containing proteins (including retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5)) detect Pathogen Associated Molecular Patterns (PAMPs) in the HCV 5' and 3' UTRs. These bind mitochondrial antiviral signaling protein (MAVS or Cardif) which recruits kinases to RIG-I. This results in activation of NFκβ and interferon regulatory factor 3 (IRF-3). Once phosphorylated, IRF-3 translocates to the nucleus where it promotes transcription of Type I

IFNs, Interferon Stimulated Genes (ISGs), in particular ISG56 and ISG15 and chemokine ligand 5 (CCL5).

(ii) **Endosomal Toll-Like Receptor (TLR) Pathway**: Products of viral replication such as dsRNA are detected via the endosomal receptor TLR3, which initiates a downstream signaling cascade mediated by the adaptor protein TRIF.

Figure 1-3 Type I and III interferons are induced via 2 main pathways in response to HCV. HCV interferes with Interferon production.



HCV binds to cell entry receptors on hepatocytes such as claudin, occludin, CD81 and Scavenger receptor type B1 (SRB-1). Products of HCV replication or apoptosed cells are also endoctyosed. Once inside the cell, HCV is detected via either 1. The Cytosolic pathway or 2. The Endosomal Toll Like Receptor (TLR) Pathway.
Cytosolic pathway: RNA and products of viral replication are detected by the cytosolic proteins retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5). These bind mitochondrial antiviral signaling protein (MAVS or Cardif) which recruits kinases to activate NFκβ and interferon response factor 3 (IRF-3). **Endosomal TLR pathway:** TLR3 detects double stranded RNA, TLR7 single stranded RNA and TLR9 unmethylated DNA with CpG motifs. This initiates downstream signaling via TRIF (Toll-IL 1 receptor domain-containing-adaptor inducing IFNβ), MyD88 and other kinases. Both pathways leading to activation of NFκβ and IRF-3 which translocate to the nucleus and promote IFN gene transcription.
HCV attempts to prevent IFN production via 3 mechanisms: 1. The HCV protease cleaves adaptor proteins such as MAVs, necessary for downstream signalling. 2.NS3/4A cleaves the promotor IRF, rendering it incapable of translocating to the nucleus for interferon gene transcription 3. HCV inhibits translation of newly transcribed IFNs through activation of Protein Kinase R (PKR).

Whilst IFNα can be produced by all nucleated cells, IFNγ is typically only produced by lymphoid derived cells such as NK cells and T cells. IFNλ is also produced by only a limited group of cell types (Coccia et al. 2004). Plasmacytoid dendritic cells are the main produces of IFNλ, however macrophages, monocyte derived DCs and hepatocytes have all been shown to produce IFNλ.

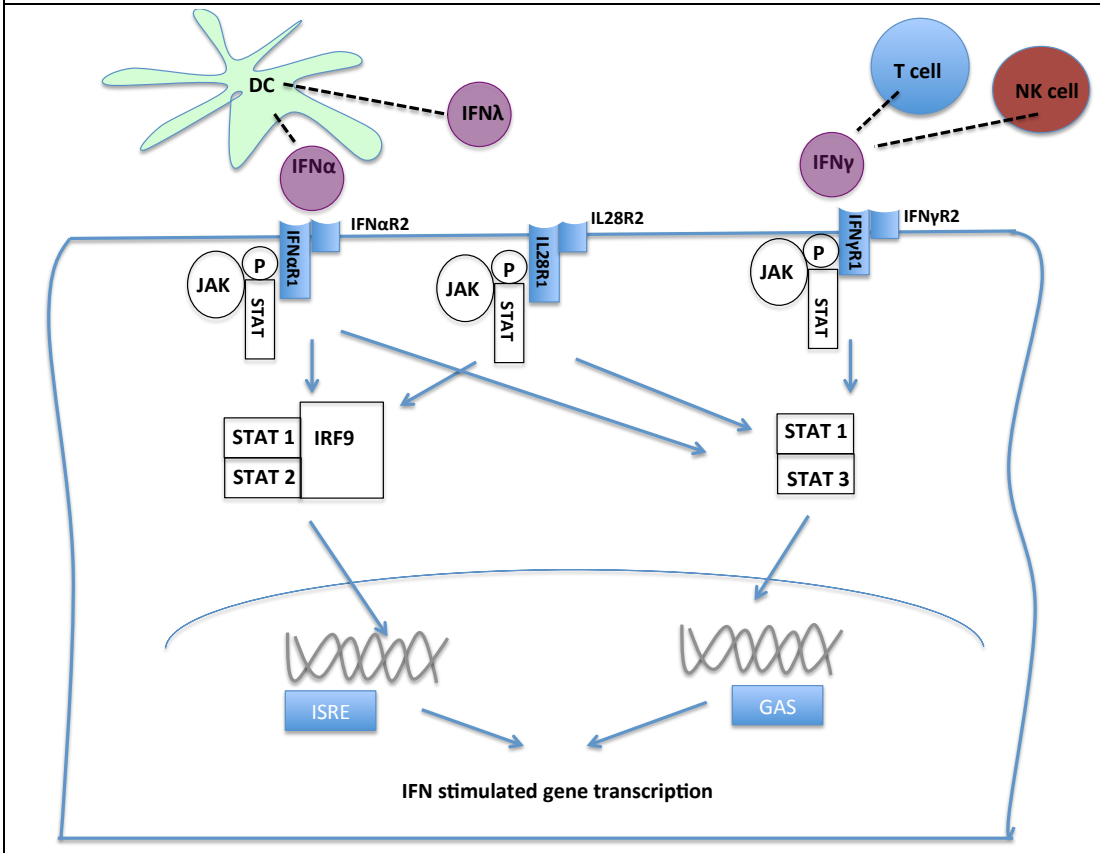
Interestingly, macrophage production of IFNλ is markedly enhanced by pre-culturing these cells with IFNα -evidence of interplay between the two pathways (Ank et al. 2006; Wolk et al. 2008).

1.5.2 Interferon Signaling

All class II cytokines are structurally related, with a shared α-helical pattern, and signal through related heterodimeric transmembrane protein receptors. However, biological activity ultimately depends on the receptor cytoplasmic domains, which are not related, and these may trigger overlapping but different biological functions. This, in addition to the pattern of receptor distribution amongst different cell types means that the different interferons (IFNs) are functionally distinct, although clearly their properties, especially those of Type I and Type III IFNs overlap. In particular, both are produced in response to viral infection and have anti-viral and immunomodulatory properties.

All class II cytokines signal through the signal transducers and activators of transcription (STATs) and janus tyrosine kinases (JAK) as shown in Figure 1-4. However, within this signaling cascade there are subtle differences between the interferons that are not fully understood. All class II cytokines appear to activate STAT1 and STAT3 and bind to IFN-gamma activated sites (GAS) to various extents in the cell nucleus, resulting in the transcription of interferon stimulated genes (ISGs). However, in addition, Type I IFNs and IFNλs also activate interferon stimulated response elements (ISRE) through the activation of STAT2 in association with STAT1 and IRF9, which again results in the production of interferon stimulated genes (Figure 1-4) (Dumoutier et al. 2004; Renauld et al. 2003).

Figure 1-4 The signaling pathways of Interferons



Dendritic Cells (DCs) make Type I and III IFNs. T cells and Natural Killer (NK) cells make Type II IFN. IFNs bind their specific receptor (R1), leading to a conformation change and recruitment of the second receptor chain. Jak1 and Tyk 2 transphophyrolate the receptor chains and form phosphyrlated tyrosine peptides on the receptor chain (P). STAT proteins bind and are phosphorylated. They can then form homo or heterodimers and migrate to the nucleus to bind gene regulatory elements (Gamma Activated Sequences or GAS). In the case of Type I and Type III IFNs they can also bind Interferon Regulatory Factor 9 (IRF9) in the cytoplasm and then migrate to the nucleus to bind Interferon Stimulated Regulatory Elements (ISREs) to regulate gene transcription.

Ultimately the interferons produce their specific effects through the stimulation of specific ISGs. Microarray experiments have shown that hundreds of ISGs are produced in response to IFN stimulation and the full spectrum of these genes for the different interferon types has not been defined. For a discussion of the differences between Type I and Type III IFNs see a review by Kelly C. et al., 2011. For the purposes of this project, the next section outlines the better

characterised downstream effects of Type I IFNs. Many of the effector genes and targets however, remain the subjects of active research.

1.5.3 Immunomodulatory effects of Type I IFNs

Type I IFNs enhance the proliferation and maturation of dendritic cells, and increase expression of HLA-II and the co-stimulatory molecules CD80 and CD86 (Luft et al. 1998). This is important, not only for antigen presentation in the innate immune system, but also for the role of DCs as priming agents of T cells in the adaptive immune system. Type I IFNs further enhance T cell priming by facilitating a process known as 'cross-priming', where $CD8^+$ T cells can be primed against exogenous antigens (Le Bon et al. 2003). Normally, exogenous antigens are presented on HLA Class II molecules and stimulate $CD4^+$ T cell responses. However dendritic cells, with the right co-stimulation, can process these endocytosed antigens so that they enter the cytosolic antigen presentation pathway and are presented on HLA-Class I molecules, thereby stimulating $CD8^+$ T cells.

IFNα also causes upregulation of HLA-I molecules (Epperson et al. 1992) and upregulates NK cell function (Bolitho et al. 2007).

1.5.4 Anti-Viral effects of Type I IFNs

Type I interferons also have direct antiviral effects involving a number of different downstream signaling pathways, described below.

(i) **ISG-15**. This prominent ISG is a ubiquitin homologue, which binds to and activates a large number of effector proteins (Loeb and Haas, 1992). There are over 158 target proteins identified thus far. These

include signaling components of the IFN pathway like JAK1 and RIG-I, leading to a positive feedback loop and further induction of the IFN system. They also include antiviral effector proteins such as MxA, PKR and RNAseL (Zhao et al. 2005). Importantly, activation by ISG-15 is reversible, and can be blocked or inhibited by certain viruses as discussed further below.

(ii) **PKR**: This is a constitutively expressed enzyme, which is further induced by Type I IFNs and activated by viral RNA. It inhibits translation of cellular and viral proteins (including HCV proteins), through the inactivation of eukaryotic translation initiation factor 2α (Sadler et al. 2008).

(iii) **OAS**: Like PKR, this is constitutively expressed in all cells, and is upregulated by type I IFNs. It is activated by viral RNA, and then binds to RNAseL and cleaves viral and cellular RNA

(iv) **Mx-GTPases**: These are located near the smooth endoplasmic reticulum in the cytoplasm and trap viral components to prevent replication and transcription (Accola et al. 2002).

1.5.5 HCV subverts the innate immune response

As described above, an effective innate anti-viral response centres around the capacity of immune cells to produce interferons. It is therefore no surprise that HCV has developed a myriad of strategies to evade host interferon responses, both by inhibiting IFN production and by interfering with its downstream effects.

Inhibition of IFN production

HCV prevents the production of Type I IFNs via three well characterised pathways, shown in Figure 1-3, p23. Firstly, the serine protease NS3/4A cleaves the adaptor proteins TRIF and MAVs, necessary for downstream signaling via IRF3 and NFκβ for IFNα production (Li et al. 2005; Meylan et al. 2005). It has also been shown in HCV infected Huh7 C5B2-3 cells, that NS3/4A cleaves IRF-3 itself, preventing nuclear translocation of this critical promotor (Foy et al. 2003). Interestingly, when these cells are treated with IFNα2b, this pathway is restored, illustrating a potential mechanism for the therapeutic effect of exogenous IFNα in patients. Recently translation silencing via enhanced PKR activity has also been described (Arnaud et al. 2010). This group demonstrated that whilst HCV infection of Huh7.25 cells decreased IFNβ mRNA production as described above, there was a greater decrease in translation, pointing to a novel mechanism of translational silencing in HCV's fight against interferons.

Interference with the actions of Interferon

HCV is also able to interfere with the downstream signaling of IFNs, the normal sequence of which is outlined in Figure 1-4, p25.

(i) **STAT1**: HCV core protein can bind STAT1 and subsequently inhibit downstream IFN signaling (Lin et al. 2006). STAT1 is also inhibited through HCV induced upregulation of protein phosphatase2A (PP2A), which is an enzyme with key roles in cell homeostasis (Duong et al. 2004). HCV core protein further interferes with this by binding

STAT1 and STAT 2. This prevents nuclear translocation of these critical signaling molecules (Mélen et al. 2004).

(ii) **PKR**: NS5A binds interferon induced PKR and prevents its activation by dsRNA (Pflugheber et al. 2002). The HCV E2 protein also inhibits PKR by means of an shared sequence with part of PKR and one of its downstream targets, the translation initiator eIF2α . This blocks PKR's inhibitory effect on cell growth and protein production (Taylor et al. 1999).

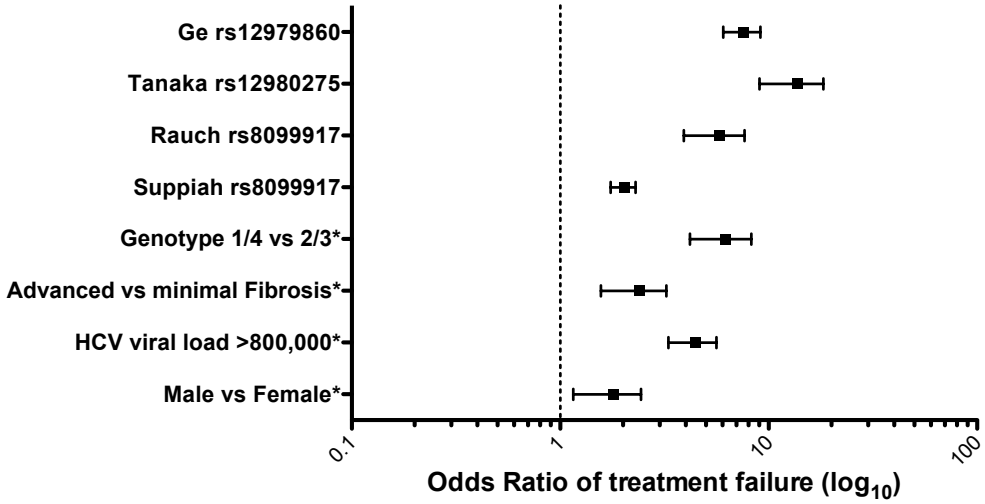
(iii) **OAS**: There is indirect evience that NS5A binds OAS and inhibits its action (Taguchi et al. 2004),

1.5.6 Polymorphisms in innate immune genes are associated with the outcome of HCV infection

Historically, most of the scientific community's efforts to understand the HCV-IFN interplay have focused on Type I interferons. However recently, Type III interferons have created a storm in the HCV arena due to research using genome wide association studies, which discovered a striking association between spontaneous clearance of HCV infection and single nucleotide polymorphisms (SNPs) linked to the gene encoding IFNλ3 (Rauch et al. 2010). The same association was seen with treatment response (SVR) by 4 groups in close succession (Ge et al. 2009; Rauch et al. 2010; Suppiah et al. 2009; Tanaka et al. 2009), demonstrating that the IFNλ pathway plays a key role in the response to IFNα as well as in natural host immunity. Indeed, in the study by Ge and others, this association was stronger than any previously identified predictor of response such as gender, baseline viral load and degree of fibrosis (see Figure

1-5). The mechanism behind how changes in these SNPs result in different immunological outcomes is unclear, although work just recently presented suggests the key may lie in a newly discovered member of this cytokine family called IFNλ4, the focus of much ongoing research endeavour (Prokunina-Olsson et al. 2013).

Figure 1-5 Odds Ratio of risk factors associated with HCV treatment failure



Risk factors for HCV treatment failure with IFN/RBV are listed on the *y axis*, IFNλ3 linked single nucleotide polymorphisms (rs12979860, rs12980275 and rs809917) are shown with the corresponding authors from published genome wide association studies Ge et al 2009; Tanaka et al. 2009; Rauch et al. 2010; Suppiah et al. 2009. *Derived from Rauch et al. 2010. Adapted from Kelly C et al. 2011.

1.6 What is the role of T cells in HCV infection?

Human and animal studies suggest that a key determinant of viral clearance in acute HCV infection is a broad and robust T cell response (Cooper et al. 1999; Lechner et al. 2000; Neumann-Haefelin et al. 2010; Thimme et al. 2001). In chimpanzee models, depletion of $CD8^+$ T cells abrogates protective immunity from previous HCV infection, while $CD4^+$ depletion prevents effective development of $CD8^+$ responses, resulting in viral persistence (Grakoui et al. 2003). In humans, the importance of HLA type has been demonstrated in single source outbreak studies. In these studies, women were infected with the same

inoculum of HCV from contaminated anti-D immunoglobulin. Approximately half of the women developed persistent infection whilst the remainder cleared the virus spontaneously, with HLA-A*03, B*27 and Cw*01 occurring more frequently in spontaneous clearers (39.5%, 14% and 9.3% respectively) than those who developed chronic infection (19.1&, 2.1% and 1.4% respectively; $p<0.005$) (McKiernan et al. 2004).

Other observational studies demonstrate a temporal relationship between the appearance of $CD8^+$ T cells and the control of viraemia in human and chimpanzee subjects (Shoukry et al. 2004; Thimme et al. 2001). In 5 subjects who developed HCV infection following a needlestick injury, 3 subjects mounted a detectable T cell response. In these individuals, a decrease in viral load was kinetically associated with the appearance of T cell responses, with the one person who spontaneously cleared the virus showing the most vigorous $CD8^+$ responses at the time of viral clearance (Thimme et al. 2001).

In contrast, the hallmark of chronic HCV infection is a narrowly focused, functionally impaired T cell response, despite the presence of circulating antigen (Lechner, Gruener et al. 2000; Missale et al. 2012). Furthermore, those T cells which can be detected are often functionally impaired, with high levels of exhaustion markers like PD-1s (Radziewicz et al. 2008), low levels of CD127 (Urbani et al. 2006) and poor effector function, demonstrated by downregulated activation markers such as HLA-DR and CD38 (Lechner et al. 2000). Indeed mouse models of chronic viral infection with lymphocytic choriomengitis virus (LCMV) suggest that constant antigenic stimulation may impair T cell function by leading to a state of functional exhaustion or anergy (Wherry, Blattman and

Ahmed 2005; Youngblood et al. 2011). These studies also suggested that it might be possible to rescue T cell responses under certain conditions. When LCMV infected mice were vaccinated with a recombinant vaccinia virus, those with lower viral loads responded better to vaccination than those with high viral loads, suggesting that attempts to induce or recover T cells in chronic HCV infection may be best performed in the context of a low viral load (Wherry, Blattman and Ahmed, 2005).

1.7 What is the role of HCV specific antibodies in HCV infection?

Antibodies are clearly an important component of the adaptive host immune response, and if induced effectively could result in sterilising immunity to HCV. This is an advantage over a T cell vaccine, which can only target cells which have already been infected. Antibodies have also been shown in some individuals to correlate with viral clearance in acute infection, and to drive selection pressure in the hypervariable E2 region (Dowd et al. 2009).

Studies in chimpanzees show that it is possible to generate neutralising HCV antibodies, which are cross-reactive to other genotypes *in vitro* (Meunier et al. 2011). However human data suggests that such antibodies can only be generated in a minority of healthy volunteers (Law et al. 2013). Furthermore, E1 and E2 proteins are technically difficult to produce in large quantities (personal correspondence Dr Antonella Folgori). Attempts at therapeutic antibody based vaccination in the post liver transplant context have been disappointing, resulting in small, transient reductions in viral load (less than $\log_{10}$) (Galun et al. 2007).

Nontheless, although conventional circulating antibodies to HCV are readily detectable within 1-3 months of infection, their presence in high titres during chronic infection demonstrates they have a limited ability to prevent or eliminate chronic disease (Bartosch et al. 2003). This is in contrast to many other viruses where vaccine-induced antibodies provide lasting sterilising immunity, for example in the cases of Hepatitis B Virus, Measles virus or even the RNA virus Rubella virus.

In order to be successful, it is likely that effective antibody based immunity to HCV will hinge upon the identification of broadly neutralising antibodies. Although there is much still to explore on this subject, a conserved antibody target has been identified in the hypervariable region. Antibodies to this region have resulted in protection from chronic HCV infection in chimeric mouse models (Law et al. 2008).

In the case of HCV it is plausible that a vaccine which does not induce antibodies could be effective against HCV. In high risk cohorts, T cell responses to HCV have been detected in the absence of seroconversion (Post et al. 2004 and Abdelwahab et al. 2012). Furthermore, studies in chimpanzees provide evidence that T cells can be induced and provide protection from chronic HCV infection without inducing HCV antibodies (Folgori et al. 2006). Thus, although ideally both antibody and T cell responses would be induced through vaccination, this may not be a pre-requisite for success.

1.8 HCV subverts the adaptive immune response

Reasons for the weak and poorly focused T cell response observed in HCV infection include host factors and intrinsic properties of HCV itself. An active

immune response also relies on the host's ability to detect the presence of pathogenic material and target appropriate virally infected cells. HCV is a hepatotropic virus although the precise mechanism which causes liver homing is not clear. The liver itself is an immunotolerant organ, being one of the few organs in the body which does not require HLA tissue matching for transplantation (Poli et al. 1998), thus once hepatocytes are infected, this relatively immune quiescent environment may permit HCV to establish chronic infection. Furthermore, it has been suggested that antigen presentation by non-professional APCs such as hepatocytes may lead to defective activation of the adaptive immune response (Zinkernagel 1996). This has been shown with other chronic human viruses, for example human papilloma virus, which when sequestered in keratinocytes fails to activate an effective T cell response until antigens are released from lysed cells and taken up by professional APCS (Tindle and Frazer 1994). Although viral antigens are expressed on the surface of hepatocytes on Class I molecules, the lack of $CD4^+$ activation (through the paucity of HLA Class II presenting cells) and the lack of co-stimulation from APCs, may partially account for the weak responses.

HCV has also developed the capacity to hide from immune cells via direct cell to cell transfer within the liver (Timpe et al. 2008) and by masking potential antibody targets by glycosylating envelope proteins on the surface of viral particles (Helle et al. 2007; Gremion and Cerny 2005).

Even if a T cell or antibody response is triggered, finding an effective, durable target in a rapidly mutating virus is a difficult task. As described previously, this rapidly mutating virus results in a swarm of divergent quasispecies being

simulataneously present in the host. These quasispecies are driven by immune pressure, with mutations in the HCV viral sequence almost 17 times more likely to occur within a T cell epitope region than without (Cox et al. 2005). Some of these, such as mutations at the HLA-B27 epitope ARMILMTHF, come at a cost to viral fitness, or produce variants which are cross-recognised by T cells (Dazert et al. 2009). However others allow viral escape through a variety of potential mechanisms, including (i) decreased recognition by T cells of either the epitope or its flanking region (ii) decreased antigen binding (iii) impaired antigen processing or presentation.

Nevertheless, there is clearly more at play here than simply the virus' ability to hide or escape from the adaptive immune system. It has been shown in chimpanzee models that $CD4^+$ epitopes are more highly conserved than $CD8^+$ epitopes and rarely escape (Fuller et al. 2010). In this work, whilst 75% of $CD8^+$ epitopes showed evidence of escape over a four year period, only 18% of $CD4^+$ epitopes did. Furthermore, despite ongoing viral sequence variation, T cells tend not to develop new epitope specificities after the first 6 months of infection (Burke and Cox 2010). Even when T cells are induced, they tend to be poorly functional, as described above (p31). T regulatory cells are enriched in the livers of patients with chronic HCV and can actively suppress $CD4^+$ and $CD8^+$ T cells (Ward et al. 2007). The lack of functional T cells implies that functional T cell development is arrested within the first year of HCV infection which begs the question: Can T cells be induced de novo, or existing responses recovered, through therapeutic vaccination of chronically infected individuals?

1.8.1 Rationale for a Therapeutic T cell vaccine

Data from human and chimpanzee studies suggest that inducing protective (although not sterilising) immunity is a realistic goal in the treatment and prevention of chronic HCV infection. Epidemiological studies have shown that in cohorts of injecting drug users, individuals have long periods of aviraemia despite ongoing risk behaviour (Villano et al. 1999) and analysis of an HCV mono-infected cohort demonstrated that individuals with previously documented infection were 12 times more likely to spontaneously clear the virus when re-infected (Mehta et al. 2002). A vaccination strategy, which mimics these natural antiviral responses, could provide protection from chronic disease and could enhance responses to treatment or reduce viral load before treatment, which would prove a major benefit to patients.

Evidence of T cell immunogenicity in healthy volunteers

Recent work by Barnes and others has demonstrated that vigorous polyfunctional T cell responses can be generated in healthy volunteers, vaccinated with adenoviral vectors expressing proteins from the non-structural HCV genome, NS3-NS5 (NSmut) (Barnes et al. 2012). In this Phase I study (called HCV001), healthy volunteers were vaccinated with escalating doses of two replication deficient adenoviral vectors: human adenovirus 6 (Ad6) and chimpanzee adenovirus 3 (ChAd3). Both vectors were safe up to a maximal dose of 7.5×10^{10}vp, and primed and boosted polyfunctional, durable CD4+ and CD8+ T cell responses. These produced IL-2, IFNγ and TNFα and recognised a broad range of T cell epitopes. Vaccine-induced T cells were detectable up to 48 weeks following vaccination and retained proliferative capacity, perforin, granzyme A

and B production. The magnitude and quality of the T cells induced was in marked contrast to those T cells described in chronic infection. If such potent T cell responses could be induced in patients with chronic HCV infection, it is possible that therapeutic vaccination could lower viral load and improve response to therapy.

The scientific rationale for a therapeutic T cell vaccine can be summarised by the following:

(i) An effective anti-HCV T cell response can control and induce spontaneous resolution of acute HCV infection. This observation suggests that T cells may be a valid therapeutic mechanism for the treatment of HCV.

(ii) Administering antigen to a peripheral site (muscle) in a novel form, such as via an adenoviral vector, could allow T cell priming outside the liver and also prevent the HCV mediated suppression of T cell responses

(iii) Animal models of chronic viral infection suggest that administering a therapeutic vaccine in the context of a low viral load may generate T cell responses more effectively than when viral loads are high. Hence vaccinations will be administered both alone and with IFNα therapy

(iv) The fact that unlike HIV, HCV can be cleared either spontaneously or with exogenous IFN/RBV therapy suggests that HCV may be more amenable to immuno-therapeutics than some other chronic viral infections.

(v) Data from healthy volunteers indicates that vigorous anti-HCV specific T cell responses can be generated through vaccination.

1.8.2 Requirements of an ideal HCV T cell vaccine

(i) **Safe**: the vaccine must be well tolerated, with minimal local and systemic adverse events, especially if administered to patients with pre-existing liver disease.

(ii) **Immunogenic:** Ability to generate long-lasting high quality T cells with proliferative and killing capacity.

(iii) **Cross-reactivity against different HCV viral variants**: Ability to generate HCV specific responses to a large range of circulating HCV viral subtypes and quasispecies, ideally across multiple genotypes.

(iv) **Applicable to a global population:** Effective in populations with multiple different HLA types. Easily administered and stored with either no cold chain required or stability in the face of an inconsistent cold chain.

1.9 HCV vaccine strategies to date

A variety of HCV vaccination strategies have been evaluated over the past decade in animal and human studies. Most of these vaccination strategies have been well tolerated, although they have been tested only in small groups and few have produced convincing evidence of immunogenicity. The major vaccine strategies in human clinical studies are summarised below.

Vaccine Type	Description	Advantages	Disadvantages
Recombinant Protein	HCV protein is cloned into bacteria, yeast or mammalian cells and injected	- can combine antigens of interest into one immunogen - technically simple	- predominantly stimulate $CD4^+$ T cell and antibody responses rather than $CD8^+$ T cells - poorly immunogenic
Peptide	Peptides, often with adjuvants, are injected in emulsion	- produce targeted immune responses to relevant antigens - easy to assess immune responses	- specific to certain HLA types - poorly immunogenic
DNA	A plasmid containing the gene of interest is injected and taken up by cells which then express HCV antigens.	- can express whole genome, thus broad range of antigenic targets	- poorly immunogenic without adjuvant methods
Viral Vector	Attenuated viruses like adenoviruses or MVA viruses carry HCV genes and transduce cells which express HCV antigens	- strong priming and boosting potential of $CD4^+$ and $CD8^+$ T cells	- Immune responses to vectors may limit immunogenicity - safety concerns about live virus

1.9.1 Peptide and Protein based vaccines

The majority of HCV vaccine studies have historically used peptide or recombinant protein approaches to generate T cell or antibody responses. These have predominantly used antigens from the envelope or core region.

Peptide based vaccines directly present antigen on HLA molecules to induce T cell responses. One limitation of this technique is that such vaccines contain only a limited repertoire of antigens, thus responses broad enough for protection may not be generated. Furthermore, although weak T cell responses and antibodies have been induced using this approach, single peptides must generally be HLA matched for T cell presentation, making this approach difficult to institute at a population level.

Antigens from the HCV envelope

The first therapeutic vaccine trial for HCV used peptides from the E1 region of the virus. There was initially a suggestion of an improvement in liver histology and ALT in patients however, this was not born out in further work and this vaccine was discontinued (Nevens et al. 2003). Whilst the envelope region is an attractive antigenic target, since it the target for antibody responses, the limitations of the host humoral response to control HCV infection, in combination with the variability of this region, suggest that envelope antigens may have limited efficacy (see 1.7, "What is the role of HCV specific antibodies in HCV infection?", p32).

However recently, data reported from a joint venture with industry (Novartis) and multiple academic sites, suggest that it may be possible to generate broadly neutralising antibodies in humans, albeit only in a small number of individuals (Law et al. 2013). In this study of vaccination with envelope glycoproteins from a genotype 1a virus, 5/13 vaccinated volunteers had sufficient antibody titres to neutralise 50% virus in *in vitro* cell culture assays (Law et al 2013). For the first time, this group showed that serum generated by genotype 1a glycoproteins could neutralise virus from other genotypes. Further studies are planned with this vaccine.

Antigens from the core region

Other peptide vaccines have included core peptides. The core region is highly conserved from a sequence perspective and thus potentially immunogenic across a broad range of genotypes. However, most known epitopes in this region

are $CD4^+$ responses, with few $CD8^+$ responses identified. Core responses have also been identified in many patients with chronic infection in the absence of viral control (Semmo et al. 2005), suggesting that the functional significance of these responses may be limited. A Japanese study designed a vaccine program based on a conserved region of the core protein (Yutani et al. 2009). IFNγ responses were measured by ELISA so are difficult to compare to other studies which have traditionally used IFNγ-ELISpot assays. Nonetheless, 17/26 patients developed detectable IFNγ levels by ELISA compared with 11 patients at baseline (range 0-780 pg/ml, no background subtracted). Only 2 patients had a decrease in viral load of 1 log during the 12 weeks vaccination follow up period.

Another peptide based vaccine, called IC41, used 5 peptides selected from core, NS3 and NS4 in HLA-A2 positive HCV infected patients (Klade et al. 2012). This vaccine was well tolerated but resulted in only a peak median IFN-γ ELISpot response of 40×10^6PBMC and a very small transient reduction in HCV viral load (0.47 log change in 34/53 patients), which does not carry clinical significance.

A larger protein vaccine, using a yeast vector to deliver a fusion protein of core/NS3/NS5B has also resulted in weak immunogenicity (GI-5005). Interim data from a Phase II study reported improved viral kinetics with vaccination + IFN/RBV, however published peer reviewed results are still awaited (Habersetzer et al. 2009).

An interesting variation on this method was attempted by an Australian group (Gowans et al. 2010) and has also been trialled in HIV (Garcia et al. 2013). Gowans and others harvested autologous dendritic cells from patients, and attempted to prime them in vitro with antigens of interest before re-infusing

them into patients. As a proof of concept study this was interesting: all six patients in the study generated HCV specific T cell responses, however this was short lived and had no effect on viral load as well as being a very labour intensive and expensive process, not easily applicable to clinical practice.

1.9.2 DNA and vector vaccines

There are two DNA vaccine studies and two viral vector strategies with published results available. The DNA vaccine trials were small, involving 15 or fewer patients. In one trial, antibody and weak T cell responses were generated in 11/15 patients, however these were not sustained and no change in viral load was observed (Alvarez-Lajonchere et al. 2009). In the second study, 2/4 patients receiving high dose vaccination developed weak T cell responses and reductions in viral load, however all patients had low viral loads prior to enrollment in the study (<800,000IU/ml) (Sallberg et al. 2008). No further development of these DNA studies into Phase II trials has been reported.

Recently results from two viral vectored vaccines have been published, one using an MVA vector in patients with HCV and one from our group in Oxford using a heterologous prime/boost vaccination strategy using adenoviral vectors in healthy volunteers (Habersetzer et al. 2011; Barnes et al. 2012). Viral vectors appear to be the most promising mechanism for inducing T cell responses out of all the strategies tried. Habersetzer and others demonstrated the generation of T cell responses in response to MVA vaccination, using HCV proteins NS3, NS4 and NS5B (TG4040). However, most of these responses were found in individuals with pre-existing T cell responses and resulted in only small and transient decreases in viral load at early stages of treatment. Furthermore there was no

analysis of viral sequence in responders, making it difficult to establish whether T cell responses were to relevant viral strains. Results from a Phase II study using this vaccine in conjunction with standard treatment (IFN/RBV) were recently presented in oral form (Wedemeyer, EASL, 2013) which showed no statistically significant difference in SVR rates between vaccinated patients and controls and only weak T cell responses (<200SFC/10^6 PBMC).

In Oxford, the Barnes/Klenerman group in collaboration with Okairos (an Italian pharmaceutical company) have been trialing a number of viral vectored HCV vaccines. The first study (HCV001) was introduced above (page 36 Rationale for a Therapeutic T cell vaccine) and demonstrated safe induction of potent T cell responses in healthy volunteers. Studies in progress from our group are listed in the next section, which summarises the Oxford HCV vaccine program (Table 1-3).

The work in this project builds on the adenoviral vector vaccine strategy from Barnes and others in healthy individuals to determine whether such responses can also be generated in the context of chronic HCV infection.

1.9.3 The HCV Vaccine Program in Oxford, UK

There are 3 HCV vaccine studies based in Oxford, which aim to test HCV vaccines in healthy volunteers and in patients with chronic HCV infection. All use the same HCV immunogen called 'NSmut' (see page 45 The Immunogen: NSmut below), delivered in a viral vector prime/boost regimen. The type of vector and the timing of vaccinations differ between the studies. These studies are outlined in Table 1-3. This thesis focuses on study HCV002, which is introduced in detail in the section to follow. Vaccine responses are compared with healthy

volunteers from HCV001, so a synopsis of the HCV001 study design is also listed below (Table 1-4) and some samples from studies HCV001 and HCV003 are used for further immunological assays in Chapter 4 (see Materials and Methods for more information).

Table 1-3 The Oxford HCV Vaccine Studies

Study	Participants	Vaccines	Immunogen
HCV001	Healthy Volunteers (n=36)	Priming with ChAd3 or Ad6, boosting with the heterologous vector	NSmut
HCV002	Patients with chronic HCV infection (n=32)	Priming vaccination with ChAd3, boosting vaccination with Ad6	NSmut
HCV003	Healthy volunteers (n=10) and patients with chronic HCV infection (n=13)	Priming vaccination with ChAd3, boosting vaccination with Modified Vaccinia Ankara (MVA)	NSmut

Table 1-4 HCV001 Study design

Group	Number	Prime (week)	Dose (vp)	Boost	Dose (vp)
1	4	Ad6 (0,4)	5×10^{8}	ChAd3 (24)	2.5×10^{10}
2	4	Ad6 (0,4)	5×10^{9}	ChAd3 (24)	2.5×10^{10}
3	6	Ad6 (0,4)	2.5×10^{10}	ChAd3 (24)	2.5×10^{10}
5	4	ChAd3 (0,4)	5×10^{8}	Ad6 (24)	2.5×10^{10}
6	4	ChAd3 (0,4)	5×10^{9}	Ad6 (24)	2.5×10^{10}
7	5	ChAd3 (0,4)	2.5×10^{10}	Ad6 (24)	2.5×10^{10}
9	5	Ad6 (0)	2.5×10^{10}	ChAd3 (8)	2.5×10^{10}
10	5	ChAd3 (0)	2.5×10^{10}	Ad6 (8)	2.5×10^{10}
11	4	ChAd3 (0)	7.5×10^{10}	Ad6 (8)	7.5×10^{10}

Number=number of healthy volunteers in each group. ChAd3= ChAd3-NSmut, Ad6= Ad6-NSmut.

1.10 HCV002TV: therapeutic vaccination in Genotype 1 HCV infection

1.10.1 Study Overview

This is a Phase I open label, dose escalation study to assess the safety and immunogenicity of ChAd3-NSmut/Ad6-NSmut vaccination in patients with

chronic Hepatitis C infection. An overview of the study design is presented in Table 1-5.

Table 1-5 Overview of Study Groups in HCV002TV

Arm	Patients (no.)	ChAd3 Prime (week)	ChAd3 second prime (week)	Ad6 Boost (week)	Vaccination dose (vp)	Follow up (months)
Patients receiving vaccination and concurrent IFNα/RBV therapy where week 0= start of therapy						
A1	2	14	-	24	5×10^{8}	18
A2	2	14	-	24	5×10^{9}	18
A3	6	14	-	24	2.5×10^{10}	18
A4	6	2	-	12	2.5×10^{10}	18
A5	4	14	18	28	2.5×10^{10}	18
A6	4	2	6	16	2.5×10^{10}	18
Patients receiving vaccination alone where week 0= start of trial						
B1	2	4	-	14	5×10^{8}	9
B2	2	4	-	14	5×10^{9}	9
B3	4	4	-	14	2.5×10^{10}	9

1.10.2 The Immunogen: NSmut

The choice of a vaccine immunogen is critical to the success of any vaccine. An immunogen must be first and foremost safe for use in humans. It also must be immunogenic and produce, in this case, T cell responses to antigens of interest contained within the immunogen. From a technical perspective, it must also be easily producible and deliverable within the chosen vaccination strategy, which for HCV002TV are the adenoviral vectors ChAd3 and Ad6, discussed below.

The non-structural (NS) portion of the HCV genome is a target for multiple HCV specific T cell responses and an attractive candidate for a T cell vaccine immunogen. According to registered epitopes on the Los Alamos Database, more than 90 $CD4^{+}$ and 70 $CD8^{+}$ T cell epitopes have been identified in this region (Yusim et al. 2005). Furthermore HLA types associated with protection from

HCV infection have been linked to epitopes which lie within the NS portion of the genome, such as the protective HLA-B27 allele, linked to the NS5B epitope ARMILMTHF (Dazert et al. 2009, McKiernan et al. 2004) and the protective HLA-A*03 linked epitope $NS3_{1080\text{-}1088}$ TVYHGAGTK (Fitzmaurice et al. 2011). Data from patients also suggest an important role for epitopes mapped to the NS region in inducing HCV specific T cells during acute infection (Bowen and Walker 2005; Lauer et al. 2002; Spada et al. 2004).

The NS region is also relatively conserved between genotypes, unlike for example the hypervariable envelope region. It represents a large portion of the HCV genome (approxiately two thirds), spanning 5 different proteins: NS3, NS4a, NS4b, NS5a and NS5b. This allows for the inclusion of multiple potential epitope targets, including longer $CD4^+$ epitopes, many of which are likely to be, as yet, undescribed. Such a broad inclusive approach to selecting an immunogen also takes into account the presence of multiple HLA types in the population, which will be capable of binding to a wide variety of different T cell epitopes. Furthermore, including a large section of the HCV genome increases the likelihood that at least some regions of the immunogen will match the sequence of different circulating viral strains.

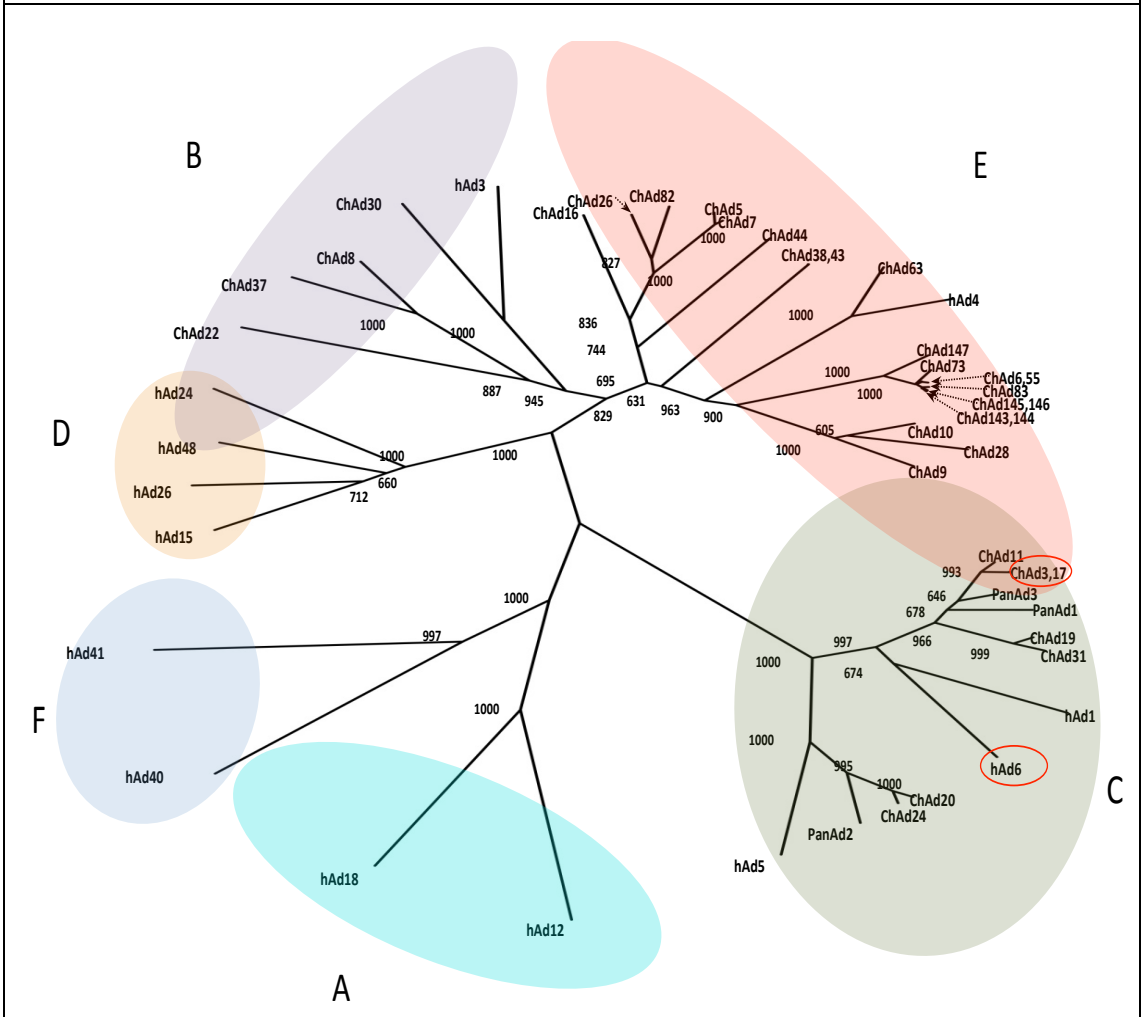
The NS insert chosen was a genotype 1b strain. Genotype 1 is the most common HCV infecting strain and represents 45% UK infections (Health Protection Authority Report UK 2012 http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317135237219 accessed 23/5/13) as well as a significant proportion of infections in the US and Japan. For an additional safety measure, a mutation was introduced in the RNA polymerase,

replacing 3 amino acids GlyAspAsp, at position 1711-1713, with the inactive AlaAlaGly (Capone, Zampaglione et al. 2006).

1.10.3 Adenoviruses as vectors

The two adenoviruses used in this trial are the human adenovirus Ad6 and the chimpanzee adenovirus ChAd3. In its natural form, Ad6 causes a respiratory illness (the common cold) in humans. ChAd3 is not known to cause illness in humans. They are distinct, but phylogenetically related viruses (Figure 1-6). For the purposes of vaccination, these viruses are modified so that they can neither replicate nor cause respiratory disease. They are replication deficient due to the deletion of the E1 protein and can only replicate in cells expressing this protein (such as the PER.C6 Cell line). The E3 gene, used by the adenovirus to combat host immune defenses, is also deleted in these vectors.

Figure 1-6 Phylogenetic tree of human and chimpanzee adenoviruses



Human adenovirus 6 and Chimpanzee adenovirus 3 (ringed in red) are both part of the Group C adenovirus family. Reproduced with permission from Colloca et al. 2012

These vectors were chosen based on a significant body of pre-clinical and clinical data demonstrating their superiority over other existing adenoviruses used in clinical trials. Prior to their appearance as vaccine candidates, adenoviral vectors have been used for many years as vehicles for gene delivery in gene therapy trials, first appearing in human trials for cystic fibrosis in 1994 (Crystal et al. 1994; Lee, Matthews and Blair 2005), as well being studied extensively in cancer immunotherapy (for a review see Larocca and Schlom 2011). Some of

the problems with using these vectors as for gene therapy are also relevant to the vaccine field. These include safety considerations, pre-existing immunity to the vectors themselves and vaccine-induced immune responses to the vectors. Other problems faced by gene therapy may prove advantageous in the context of vaccination. These include the short duration of expression of the transgene, reported to be up to 40 days in one cystic fibrosis study and local inflammation; potentially beneficial to vaccination at a peripheral site (Lee, Matthews and Blair 2005).

There is extensive experience in the HIV and malaria vaccine fields using genetically modified viral vectors to deliver antigens of interest for vaccination. Chimpanzee adenovirus ChAd63 has been trialed in multiple malaria vaccine studies since 2007, with an excellent safety and immunogenicity profile (O'Hara et. al. 2012). Trials using this and other viral vectors are ongoing within the Hill group in Oxford, UK (Ogwang et al. 2013; Sheehy et al. 2012). Human adenovirus 5 (Ad5) has been used in multiple HIV vaccines, with promising results in Macaques (Shiver et al. 2002). This was followed by two Phase II trials in humans in South Africa (Gray et al. 2011) and in the Americas/Euroasia (the STEP trial). However, these trials were stopped early due to futility. Interim analysis of the STEP trial suggested that vaccination of uncircumcised Ad5 seropositive individuals was associated with an increased risk of HIV infection, (discussed further below on page 56 Risks to the individual) (Buchbinder et al. 2008). In May 2013 a further Ad5 based HIV study from the US (National Institute of Allergy and Infectious Diseases) was also suspended due to futility. There also initially appeared to be a trend towards more HIV infections in the

vaccinated group (clinical trials no. NCT00865566 http://www.clinicaltrials.gov/ct2/show/NCT00865566?term=hvtn+505&rank=1 accessed 28/5/13), however a published statement from the NIH is awaited, following a summit in September of this year on the status of Ad5-HIV vaccines (http://www.niaid.nih.gov/topics/HIVAIDS/Research/vaccines/Pages/adDraftAgenda.aspx accessed 23/10/13).

One problem with using the potent adenoviral vector Ad5 is pre-existing immunity, which can limit immunogenicity or even be a cause for safety concern as observed in the STEP study mentioned above. Other less common adenoviral serotypes, including related but genetically distinct chimpanzee adenoviruses could conceivably circumvent this problem. Our collaborators at Okairos have previously constructed different replication deficient adenoviral vectors encoding different HCV proteins (Capone, Meola et al. 2006). This work in mice and non-human primates demonstrated that Ad6 -NSmut expressing vectors produced equivalent immunogenicity to Ad5- NSmut expressing vectors. Furthermore, human adenovirus 6 (Ad6) has a lower pre-existing seroprevalence than Ad5; 38% versus 48% of healthy Europeans respectively, and the neutralising antibodies detected were not cross-reactive between Ad5 and Ad6. When nAb titres >200 were assessed, pre-existing immunity to Ad6 was even less common- 11/93 people versus 31/93 for Ad5.

Further work in this group identified ChAd3 as a potent immunogenic vector when encoding HIV antigens, in mice and Macaques, again with low levels of pre-existing antibodies (<10% of humans tested had nAb titres >200) (Colloca et al. 2012). The immunogenicity of these two vectors was verified in human

participants in study HCV001 (see page 36 Rationale for a Therapeutic T cell vaccine). For this reason the ChAd3 and Ad6 vectors were chosen for ongoing clinical development in study HCV002TV.

1.10.4 Rationale for the Oxford therapeutic T cell trial design:

1.10.4.1 Arm A receives vaccination with concurrent IFN/RBV

T cell responses may be generated more effectively when viral loads are low. This has been observed, not only in mouse models of vaccination in the context of chronic infection, but also when outcomes from patients with chronic HCV are assessed. In resolved HCV infection, CD4+ and CD8+ T cells are frequently detectable for decades, whereas in chronic HCV infection, T cell responses are difficult to identify in peripheral blood (Lauer et al. 2004). Therefore, one arm of this study (Arm A of study HCV002TV) was designed to include patients receiving concurrent IFN/RBV to suppress viral load for up to 14 weeks prior to vaccination (see study design Table 2-2, p63).

The relationship between IFN therapy and HCV specific T cell responses is however, not clearly defined. The immunomodulatory and antiviral effects of IFN therapy may enhance or suppress T cell responses. This has the potential to alter or confound T cell responses to therapeutic vaccination. Efforts to explore the effect of IFN therapy on T cell responses have resulted in disparate results between studies. The use of different assays, different peptide sets and the detection of weak or historical T cell responses are likely to have contributed to conflicting data. Whilst it has been shown that the magnitude of T cell response correlates with SVR in some studies, others have shown that this is not the case

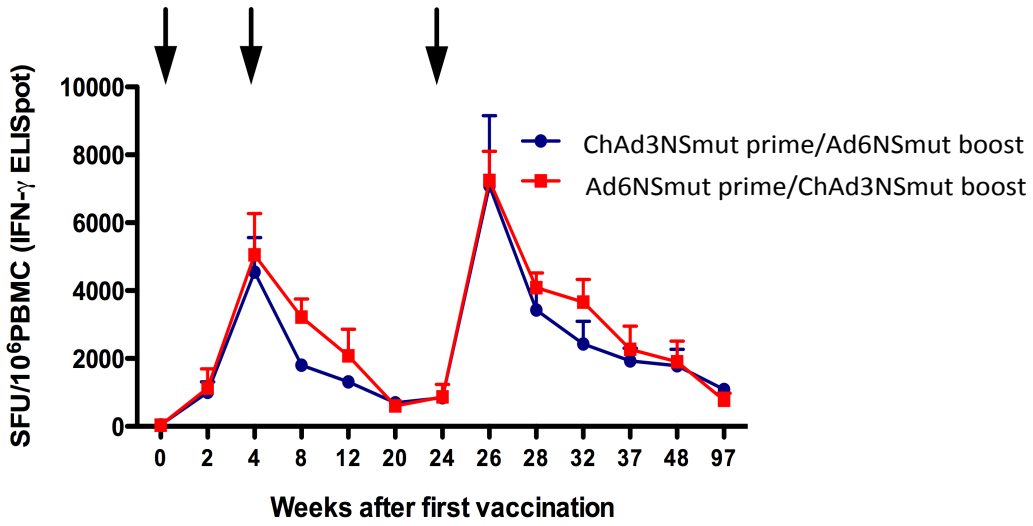
(Kamal et al. 2002; Barnes et al. 2009). In order to address this, a control arm, of patients who receive IFN/RBV alone, without vaccination has been included in this study, to detect fluctuations in T cell responses throughout treatment. In addition, a separate arm (Arm B) has been included, in which patients receive vaccination alone, to observe whether the immunomodulatory effects of IFN may be detrimental to vaccine responses.

1.10.4.2 Both Arms receive heterologous prime/boost vaccination

A heterologous prime/boost strategy, whereby different vaccine vectors are used for priming and boosting, is designed to allow for repeated administration of an immunogen without inducing immune responses to the vector which might inhibit vaccine efficacy. Furthermore it is likely that some vectors are better for priming and others for boosting.

Okairos have previously shown that heterologous prime/boost vaccination using adenoviral vectors from different serotypes can successfully induce T cells against NSmut in rhesus macaques (Fattori et al. 2006). The same group has tested the vectors proposed in HCV002TV (Ad6 and ChAd3) in rhesus macaques and shown that these vectors induce potent, long lasting T cell responses (Folgori personal communication, Figure 1-7).

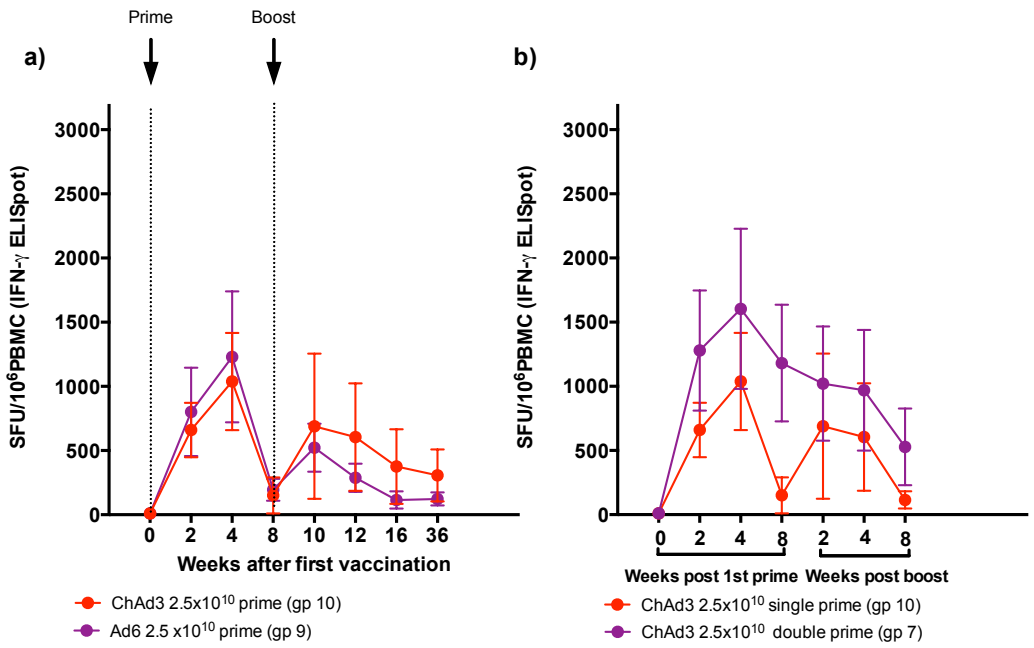
Figure 1-7 Vaccination of Macaques using ChAd3-NSmut and Ad6-NSmut



Two vaccination regimens are shown: Double prime with ChAd3-NSmut followed by Ad6 boost (blue) and double prime with Ad6-NSmut followed by ChAd3-NSmut boost (red). Magnitude of T cell response by IFN-γ ELISpot assays is shown (y axis). Vaccinations are indicated by arrows. Data reproduced with permission from Dr A. Folgori, Okairos Italy.

Human studies also support this prime/boost regimen. In HCV001, healthy volunteers received a priming vaccination with either ChAd3 or Ad6 followed by a boosting vaccination of an alternative vector. As described above, this was a highly immunogenic regimen with an excellent safety profile. Analysis of the different regimens used suggested that the optimal order of vaccination was priming with ChAd3 and boosting with Ad6, which achieved higher peak boosting responses than the alternative regimen (Ad6 prime/ChAd3 boost) (Figure 1-8a). This study also suggested that double priming, that is, two injections of the priming vector, could lead to higher and more sustained IFNγ T cell responses compared with a single priming injection (Figure 1-8b). For this reason, single and double priming regimens were included in the trial design for HCV002TV.

Figure 1-8 Vaccination with a heterologous prime/boost regimen in healthy volunteers from study HCV001.



a) Healthy volunteers received priming vaccination with ChAd3-NSmut (red) or Ad6-NSmut (purple) followed by heterologous boosting vaccination**. b)** Volunteers received a single prime (red) or double prime (purple) vaccination with ChAd3-NSmut, followed by boosting with Ad6-NSmut. Group means are shown with s.e.m.. Data adapted from study HCV001 (Barnes et al. 2012) with corresponding author's permission.

1.10.5 Route of injection

Intramuscular injection was chosen as the route of vaccine administration. Local and systemic side effects using this method of vaccination were well tolerated by healthy volunteers in HCV001, supporting its continuation into HCV002TV. It is assumed that no co-infection with wild type adenoviruses could occur at this site (see page 59 Risks to the community). This is also a conventional and familiar method of vaccine administration for clinicians and patients.

1.10.6 Dose of ChAd3 and Ad6

Studies of acute HCV infection suggest that the stronger and broader the T cell response, the more likely it is that acute HCV infection will result in clearance (Spada et al. 2004; Folgori et al. 2006). Therefore the magnitude, breadth and

perhaps duration of T cell response can be considered as immune correlates for protection against chronic HCV infection.

The necessary and sufficient dose to achieve T cell responses in HCV002TV was determined using data from both non-human primate and human studies. When rhesus macaques were injected with adenoviral vectors containing the NSmut antigen at doses of 10^8-10^{11} vp, all animals receiving 10^{10} vp or higher produced a T cell response. Vaccination with the lower dose (10^8 vp) was less efficient, with only 4 out of 5 macaques responding (Capone, Meola et al. 2006). There is also a large body of safety and immunogenicity data from HCV001, where healthy people were vaccinated with the same vaccines proposed for HCV002TV. The dose range tested in HCV001 included all doses proposed for HCV002TV (5×10^8, 5×10^9 and 2.5×10^{10} vp), as well as an additional extra high dose (7.5×10^{10} vp) (Table 1-4 p44). This study demonstrated that vaccination was well tolerated at all doses, although adverse events were more common as dose increased. At 2.5×10^{10} vp all individuals mounted T cell responses however there appeared to be a ceiling effect on the magnitude of T cells which could be induced, where no increased immunogenicity was observed with the extra high dose 7.5×10^{10} vp. This suggested that while the lower doses would be necessary for assessment of safety, the optimal dose for HCV002TV would be 2.5×10^{10} vp. The largest numbers of patients were hence enrolled in those groups to obtain the most relevant safety and efficacy data.

1.10.7 Potential Safety Risks

1.10.7.1 Risks to the individual

Although there is a considerable amount of safety data regarding vaccination with ChAd3-NSmut/Ad6 boost in healthy volunteers, these vaccines have never been administered to patients with concurrent HCV infection. Additionally, some of these patients face the additional complication of concurrent therapy with IFN/RBV which have their own side effect profiles. Safety is therefore a primary endpoint in this study.

Phlebotomy: The volume of blood drawn throughout the study should not compromise patients. It is less than the recommend amount acceptable via blood donations in the UK which is 1527ml/year for women or 1880ml/year for men (http://www.blood.co.uk/giving-blood/faqs/ accessed 29/5/13). In Arm A of the study patients will be treated with IFN/RBV concurrently, and can develop anaemia while taking ribavirin. This will be monitored closely, and the dose of ribavirin adjusted accordingly. Volumes of blood drawn in each group are outlined below (Table 1-6). It is possible that venepuncture may be challenging in some individuals, particularly those with a history of IV drug use (IVDU).

Table 1-6 Blood volumes taken during the study period, per patient group in HCV002TV

Group	Total blood volume (ml)	Time period
A1-A3	1123	18 months
A4	1214	18 months
A5	1273	18 months
A6	1272	18 months
B1-B3	1208	9 months

Local reactions to most vaccinations are common due to local immune responses. These include erythema, local pain, itch and swelling. Based on the safety data from the preceding HCV001 trial, where the same vaccines at the same dose have been administered to healthy volunteers, it is likely that local reactions will be mild in severity and of short duration (Barnes et al. 2012).

Systemic responses to vaccination such as arthralgia, myalgia and feeling feverish are also likely, however again it is possible to predict that these will be mild or moderate, as in HCV001. Those patients receiving concurrent IFN/RBV (Arm A) may also suffer from these side effects as a consequences of IFN treatment, thus the effect of these additional symptoms may be more burdensome in this group. Any vaccine also carries the risk of generalised immune mediated reactions such as Guillain-Barré syndrome, a demyelinating polyneuropathy which can result in paralysis or death. Allergy or anaphylaxis to the vaccine or to substances used in its composition could also occur, although this is a rare complication of vaccination and all vaccines are administered in hospital by adequately trained clinical staff.

There exists a theoretical possibility that T cells generated by vaccination could target HCV infected hepatocytes, cause hepatocyte death and ultimately, if severe, hepatic necrosis and liver failure. This would be reflected both in a clinical deterioration and in a rise of liver enzymes, in particular Alanine Transaminase (ALT). This is carefully monitored during the study and participants excluded from vaccination in the presence of pre-existing liver inflammation if ALT and bilirubin are raised; >3 Upper limited of normal (ULN) and >2ULN respectively.

There is strong evidence that natural T cell mediated immune responses to HCV infection do not result in the above scenario, even where HCV is spontaneously 'cleared' by the immune system. In acute infection, there is usually an elevation in liver enzymes from baseline, however this rarely results in fulminant liver failure, probably because only a minority of hepatocytes are infected with HCV, estimated at 7-20% (Liang et al. 2009). It has been shown in studies of acute infection that although ALT rises after HCV infection (to a mean level of 360 IU/ml, 9 times the ULN) it does not correlate with HCV viral load or viral persistence (Cox et al. 2005). Furthermore, this same group showed that it is possible to spontaneously clear HCV without a significant rise in ALT at all.

Studies from non-human primates further support the concept that HCV-specific T cell responses can control HCV viraemia without hepatotoxicity. 4/5 chimpanzees vaccinated with an HCV candidate vaccine developed HCV specific T cell responses which were associated with protection from acute viraemia (Folgori et al. 2006). These chimpanzees did not develop a rise in ALT when challenged with HCV, however the unvaccinated control arm did. This setting is slightly different to that in study HCV002TV, where patients are infected with HCV first, then vaccinated, however suggests that viral control is possible without significant liver inflammation. There is however, evidence that bilirubin level is linked to spontaneous clearance and that ALT flares can correlate with viral clearance in some individuals (Thomson et al. 2011). Therefore monitoring ALT levels is an important part of any therapeutic HCV trial.

It is noteworthy that there have been two severe complications from previous adenoviral vector studies. One of these involved using adenoviruses as gene

transfer vehicles to cure a metabolic defect (ornithine transcarbamylase deficiency) and tragically resulted in a fatality due to an acute inflammatory response and multi-organ failure (Raper et al. 2002). In this study however, the vectors were injected directly into the hepatic artery (rather than IM) at much higher doses than those proposed here (up to 6×10^{11}/kg).

The second serious complication arose from the STEP trial, which assessed the effectiveness of an Ad5 based HIV vaccine. In this study, vaccinated individuals with Ad5 nAbs were initially found to be at a statistically higher risk of contracting HIV than those who had not been vaccinated. Although the reasons for this are still unclear, one explanation proposed at the time, was that those volunteers who had previously been exposed to Ad5, had higher levels of vector specific T cells, thus providing more potential T cells for HIV to infect (Buchbinder et al. 2008). This is quite different to HCV, where T cells are not a target of infection. Furthermore, in the proposed model of HCV002TV, patients are already infected with HCV, although this consideration is important to consider when vaccinating healthy volunteers.

1.10.7.2 Risks to the community

Should this work lead to a successful therapeutic or prophylactic HCV vaccine there are considerable benefits to community health as well as decreased costs to the health care system. There are minimal community risks. Rendering the viral vectors replication deficient (through deletion of the E1 and E3 portion of their genome) means they cannot be replicate in the host and spread to other people in the community. There exists the unlikely possibility that they could recombine with wildtype adenoviruses and acquire E1 and E3 and become a

recombinant replication competent adenovirus. This scenario is however unlikely since the Ad6 and ChAd3 used in these vaccines are 'double deleted' (i.e. are missing both E1 and E3 genes) and such a scenario would require wild type adenoviruses to infect the same cells as those at the site of vaccination.

1.11 Aims of this work

This thesis aims to examine the host-virus interaction in HCV infection, exploring how cell mediated immunity can be altered through vaccination.

1. To determine the effect of therapeutic vaccination in patients with chronic HCV infection. I hypothesise that
    a. HCV specific T cells can be safely induced in patients with chronic HCV infection.
    b. HCV specific T cells induced by vaccination are immunologically functional, with markers of killing capacity, activation and effector memory phenotypes.
    c. HCV specific T cells can target circulating autologous virus in patients with chronic HCV.
    d. There is a relationship between viral sequence and T cell responses to vaccination.
2. To explore T cell responses to HCV epitopes in the context of the highly variable HCV population. I hypothesise that
    a. Immune responses to the vaccine immunogen will cover a wide range of circulating viral sequence variants.
    b. There is an optimal immunogen which will produce responses to a broad range of HCV viral variants.

2 Materials and Methods

2.1 Vaccination of patients with chronic HCV

2.1.1 Patient enrolment

Eligible patients were males and females with treatment naïve chronic genotype 1 HCV infection, defined as the presence of HCV RNA in two plasma samples at least 6 months apart. Patients were aged 18-65 years (inclusive) and had no clinical, biochemical or histological evidence of liver cirrhosis. Patients were excluded if they were co-infected with Hepatitis B Virus (HBV) or Human Immunodeficiency Virus (HIV) or were seropositive for Ad6 or ChAd3 viruses (titre >200). A control cohort of patients receiving IFN/RBV treatment alone was recruited first followed by recruitment of patients receiving vaccination (characteristics of enrolled patients are outlined in Table 3-3 and Table 3-4 p90). Patients were recruited at the John Radcliffe Hospital, Oxford UK and at the Queen Elizabeth Hospital in Birmingham UK according to the inclusion and exclusion criteria in Table 2-1. All patients gave written informed consent before participation, and the study was conducted according to the principles of the Declaration of Helsinki and in accordance with Good Clinical Practice.

Table 2-1 Inclusion and exclusion criteria for HCV002TV therapeutic vaccination trial

Inclusion Criteria for HCV002TV
HCV genotype 1 infection
Age 18-65 years old inclusive
In groups A1, A2, A3 and A5 patients will only be vaccinated if they have a >2 log drop in viral load at week 12 of IFN/RBV therapy.
Resident in /near the trial site for the duration of the study
Able to comply with the study requirements
For women of child bearing potential: willingness to practice effective contraception during the study and a negative pregnancy test on the day of vaccination
For men: to use barrier contraception until 3 months after the last vaccination
Written informed consent

Exclusion Criteria for HCV002TV
Participation in another research study involving an investigational product within 30 days preceding enrolment
Prior receipt of recombinant simian or human adenoviral vaccine
Clinical, biochemical, ultrasonographic or liver biopsy (histology) evidence of cirrhosis or portal hypertension
Inability to provide written informed consent
Confirmed or suspected immunosuppressive or immunodeficient state, including HIV infection; asplenia; recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the last 6 months (inhaled and topical steroids are allowed)
Patients likely to have been infected with HCV within the last 12 months
History of allergic disease or reactions likely to be exacerbated by an component of the vaccine eg Kathon
History of clinically significant contact dermatitis
Any history of anaphylaxis in response to vaccination
Pregnancy, lactation or intention to become pregnant during the study
History of cancer (except basal cell carcinoma of the skin and cervical carcinoma in situ)
Any other serious chronic illness requiring hospital specialist supervision
Suspected or known current alcohol abuse defined as >42 units per week
Suspected or known injecting drug use
Seropositive for Hepatitis B surface antigen
Seropositive for HIV
Seropositive for simian adenovirus 3 (ChAd3) titres >200 at screening
Seropositive for human adenovirus 6 (Ad6) titres >200 at screening
Temperature >38°C in the 3 days preceding vaccination
Any finding which, in the opinion of the Investigator, may either put the patient at risk or may influence the result of the study

2.2 Vaccines Ad6-NSmut and ChAd3-NSmut

The Ad6 and ChAd3 vectors are replication deficient adenoviruses, encoding the NS3-5B region of genotype 1B (based on sequence accession number M58335) (Colloca et al. 2012). Ad6 is a rare human serotype, and ChAd3 is a chimpanzee adenovirus. Construction of the HCV immunogen insert (NSmut) by Okairos has been described elsewhere (Capone et al. 2006). Vaccines were administered intramuscularly, and the trial group protocols are detailed in Table 2-2.

Table 2-2 Study Design HCV002TV

Arm	Patients (no.)	ChAd3 Prime (week)	ChAd3 second prime (week)	Ad6 Boost (week)	Vaccination dose (vp)	Follow up (months)
Patients receiving vaccination and concurrent IFNα/RBV therapy where week 0= start of therapy						
A1	2	14	-	24	5×10^{8}	18
A2	2	14	-	24	5×10^{9}	18
A3	6	14	-	24	2.5×10^{10}	18
A4	6	2	-	12	2.5×10^{10}	18
A5	4	14	18	28	2.5×10^{10}	18
A6	4	2	6	16	2.5×10^{10}	18
Patients receiving vaccination alone where week 0= start of trial						
B1	2	4	-	14	5×10^{8}	9
B2	2	4	-	14	5×10^{9}	9
B3	4	4	-	14	2.5×10^{10}	9

2.3 Healthy Volunteers from other vaccine trials

Samples from healthy volunteers vaccinated with the same immunogen (NSmut) in studies HCV001 and HCV003 were used in some assays.

HCV001

In this study, healthy volunteers were vaccinated with ChAd3-NSmut and Ad6-NSmut (Barnes et al. 2012). The overall trial design is outlined in the

Introduction in Table 1-4 p44. Certain volunteers from this trial were selected for further assays in Chapter 4, p143, below. The vaccine regimen of these volunteers is described below.

Subjects	Prime (2.5×10^{10} vp)	Boost (2.5×10^{10} vp)
001-032 **001-038**	ChAd3-NSmut Week 0 and Week 4	Ad6-NSmut Week 24
001-102	Ad6-NSmut Week 0	ChAd3-NSmut Week 8

HCV003

Healthy volunteers from this study were vaccinated with ChAd3-NSmut prime followed by MVA-NSmut boost 8 weeks later (manuscript in preparation). Some volunteers were chosen for further assays in Chapter 4, p143. Samples from volunteers from study HCV003, discussed in this thesis are listed below, all vaccinated with ChAd3-NSmut prime (2.5×10^{10} vp) (Wk0), MVA-NSmut boost (2.5×10^{8} pfu) (Wk 8):

Subjects from HCV003 used in comparative assays
003-308, 003-310, 003-328, 003-319, 003-322, 003-305, 003-324

2.4 Safety: Classification of adverse events

Adverse events were recorded by patients using a diary card for 7 days following each vaccination and then by clinician or study nurses on subsequent monitoring

visits. A uniform scale for grading AE severity was determined based on mild, moderate and severe features (Table 2-3).

Table 2-3 Scale for assessing the severity of AEs

Adverse Event	Grade	Definition
Pain	Mild	Painful to touch. Easily tolerated
	Moderate	Painful when limb is moved, interferes with daily activity
	Severe	Severe pain at rest, prevents daily activity
Erythema (Diameter)	Mild	3-50mm
	Moderate	51-100mm
	Severe	>100mm
Swelling	Mild	1-20mm
	Moderate	21-50mm
	Severe	>50mm
Fever (oral temperature)	Mild	37.6°C -38°C
	Moderate	38.1°C -39°C
	Severe	>39°C
Other AEs	Mild	Awareness of symptom but tolerated. Little or no medical intervention.
	Moderate	Some limitation of usual activity, some medical intervention required
	Severe	Incapacitating, bed rest required.

Serious AEs were defined as any AE that resulted in death, significant disability of incapacity, hospitalisation, an important medical event that may jeopardise the patient in the opinion of the clinician or a congenital abnormality. Sudden unexpected Serious Adverse Reactions (SUSAR) were defined as any serious AE which was unexpected and thought to be related to vaccination. A SUSAR would result in suspension of the trial until a safety review. The relationship of an AE to vaccination was determined using Table 2-4.

Table 2-4 Guidelines for assessing the relationship of vaccine administration to an Adverse Event

No relationship	No temporal relationship to study product **and** Alternate aetiology likely **and** Does not follow known pattern of response to the vaccine
Unlikely	Unlikely temporal relationship to study product **and** Alternate aetiology likely **and** Does not follow known 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
Probable	Reasonable temporal relationship to study product **and** Event not readily produced by clinical state, environmental or other interventions **or** Known pattern of response to that seen with other vaccines
Definite	Reasonable temporal relationship to study product **and** Event not readily produced by clinical state, environmental or other interventions **and** Known pattern of response to that seen with other vaccines

2.5 Cell Handling

2.5.1 Blood Separation and PBMC Isolation

PBMC were separated via density gradient separation. Whole blood was layered onto lymphoprep and centrifuged (2200rpm for 24mins without brake). The buffy layer was removed and transferred to RPMI. Cells were washed by centrifugation at 1800rpm for 10minutes, then at 1600 rpm for 7 minutes, resuspending in R10 each time.

2.5.2 Plasma Isolation

Whole blood from an Ethylediaminetetraacetic acid (EDTA) tube was centrifuged (2200rpm ×10minutes). Supernatant (plasma) was aspirated and stored at -20°C.

2.5.3 Preparation of R10:

The following reagents were added to RPMI medium (GibcoBRL 11875-093) to a final concentration as listed: Fetal bovine serum (FCS) (HyClone SH30070.03) 1:10, 200 mM L-glutamine (GibcoBRL 25030-081) 1:100 and 100 X penicillin-streptomycin solution (GibcoBRL 15140-122) 1:100.

2.5.4 Cell counting

Cells were counted using a Guava Personal Cell Analyser (Catalogue no. 0100-1430, Guava Technologies Inc, California). The machine was calibrated daily according to manufacturer's instructions using Guava check beads (Guava 4200-0070 and Guava Check Diluent (Guava 1 4200-0050). Cells were then counted using Guava ViaCount (Guava 1 4000-0040) at 1:20 and analysed using Guava CytoSoft Software, Version 2.1.5 (Guava 0500-1030).

2.5.5 Cell Preservation

Cells were cryopreserved in liquid nitrogen in 1 ml aliquots at 10-12 × 10^6 cells/ml in freezing medium (DMSO 20% and RPMI 80%) and FCS in equal parts to a final concentration of 10-12 ×10^6 cells/ml. 1ml aliquots were stored in a "Mr Freezy" and kept at -80 for 2-3 days, before being transferred to liquid nitrogen storage.

2.5.6 Cell Thawing

Cells were warmed in a water bath at 37˚C for 15seconds until partially thawed. Warmed thawing medium (RPMI 9ml, CTL 1ml, DNAse 100µl per sample) was then added gradually, to a total volume of 10mls. The sample was centrifuged and resuspended in R10.

2.6 Peptides and Antigens

IFN-γ ELISpot assays were performed using a set of 494 overlapping peptides spanning the HCV genome of HCV genotype 1B strain BK based on sequence accession number M58335, supplied by BEI Resources and listed in the Appendix p258. These were divided into pools with 6 pools (F-M) representing the open reading frame from NS3-NS5B (mean 82; range 73 to 112 peptides per pool).

2.7 Evaluation of IFN-γ producing cells following HCV peptide stimulation, by IFN-γ ELISpot

IFN-γ ELISpot assays were performed according to manufacturer instructions (Mabtech) on freshly isolated PBMC or thawed PBMC rested overnight. Millipore AIP plates were coated with 100µl of anti-human IFN-γ antibody at 5µg/ml in PBS overnight at 4˚C. Antibody was washed off with PBS, and plates blocked with R10 (200µl/well) and rested at 37˚C for 2 hours. Cells were plated at 2 × 10^5 PBMC/well in the plate in duplicate or triplicate (depending on cell numbers). These were co-cultured with HCV specific peptides in overlapping pools (see section 2.6 and Appendix for a full list of peptides) overnight at a final concentration of 3µg/ml. Internal controls were: DMSO (negative control) and two positive controls: Concavalin A and FEC (mixed HLA class I-restricted peptides from influenza, Epstein-Barr virus, and CMV). Plates were washed 7 times with PBS/Tween wash buffer using a plate washer and blotted dry after each wash. 50µl/well of biotinylated mouse anti-human IFN-γ diluted to 0.5µg/ml in PBS was added and plates incubated at room temperature for 2-4 hours. Plates were washed with PBS/Tween wash buffer 4 times, then 50 µl of alkaline phosphatase antibiotin antibody added (diluted 1:750 in 0.5% BSA/PBS)

and plates left to incubate for 2 hours. Plates were washed a further 4 times with PBS/Tween wash buffer, then 50µl of BCIP/NBT solution added to each well. Each plate was then incubated at room temperature for 15 minutes, and plates rinsed again. Plates were left to dry overnight at room temperature. Plates were read manually using an ELISpot reader. Total NS response was calculated by summing the mean responses to pools F-M and subtracting the mean DMSO response. A positive response was defined as a summed T cell response to NS proteins greater than 39 SFU/10^6PBMC (see 2.7.2). Unless specified, all peptides were used at a final concentration of 3µg/ml.

2.7.1 CD8+ Depletion IFN-γ ELISpot Assay

In order to determine whether novel epitopes were CD8+ or CD4+ restricted, CD8+ depletion IFN-γ ELISpots were performed in selected patients. 50µl of CD8+ beads per 5×10^6PBMC were washed three times in 3mls of PBS/2%FCS to remove sodium azide. PBMC were resuspended in 2ml chilled PBS/2%FCS and incubated with prepared beads at 4˚C for 20mins. The solution was placed in the Dynal magnet and liquid removed. The procedure was repeated using a second tube with fresh beads. The CD8+ depleted cell suspension was plated for an ELISpot assay as described previously (section 2.7).

2.7.2 Definition of a positive IFN-γ ELIspot response

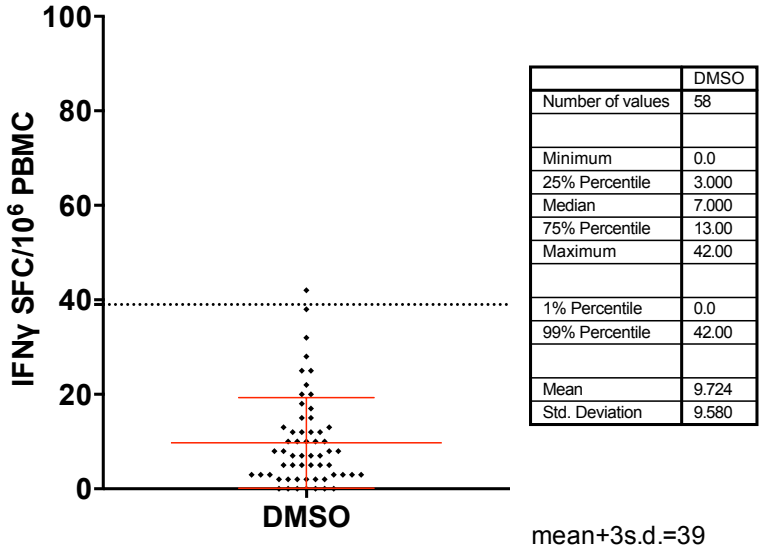
A peptide pool was defined as 'positive' if

(i) it contained ≥39SFU/10^6PBMC **and**

(ii) was ≥3 times the background level (measured in DMSO)

This cut-off was determined based on the mean + 3 standard deviations of background IFN-γ ELISpot responses in 58 patients with chronic HCV, measured in the Barnes group, using the same protocol.

Figure 2-1 Background IFN-γ ELISpot response to DMSO in patients with chronic HCV



	DMSO
Number of values	58
Minimum	0.0
25% Percentile	3.000
Median	7.000
75% Percentile	13.00
Maximum	42.00
1% Percentile	0.0
99% Percentile	42.00
Mean	9.724
Std. Deviation	9.580

Magnitude of IFN-γ ELISpot responses to DMSO in 58 patients with chronic HCV infection, not on treatment. Assays performed by research assistants at the Barnes lab.

Positive pools were then summed and the mean background from DMSO wells in triplicate was subtracted to give a total summed NS response.

A vaccine related T cell response was defined as a positive pool which was either

(i) new (i.e. not present in the screening ELISpots performed prior to vaccination) or

(ii) which represented an increase in response of >30% from a pre-existing positive response.

A patient who responded to vaccination was defined as any individual with a positive IFN-γ ELISpot response post vaccination, which was either

(i) a new T cell response

(ii) an increase in the magnitude of T cell response by ≥30% from baseline, occurring within 2 weeks of vaccination.

2.7.3 Identifying T cell epitopes through peptide mapping

Where possible, the individual peptide to which T cells responded was identified through deconvolution of peptide pools. Positive peptide pools were divided into minipools of approximately 10 peptides each, then these were mapped to single 15 amino acid long peptides and smaller optimal length peptides where possible.

2.8 Population variation at key epitopes

To determine the variability at a population level of immunodominant T cell epitopes in response to vaccination, all available genotype 1 sequences for a given region of the HCV genome were downloaded from Los Alamos database (http://hcv.lanl.gov/content/index). The median number of sequences analysed was 2000 (range 1131-3896). Single variants with a prevalence of >5% were identified at each epitope. Incomplete sequences and non-genotype 1a or 1b sequences were excluded.

2.9 Intracellular Cytokine Staining (ICS)

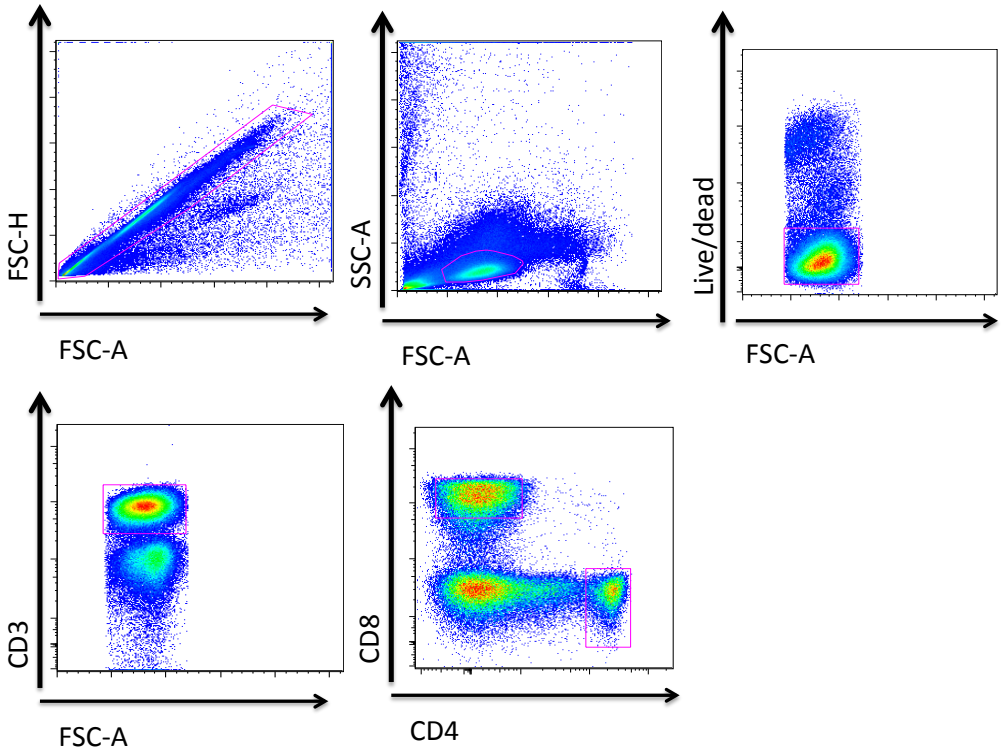
Fresh or thawed PBMC (rested overnight) were plated into 96 well plates at 1 $\times 10^6$ cells/100µl and stimulated with either (i) single peptides where the target epitope had been identified at 1µg/ml, (obtained from BEI resources and

Proimmune Pty Ltd) or (ii) pools of peptides (F+G=NS3/4, I + L+ M=NS5A/B). There were 2 control pools: (i) positive control PMA (phorbol 12-myristate 13-acetate)/ionomycin at 500ng/ml and (ii) unstimulated cells with dimethyl sulfoxide (DMSO) at 5ng/ml. Antibody CD107a PECy5.5 was added at time 0, then 2 hours later brefeldin A was added at 10μg/ml and cells incubated overnight at 37°C. Cells were then stained with live/dead antibody, fixed and permeabilised (Perm buffer BD) and stained with the following panel of antibodies in perm buffer for 30minutes in darkness: CD3-PO (Pacific Orange) (1:50), CD4- Qdot605 (1:200), CD8-PB (Pacific Blue) (1:200), IFN-AlexaFluor700 (1:50), IL2-APC (allophycocyanin) (1:25), TNFα-PE (phycoerythrin)-Cy7 (1:25), and either MIP-1β-PE (1:100) (if single peptide) or IL17-PE (1:25) (if pooled peptides). Antibodies were obtained from Becton Dickson Sciences and Invitrogen. Compensation beads (BD) were stained with 2μl of the relevant antibody above, and run on the day of flow cytometry on a BD LSRII machine. FlowJo software (Treestar) v. 9.5.2 was used to analyse results.

2.9.1 Gating Strategy

Samples were gated sequentially in Flow Jo, as demonstrated below in a representative patient (Figure 2-2). Cytokine stains were then assessed on CD4+ and CD8+ T cells.

Figure 2-2. Gating strategy for Intracellular cytokine staining



Staining in a representative individual is shown (001-032, TW8 stimulated with peptide CINGVCWTV). Each plot shows sequential gating: Single cells ➜ Lymphocytes ➜ Live cells ➜ $CD3^+$ ➜ $CD4^+$ and $CD8^+$ cells. FSC-H= Forward Scatter Height FSC-A=Forward Scatter Area, SSC=Side Scatter Area.

2.10 HLA Class I Pentamer Staining

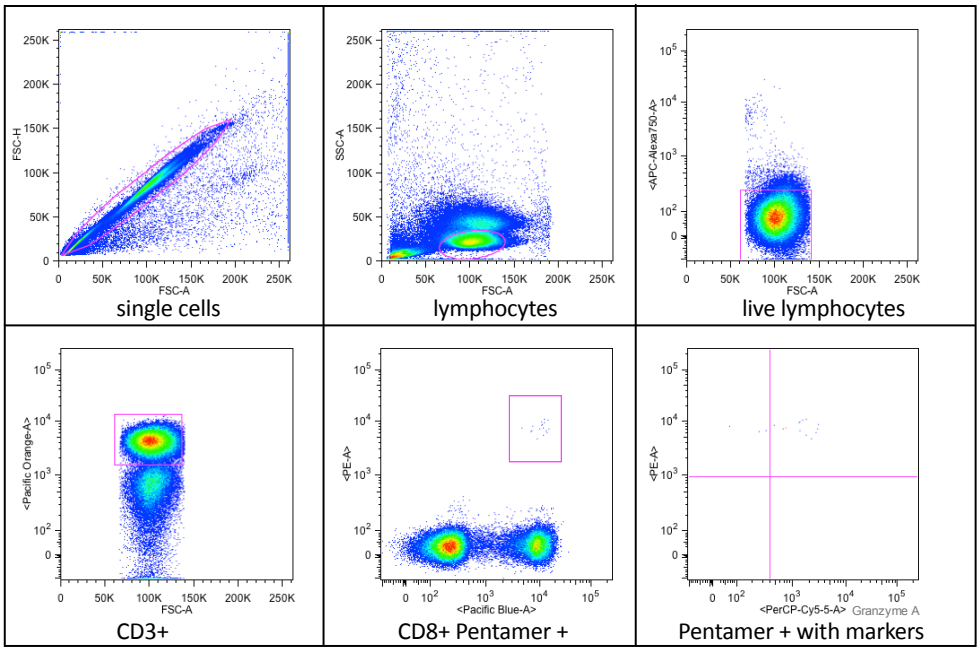
HLA*0201 matched pentamers labeled with PE were obtained from Proimmune Pty Ltd. These were centrifuged at 4˚C (1400gX10mins) and cells stained for 20 minutes at 1:50 at 37˚C . Cells were stained with a live/dead antibody (L/D: Alexa750) then fixed (1% formaldehyde in PBS) and stained with antibodies for gating (CD3-PO, CD8-PB) either with antibodies in panel 1 below or permeabilised (Foxp3 Perm Buffer 1:10) and stained with antibodies in Panel 2 (Table 2-5). Cells were analysed by flow cytometry as described above.

Table 2-5 Panels of Antibodies to characterise HLA+0201 pentamer positive T cells

	PECy7	FITC*	APC	PER CP Cy 5.5§	Alexa 700
Panel 1	**CCR7 1:50**	**CD45RA 1:50**	**CD127 1:50**	**CD38 1:50**	**HLA-DR 1:50**
Panel 1 Purpose	Central memory, LN homing	Antigen exposure. Re-expressed on late terminally differentiated CD8+T cells	Memory and antigen exposure	Activation	Activation
Panel 2	**PD-1 1:50**	**Perforin 1:50**	**CD161 1:100**	**GzA 1:100**	**Gz B 1:50**
Panel 2 Purpose	Exhaustion or activation	Cytotoxicity	Liver homing	Cytotoxicity	Cytotoxicity

FITC= fluorescein isothiocyanate §PerCP =peridinin chlorophyll protein

Figure 2-3 Gating strategy for pentamer staining



A representative patient is shown from study HCV002TV (patient 55), stained with pentamer $NS3_{1406}$ at TW32. TW= treatment week. Pt 55 was vaccinated with ChAd3-NSmut (Wk14 and Wk18) followed by Ad6-NSmut (Wk28) 2.5×10^{10} vp.

2.11 Proliferation Assays

These assays were performed by Anthony Brown (Research assistant at the Barnes group). Conventional thymidine incorporation assays were performed ex-vivo, on freshly isolated PBMC, 2×10^5 PBMC/well (in triplicate). Recombinant HCV proteins (from Mikrogen) were added at 1µg/ml, and results reported as stimulation index (SI) An SI ≥ 3 was considered positive.

2.12 Sequencing of autologous virus in patients with HCV

2.12.1 RNA extraction

Plasma was separated from whole blood from serum tubes, and stored at -80˚C. Thawed plasma (500µl) was pelleted by centrifugation (23,600g at 4˚C for 60minutes) and resuspended in 140µl of spun plasma. Viral RNA was extracted using QIAamp Viral RNA mini kit (Qiagen 52904), eluted in 60µl of AVE elution buffer and stored at -80˚C.

2.12.2 PCR

RNA was reverse transcribed and 1st round PCR performed using Superscript III One-Step RT-PCR (Invitrogen 12574-035), with specific primers (Table 2-6). Second round internal amplification was performed using Expand High Fidelity PCR System (Roche, 11732650001). PCR cycling conditions were primer specific and outlined in Table 2-6. All primers were sourced from Kuntzen and others (Kuntzen et al. 2007) except those used to sequence epitopes CVNGVCWTV and ARMILMTHF which were sourced from Klenerman and others (Komatsu et al. 2006). PCR cycle conditions were designed by me except for the

cycle used to sequence epitope RPRWFMCLLL which was designed by Dr John Halliday (Klenerman/Barnes group).

Table 2-6 Primers and PCR conditions for viral sequencing of patients in HCV002TV. Primers from Kuntzen et al. 2007 except those with "*" which are from Komatsu et al. 2006

Primers 5'-3' (Ext=external Int=internal)	1stR PCR cycle (external primers)	2ndR PCR cycle (internal primers)	Length (bp)	Epitope
Ext: ATCACGGCGTACGCCCAGCAGA* Ext: ATCGGCGTGCCTCGTGACCA Int: AGCCTAACTGGCCGGGACAA TCCGAGGAGCCGCAAGTGCA	50˚C X30mins, 94˚C X 2mins, 39 cycles of 94˚C X15secs, 52˚C X 30secs, 68˚C X 30secs, final elongation at 68˚C X 5mins	94˚C X 2mins then 39 cycles of 94˚C X 15secs, 55˚C X30secs, 72˚C X30secs then final elongation at 72˚C X 2mins	250	CVNGVCWTV
Ext: GACAAAAACCAAGTGGAGGG Ext: GAGGACCTTCCCCAGTCC	50˚C X45mins, 94˚C X 2mins, 39 cycles of 94˚C X30secs, 52˚C X 30secs, 68˚C X 2mins, final elongation at 68˚C X 10mins			
Int: CCTACGGCAAGTTCCTTGC AGCGTGRTTGTCTCAATGG		94˚C X 2mins then 39 cycles of 94˚C X 30secs, 52˚C X 30secs, 72˚C X30secs, final elongation at 72˚C X 5mins	528	KLSGLGINAV, HSKKKCDEL, ATDALMTGY, TLTHPVTK
Int: TGTGTCACYCAGACAGTCG GGGCCCTTCTGCTTGAACTGC		94˚C X 2mins then 39 cycles of 94˚C X 30secs, 57˚C X30secs, 72˚C X 1min, final elongation at 72˚C X 5mins	747	SVVIVGRIIL QEFDEMEECASHLPY
Ext: GCTCGCYGAGCAGTTCAAGC Ext: TYGACCATGACCCGTCGC	50˚C X45mins, 94˚C X 2mins, 39 cycles of 94˚C X30secs, 54˚C X 30secs, 68˚C X 2mins, final elongation at 68˚C X 10mins			
Int: GCTCGYGAGCAGTTCAAGC GGCTATYAGCCCGGTTCATCC		94˚C X 2mins then 39 cycles of 94˚C X 30secs, 55˚C X30secs, 72˚C X 1min, final elongation at 72˚C X 5mins	575	Geno1a: ILAGYGAGV ALVVGVVCAA
Int: AGGAACATGTGGAGTGGG GTTYTCTGACTCAACCCTGG		94˚C X 2mins then 39 cycles of 94˚C X 30secs, 53˚C X30secs, 72˚C X 45secs, final elongation at 72˚C X 5mins	570	Geno1a APNYSRAL FQVGLNQYLVGSQLP
Ext: GTGGAAGTGTCTCAYACG Ext: ATGTTYCCGCCCATCTCCTGCCG	50˚C X45mins, 94˚C X 2mins, 39 cycles of 94˚C X30secs, 54˚C X 30secs, 68˚C X 2mins, final elongation at 68˚C X 10mins			
Int: CGARCAGGGAATGCAGCTCGC AGCCGGTTCATCCACTGC		94˚C X 2mins then 39 cycles of 94˚C X 30secs, 55˚C X30secs, 72˚C X 45secs, final elongation at 72˚C X 5mins	550	Geno1b ILAGYGAGV ALVVGVVCAA
Int: CACCTGCCCATGTGGAGC AGGGGTCGGTRAGCATG		As above	430	Geno1b APNYSRAL FQVGLNQYLVGSQLP
Ext: CCACATCAACTCCGTGTGG Ext: TTCATCGGTTGGGGAGGAGG Int: CCACATCAACTCCGTGTGG CCACACACGAGCATGGTGC	50˚C X45mins, 94˚C X 2mins, 39 cycles of 94˚C X30secs, 53˚C X 30secs, 68˚C X 2mins, final elongation at 68˚C X 10mins	94˚C X 2mins then 39 cycles of 94˚C X 30secs, 53˚C X30secs, 72˚C X 45secs then final elongation at 72˚C X 7mins	580	AYDVVSTL
Ext: GGCGACGACTTAGTCGTTATCTGTG* Ext: TTCAAGCTGGTCCCTGGCTATAAGG Int:GTTATCTGTGAAAGTGCGGGGTCC GGCTATAAGGACGCTAAAGAAATGG	50˚C X30mins, 94˚C X 2mins, 39 cycles of 94˚C X15secs, 55˚C X 30secs, 68˚C X 30secs, final elongation at 68˚C X 5mins	94˚C X 2mins then 39 cycles of 94˚C X 15secs, 57˚C X30secs, 72˚C X 30secs then final elongation at 72˚C X 2mins	288bp	LTRDPTTPLAR, ARMILMTHF
Ext: CCACATCAACTCCGTGTGG Ext: TTCATCGGTTGGGGAGGAGG Int: GCCTGCTACTCCATAGAACC TTCATCGGTTGGGGAGGAG	50˚C X45mins, 94˚C X 2mins, 39 cycles of 94˚C X30secs, 53˚C X 30secs, 68˚C X 2mins, final elongation at 68˚C X 10mins	94˚C X 2mins then 39 cycles of 94˚C X 30secs, 55˚C X30secs, 72˚C X 40secs then final elongation at 72˚C X 4mins	390	RPRWMFCLLL

2.12.3 Extraction of PCR products

PCR products were analysed on 2% agarose gels containing ethidium bromide and DNA products were either gel or PCR purified (QIAquick Gel Extraction Kit, Qiagen 28704 or QIAquick PCR Purification Kit Qiagen 28104).

2.12.4 Direct Sequencing

Products were sequenced bidirectionally using 2nd round internal primers and Prism Big Dye (Applied Biosystems) on an ABI 3100 automated sequencer. Cycling conditions were: 96˚C × 1min, followed by 30 cycles of 96˚C × 15secs, 50˚C × 10secs, 60˚C × 4mins. Sequences were analysed and aligned using Sequencher and Se-Al software (http://tree.bio.ed.ac.uk/software/) .

2.13 Neutralising Antibodies to Adenoviral Vectors

Neutralising antibody assays to adenoviral vectors were performed by Okairos (via dei Castelli Romani 22, Pomezia, 00040 Rome, Italy) as described by their group previously. (Aste-Amézaga et al. 2004). Briefly, recombinant adenoviruses expressing SEAP (secreted alkaline phosphatase) as a reporter gene were incubated for 1 hour at 37°C alone, or with serial dilutions from subject serum samples. These were added to human embryonic kidney cell lines (HEK), seeded at 293 cells/well in a 96 well plate. SEAP expression was measured 24 hours later with the chemiluminescent substrate (CSPD) from the Phospha-Light kit (Tropix) without heat inactivation. Light emission was monitored 45mins after the addition of the CSPD substrate with the Envision 2102 Multi-label reader (Perkin Elmer).

2.14 IL28B genotyping

IL28B genotyping was performed by Rachel Townsend (research assistant in he Klenerman/Barnes lab). DNA was purified from whole blood using the Qiagen Gentra Puregene kit (1068994) and tested for polymorphism rs8099917 using the ABI TaqMan allelic discrimination kit from Applied Biosystems (C_11710096). The possible genotypes for this polymorphism are GG, GT and TT, where the G allele has been associated with decreased viral clearance in acute HCV infection and a poorer response to treatment with IFN/RBV than the T allele.

2.15 Priming naïve T cells using monocyte derived dendritic cells

An overview of this *in vitro* priming model is shown in Chapter 5, Figure 5-1 p184.

2.15.1 Generation of immature Dendritic Cells

Whole blood cones from healthy donors, negative for HIV Ab, HCV Ab, HBV sAg were obtained from The John Radcliffe Hospital, Oxford, UK. Whole blood stains were performed using HLA-A2-FITC antibody (1:50) and samples analysed on LSR2. PBMC from HLA-A2 positive samples were isolated via density gradient separation as previously described (2.5.1 p.66). Monocytes were purified by positive enrichment using anti-$CD14^+$ magnetic beads (Milteni Biotec 130-050-201). The negative fraction was frozen for later use. Immature DCs were generated by culturing monocytes for 4 days at 500,000cells/ml in 6 well plates (Costar Cat No. 3516) in Cellgenix DC medium, supplemented with Rh10 (10%), IL4 (1000IU/ml) and GM-CSF (800IU/ml) (Peprotech AF-HDC).

2.15.2 Maturation of Immature dendritic cells

On day 4, cells were matured by adding LPS 10ng/ml (Sigma, from E Coli 055:B56). Media was supplemented with fresh Rh10, IL4 and GM-CSF. Parallel to this cells were incubated with peptide (final concentration 10µg/ml, 0.017% DMSO), producing 5 different peptide specific DC lines. 36 hours later cells were harvested as mature DCs and used for T cell stimulation.

2.15.3 T cell stimulation by autologous DCs

On day 6, the negative PBMC fraction (following monocyte separation on day 1) was thawed and rested in RPMI at 37°C for 3-5 hours. Mature, peptide pulsed DCs were harvested, washed and replated in 24 well plates (Costar Corning) at 250,000 cells/ml/well. Autologous PBCMs were added at 1:20 (DC:PBMC) (1 ml of $5X10^6$ cells/ml/well). Cells were incubated in complete T cell medium (Rh10 + 12.5mM HEPES (Invitrogen) +50µM 2-Mercaptoethanol (Sigma-Aldrich). On day 10, IL2 (100IU/ml, Roche) and IL7 (5ng/ml, R and D) were added. Fresh media and cytokines were added every 2-3 days until harvesting.

2.15.4 Restimulation of cell lines

7-9 days after priming, peptide was added to cultured cell lines at (10µg/ml). For the subsequent restimulations cell lines were harvested and replated in 6 well plates in 1ml of T cell medium. Frozen autologous PBMC were thawed and pulsed with peptide for 3 hours at 37°C then added to plates at 1×10^7/ml/well.

2.15.5 Phenotypic Analysis of Dendritic Cells

The phenotype of immature and mature dendritic cells was assessed using surface stains with the following antibody panel:

Antibody Colour	Marker	concentration
APC-Cy7	Live/Dead	1:50
PE	CD1a	1:50
PerCPCy5.5	CD86	1:100
PECy7	CD80	1:50
Brilliant Violet 421	CD83	1:50
V500	CD14	1:50
APC	HLAII	1:50

Samples were run on a MACSQuant machine and analysed using FlowJo v9.6.1.

2.15.6 Peptides and tetramers for DC experiments

All peptides and tetramers were synthesised by Proimmune, Oxford. Peptides were dissolved in DSMO then diluted in Rh10.

2.16 Statistical Analysis

Data was assumed to have a non-Gaussian distribution, so non-parametric tests were used throughout. For comparison within an individual a paired test was used (Wilcoxon matched pairs signed rank test). For comparison between groups an unpaired test was used (Mann-Whitney). For correlation between two variables, a Spearman's r test was used. 2 tailed p values were used throughout, and a p value <0.05 considered significant. Prism (v. 5.0d for Mac) was used throughout.

Study HCV002TV was an observational and descriptive safety study thus sample size chosen based on sufficient numbers to allow determination of safety and proof of concept of immunogenicity, rather than to obtain statistical significance.

2.17 Funding

Christabel Kelly was funded by The Wellcome Trust, UK.

Study HCV002TV was funded by the Medical Research Council UK through an experimental medicine award. BEI Resources provided the peptides used.

2.18 Work done by others

IFN-γ ELISpot assays performed at study protocol timepoints, using pooled peptides, were performed by research assistants Tony Brown, Leo Swadling and Rachel Townsend in the Klenerman and Barnes group. These were performed using standard operating procedures and provided data used in Figure 3-8, Figure 3-9, Figure 3-10, Figure 3-11, Figure 3-12, Figure 3-13, Figure 3-18, Figure 3-19, Figure 3-20.

Proliferation assays were performed by Tony Brown.
IL28B assays were performed by Rachel Townsend.
Neutralising antibody assays were performed by Okairos.

Clinical care of patients in the study was provided by the clinical team at the John Radcliffe Hospital/ University of Oxford: Dr Ellie Barnes (consultant), Dr Jane Collier (consultant), Dr Christabel Kelly (clinical research fellow), Dr John Halliday (clinical research fellow), Ms Lizzie Stafford (nurse), Ms Denise O'Donnell (nurse).

3 Is therapeutic vaccination safe and immunogenic?

3.1 Aims

To evaluate the safety and immunogenicity of ChAd3-NSmut and Ad6-NSmut in patients with chronic HCV

3.2 Background

These vaccines have never before been used in patients with chronic HCV infection, therefore the most important initial primary outcome measure of this study was safety. Although the identical vaccine regimen was well tolerated in healthy volunteers, described by Barnes and others, patients with chronic HCV bring a unique set of comorbidities and a different immunological milieu, where they have memory T cell populations to previous and concurrent viral quasi-species and potentially diminished hepatic reserve (Barnes et al. 2012). Patients in Arm A of this study also received concurrent IFN/RBV, which carry a significant side effect profile of their own and may influence tolerability of adverse events associated with vaccination (Fried et al. 2002). The first section of this chapter outlines the enrollment process for each study group and analyses the local and systemic adverse events possibly related to vaccination, determined as described in *Materials and Methods 2.4 p64* above.

The second key outcome from the study was immunogenicity, measured here using a range of T cell assays: IFN-γ ELISpot assays, proliferation assays, pentamer stains and ICS. The effect of vaccination in three different contexts was assessed: (i) vaccination with concurrent IFN/RBV versus vaccination alone, (ii) vaccination with a high versus low viral load and (iii) double priming

vaccination compared with single priming. T cell responses in a control cohort receiving IFN/RBV alone (without vaccination) was used for comparison, given the conflicting reports in the literature regarding the effect of IFN on T cell responses (see *Introduction p51*). The study was not designed to show a difference in viral clearance with IFN/RBV treatment (SVR) between patients who made a T cell response to vaccination and those who did not, however viral loads were followed over time in all patients.

3.3 Questions

(i) Can T cells be safely induced or recovered in patients with chronic HCV infection?

(ii) What is the phenotype and functionality of T cells detected after vaccination with ChAd3-NSmut and Ad6-NSmut?

(iii) Is immunogenicity enhanced by vaccination after viral suppression with IFN?

(iv) What is the effect of vaccination on viral load?

3.4 Enrollment and clinical course of patients in HCV002TV

Patients with chronic HCV were identified in outpatient clinics and enrolled as described in the section *Materials and Methods.* One group with 12 patients, who received IFN/RBV treatment alone, was recruited as a control to compare the effect of IFN/RBV alone on T cell responses. Their characteristics are shown in Table 3-4. The other patients were stratified to the two vaccination arms of the study, concurrent treatment (Arm A) and non-treatment (Arm B), according to physician and patient preference. A schema of the enrollment process for vaccinated patients is shown in Figure 3-1.

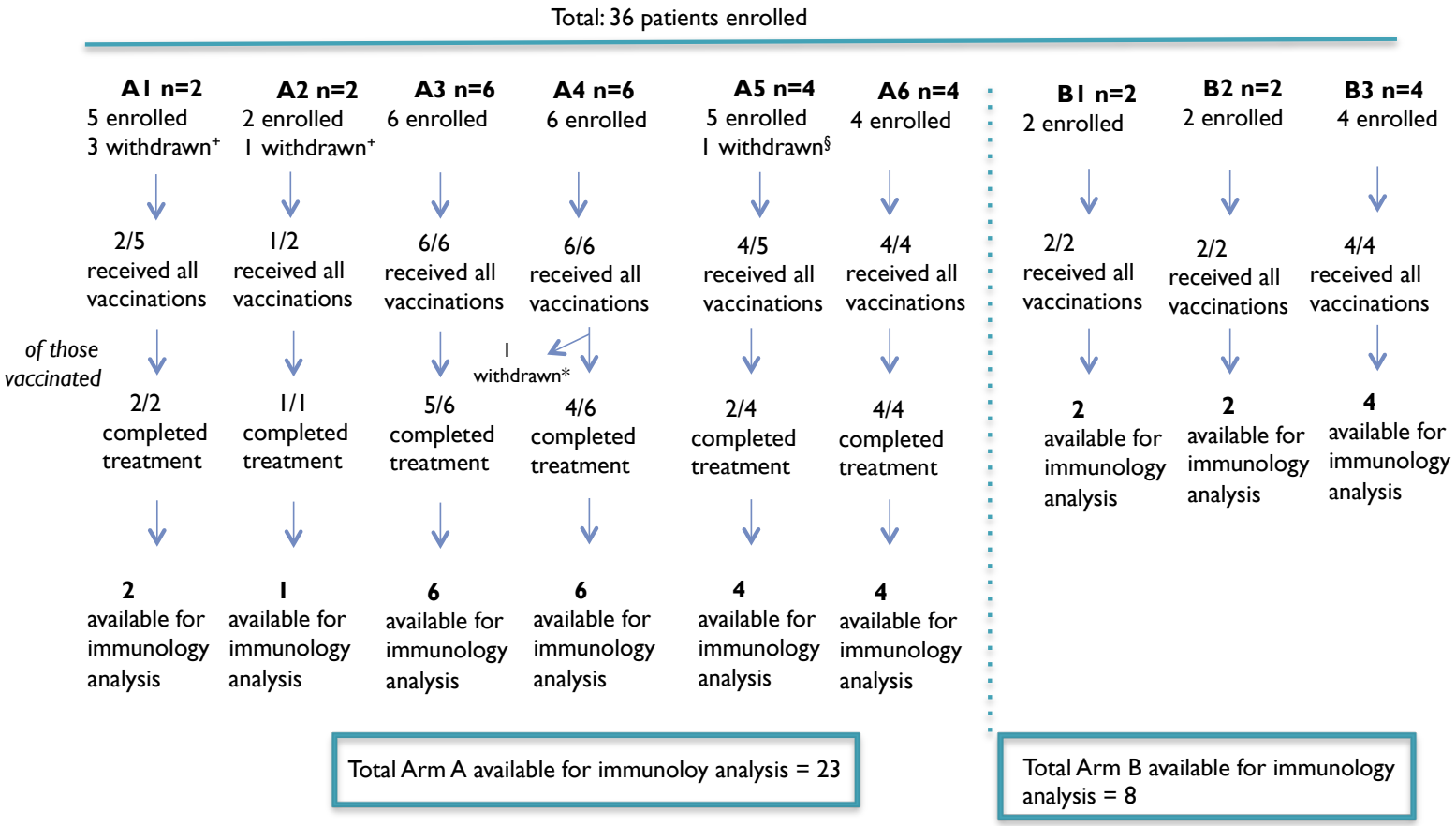
In total, 36 patients were enrolled in Arms A and B, of whom 31 had samples available for final immunology analysis (23 patients in Arm A and 8 patients in Arm B). 5 patients were withdrawn from the study and 2 patients were lost to follow up: 1 after screening (patient 108) and 1 at the final trial visit (patient 100) (Table 3-1). Of the patients who withdrew, patient 002-021 in Arm A1 had a rash likely related to priming vaccination and was replaced in order to establish that it was safe to proceed to the next dose group in Arm A2. Patient 002-031 in Arm A2 withdrew due to stress related to venesection. One further patient (002-045, Arm A4) withdrew late in the study after developing thyrotoxicosis related to IFN/RBV therapy, however was included in immunology analysis, as this patient continued to provide samples for immunology up until week 32 of IFN/RBV treatment and had completed the vaccination protocol.

Characteristics of the enrolled vaccinated patients are outlined in Table 3-3. All patients were of Caucasian origin. The median age at enrollment of patients who received at least one vaccination was 43 years (range 28-61). 21 (61.7%) were infected with genotype 1a, 11 (32.4%) with genotype 1b and 2 (0.06%) had an indeterminate genotype 1 subtype. The majority of patients (74%) were male.

Table 3-1 Patients withdrawn or lost to follow up in HCV002TV

Patient	Arm	Week withdrawn	Reason	Vaccinations received	Included in immunology analysis
21	A1	14	Rash possibly due to vaccination	ChAd3 (5×10^8vp) prime	N
23	A1	11	Patient choice	None	N
32	A1	12	Pyschotic episode	None	N
31	A2	22	Difficulty with venesection	ChAd3 (5×10^9vp) prime	N
100	A3	60	Lost to follow up at TW60	All	Y
45	A4	32	Following complications from IFN/RBV,	All	Y
108	A5	1	Failed to attend any further visits after screening	None	N

Figure 3-1 Patient enrollment in study HCV002TV



+1 patient from A1 and 1 patient from A2 were withdrawn following priming vaccination. 2 patients from A1 and 1 patient from A5 withdrew prior to receiving any vaccinations. * Withdrawn late in the study following complications from IFN/RBV treatment. Immunology samples availabile up until TW32 so included in final analysis.

3.4.1 Treatment Outcomes in Arm A

It was expected, based on clinical experience that not all patients in Arm A would complete therapy. Out of 23 patients who completed the vaccination protocol in Arm A, 5 stopped treatment early as detailed in Table 3-2 below. SVR results were available for 22/23 patients. Results for one patient (002-100) who was lost to follow up at week 48 were not available . RVR, EVR and SVR rates were 37.5%, 65.2% and 56.5% respectively (as defined in Table 1-1 p15), comparable to the treatment response rates in the published literature for genotype 1 infection (Fried et al. 2002). This was comparable to the control arm where RVR, EVR and SVR rates were 25%, 67% and 58% respectively. There was a higher treatment discontinuation rate in the control arm, where 5/12 patients did not complete 48 weeks of therapy (Table 3-4 p91).

Of the 10 patients in Arm A of HCV002TV who did not achieve an SVR, only 1 had virological breakthrough while on therapy (patient 002-101). 1 patient who stopped treatment after only 16 weeks (002-045) relapsed 8 weeks later. The remainder of these patients relapsed after the end of treatment, despite completing 48 weeks of therapy.

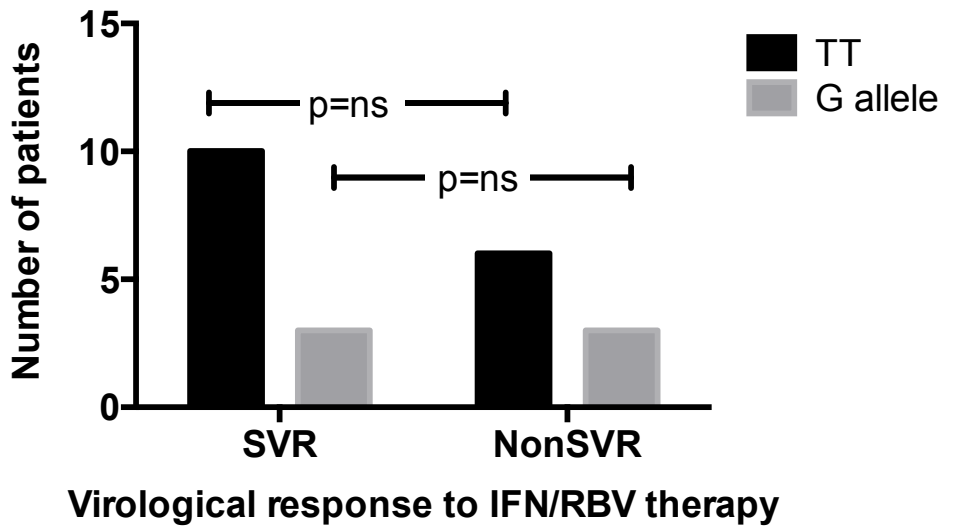
Table 3-2 Patients who stopped treatment with IFN/RBV early

Patient	Arm	Week stopped therapy	Reason
002-036	A3	30	Neutropenia and recurrent infections
002-101	A4	24	Virological breakthrough on treatment
002-045	A4	16	Thyrotoxicosis
002-055	A5	24	Social reasons
002-056	A5	24*	Severe rash

* stopped ribavirin at week 19

There was no statistically significant difference in treatment outcome between patients carrying the IL28B risk allele (GT or GG) and those homozygous for the protective allele (SVR rates of 50% versus 63% respectively, p=ns by Fisher's exact test) (Figure 3-2). There were however only 6 out of the 23 treated patients with SVR results, who carried the risk allele and there were no homozygotes (GG) (Table 3-3).

Figure 3-2 SVR rates according to IL28B genotype in patients in Study HCV002TV



SVR (sustained virological response) rates in 23 patients receiving IFN/RBV therapy and vaccination (Arm A from study HCV002TV). IL28B status is shown, based on SNP rs8099917 where G is the risk allele and T is associated with HCV clearance.

Table 3-3 Characteristics of patients enrolled in HCV002TV Arms A and B

Patient	Group	Sex	Age	HCV subtype	Fibrosis Score[+]	ALT	Treatment Duration (weeks)	Baseline Viral load (IU/ml)	RVR	EVR	SVR	IL28B
32	A1	M	51	nd	1	40	WD	4.57×10^6	Y	Y	na	nd
23	A1	M	46	nd	1	49	WD	72680	Y	Y	na	nd
20	A1	M	54	1	3	40	48	1.54×10^6	N	Y	Y	TT
21	A1	F	65	1a	3	28	WD	1.98×10^6	Y	Y	na	nd
30	A1	M	56	1a	4	28	48	15995	Y	Y	Y	TT
24	A2	M	42	1b	3	69	48	11.3×10^6	Y	Y	Y	TT
31	A2	M	36	1a	2	56	WD	6.3×10^6	N	N	na	TT
27	A3	F	33	1	2	18	48	22228	Y	Y	Y	GT
28	A3	M	58	1a	2	144	48	4.14×10^6	N	N	N	TT
36	A3	F	32	1b	nd	16	30	9199	Y	Y	Y	TT
100	A3	M	24	1b	7.5*	54	48	1.31×10^6	N	Y	nd	GG
48	A3	M	53	1a	4	84	48	2.93×10^6	N	N	N	TT
50	A3	M	50	1a	1	42	48	761224	N	N	N	GT
39	A4	M	31	1b	1	72	48	11.5×10^6	N	Y	Y	GT
101	A4	M	43	1a	6*	71	24	1.89×10^6	N	N	N	GT
102	A4	M	30	1b	**1**	151	48	584666	N	Y	Y	GT
45	A4	F	35	1a	2	74	WD[§]	343163	N	nd	na	TT
106	A4	M	44	1a	5.4*	26	48	3.08×10^6	N	N	N	TT
54	A4	M	44	1a	0	44	48	824830	N	N	N	GT
37	A5	M	35	1b	3	113	48	10.2×10^6	Y	Y	Y	TT
38	A5	M	28	1b	2[+]	69	48	631606	Y	Y	Y	TT
55	A5	M	35	1a	1	76	24	398842	Y	Y	Y	TT
108	A5	M	36	1a	4.8*	nd	WD	993615	nd	nd	nd	TT
56	A5	F	61	1b	4	36	24	31025	Y	Y	Y	TT
40	A6	M	39	1b	2	26	48	2.45×10^6	N	N	N	TT
43	A6	M	54	1a	2	77	48	11.65×10^6	N	Y	Y	TT
103	A6	M	36	1b	7.3*	59	48	36678	Y	Y	Y	TT
25	A6	M	43	1a	2	56	48	8.40×10^6	N	Y	N	TT
34	B1	M	55	1a	1	41	NA	9.03×10^6	NA	NA	NA	TT
29	B1	F	49	1a	1	47	NA	392438	NA	NA	NA	TT
35	B2	F	55	1a	1	35	NA	831657	NA	NA	NA	GT
44	B2	F	33	1b	1	51	NA	1.69×10^6	NA	NA	NA	TT
46	B3	M	53	1a	1	101	NA	2.79×10^6	NA	NA	NA	GT
51	B3	F	43	1a	1	76	NA	1.65×10^6	NA	NA	NA	TT
52	B3	M	59	1a	3	46	NA	2.55×10^6	NA	NA	NA	GT
53	B3	M	49	1a	2	31	NA	3868	NA	NA	NA	TT

+= fibrosis was determined through histology (Ishak score) or non-invasive methods (fibroscan)*. Ishak score ranges from 0-6 where 6=cirrhosis. WD= withdrawn na= not applicable. Nd=not done. §=Pt 045 ceased treatment at week 16 due to thyrotoxicosis, then was retreated at a later timepoint. Results here are based on the first treatment course. IL28B status is based on SNP rs8099917 where G is the risk allele and T is associated with HCV clearance. RVR= undetectable viral load after 4 weeks of treatment. EVR= undetectable viral load after 12 weeks of treatment. SVR= undetectable viral load 6 months post end of treatment.

Table 3-4 Characteristics of patients with chronic HCV infection in control cohort

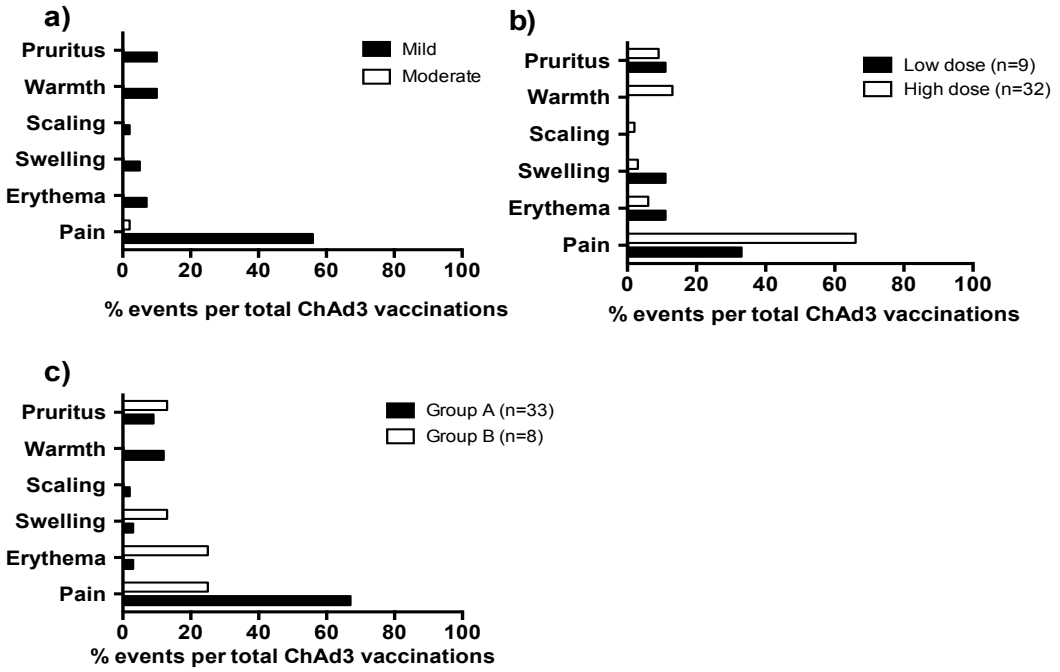
Patient	Sex	Age	Fibrosis score	Treatment Duration (weeks)	Baseline Viral load IU/ml	RVR	EVR	Treatment Outcome	IL28B	CMV +ve at screen	FEC +ve at screen
001	M	54	2/6	12	$1.19x10^6$	N	N	NVR	GT	N	Y
002	M	42	1/6	48	$3.12x10^6$	N	Y	SVR	GT	Y	Y
003	F	48	2/6	48	$1.53x10^6$	N	Y	SVR	TT	Y	Y
004	F	42	3/6	48	531000	Y	Y	SVR	TT	nd	nd
005	M	46	2/6	48	334000	Y	Y	SVR	TT	nd	nd
006	M	68	2/6	12	156000	N	Y	SVR	TT	Y	Y
007	M	44	2/6	12	$9.77x10^6$	N	N	NVR	TT	N	Y
008	F	41	1/6	48	$5.57x10^6$	N	Y	SVR	TT	N	Y
009	M	30	nd	48	$1.34x10^6$	N	Y	NVR	TT	Y	N
010	F	50	3/6	22	838000	N	N	NVR	GT	Y	Y
011	M	46	4/6	14	32261	N	N	NVR	GT	Y	N
012	M	43	3/6	48	17784	Y	Y	SVR	GT	Y	N

nd= not done RVR= Rapid Virological Response (viral load<50IU/ml at Wk 4). EVR= Early Virological Response (undetectable viral load at Wk 12). SVR= Sustained Virological Response (undetectable viral load 6 months following treatment completion). Fibrosis score determined by liver biopsy prior to start of treatment *IL28B genotype based on SNP rs8099917 T=protective allele G=risk allele

3.5 Is therapeutic vaccination in HCV002TV safe?

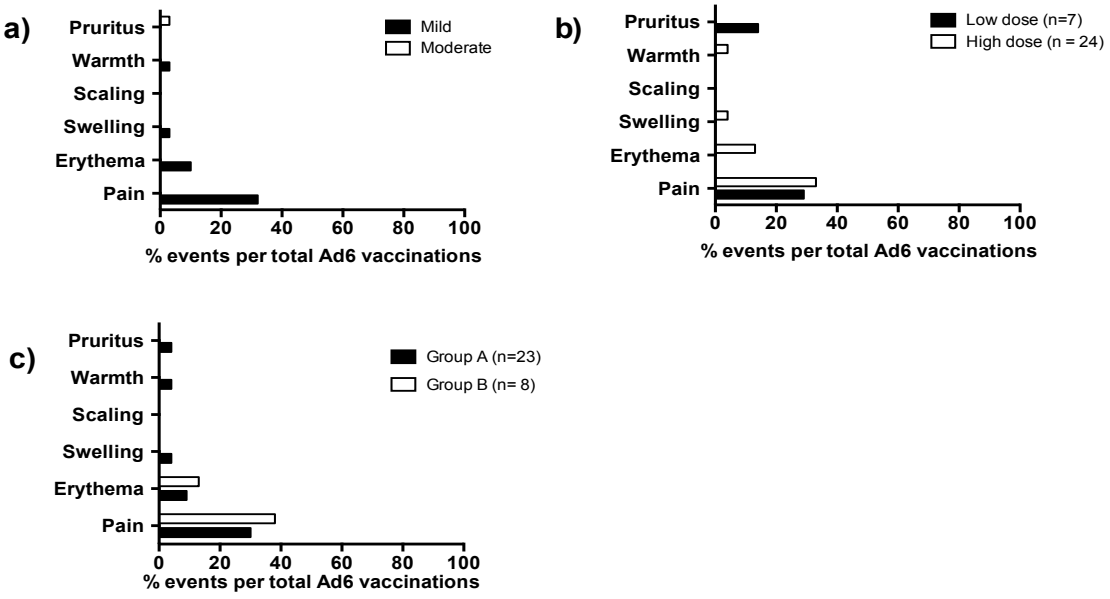
Vaccination was well tolerated. A dose escalation strategy of both the priming vector (ChAd3) and the boosting vector (Ad6) was used (low 5×10^8vp, medium 5×10^9 vp and high 2.5×10^{10}vp). Mild local and systemic effects were consistent with those reported in previous adenoviral vector trials. Figure 3-3 and Figure 3-4 detail local adverse events following vaccination. The most common adverse event was localised pain following vaccination in 56% and 29% of patients receiving ChAd3-NSmut prime and Ad6-NSmut boost respectively. This was mild in all but one individual who reported moderate discomfort. Local pain was more common as ChAd3 dose increased, with 33% of patients reporting pain following low or medium dose priming vaccination compared with 66% following high dose vaccination (Figure 3-3b).

Figure 3-3 Local adverse events (AEs) possibly, probably or definitely associated with vaccination with ChAd3-NSmut.



Total number of vaccines given in each group is in brackets. **a)** local AEs by severity. **b)** comparison of AEs after lower dose (5×10^8vp, and 5×10^9 vp) and high dose (2.5×10^{10}vp). **c)** comparison of patients receiving concurrent IFN/RBV therapy (Group A) with those receiving vaccination alone (Group B).

Figure 3-4 Local adverse events (AEs) possibly, probably or definitely associated with vaccination with Ad6-NSmut.



Total number of vaccines given in each group is in brackets. **a)** local AEs by severity. **b)** comparison of AEs after lower dose (5×10^8vp, and 5×10^9 vp) and high dose (2.5×10^{10}vp). **c)** comparison of patients receiving concurrent IFN/RBV therapy (Group A) with those receiving vaccination alone (Group B).

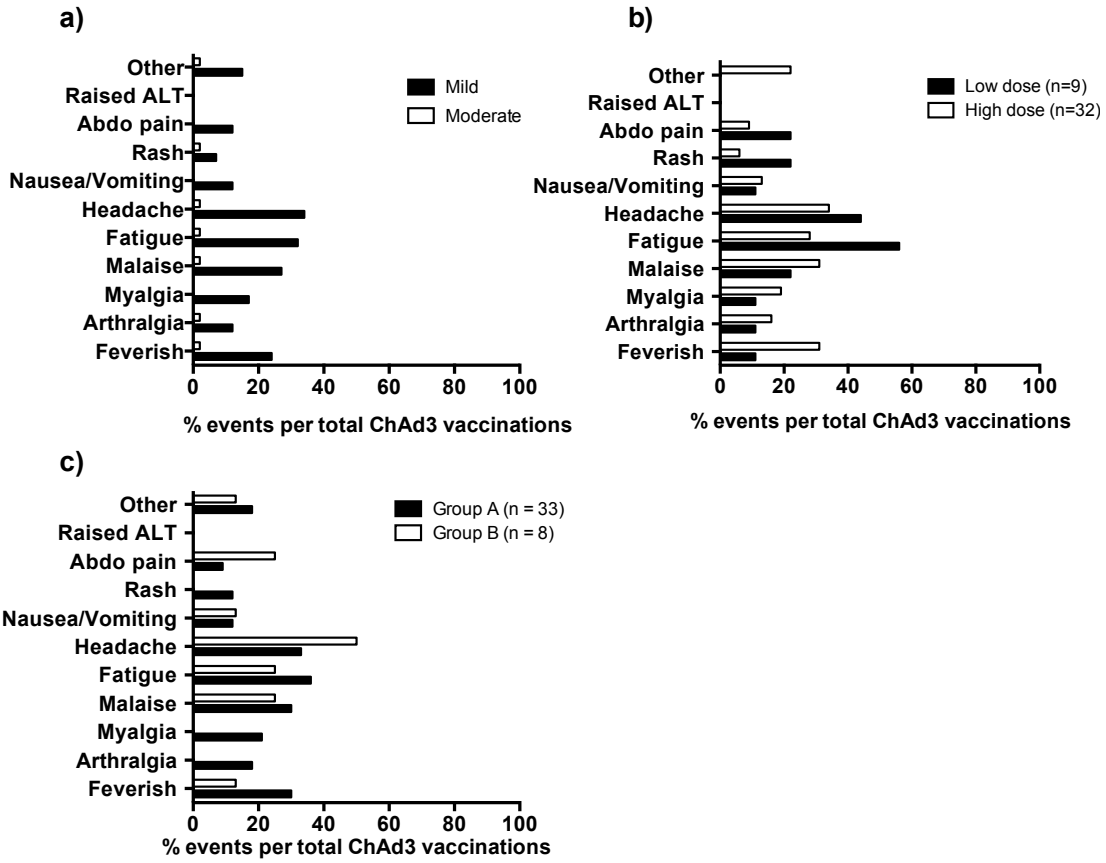
Systemic adverse events following vaccination are detailed in Figure 3-5 and Figure 3-6. These were generally mild in nature. Malaise, fatigue and headache were the most common systemic adverse events following ChAd3 priming, present in 29%, 34% and 36% of patients respectively. These were less common following boosting with Ad6-NSmut, being reported after 13%, 16% and 16% of vaccinations respectively. A rash was reported in 9% of individuals following ChAd3 vaccination and was moderate in severity in one case, causing the patient to be withdrawn from the study (patient 002-021). These all occurred in patients on concurrent IFN/RBV therapy. No rashes were reported following Ad6-NSmut vaccination.

Overall, although there was no statistically significant difference between the number of adverse events between Arms A and B, there was a trend towards more adverse events in Arm A, many of which were also common side effects of IFN/RBV. I tried to account for the effect of concurrent IFN/RBV by only recording an increase in symptoms from a pre-vaccine baseline, however given the ongoing use of IFN/RBV in Arm A patients it is difficult to clearly attribute the cause of some adverse events to vaccination.

Systemic adverse events were predominantly mild. Systemic AEs classed as moderate following ChAd3 vaccination were arthralgia, diarrhoea, depression, feeling feverish and headache in separate individuals. Moderate AEs following Ad6 vaccination were generalised pruritus, sore throat, abdominal pain, knee swelling and a significantly raised ALT in one individual. Changes in ALT are discussed further in the following section.

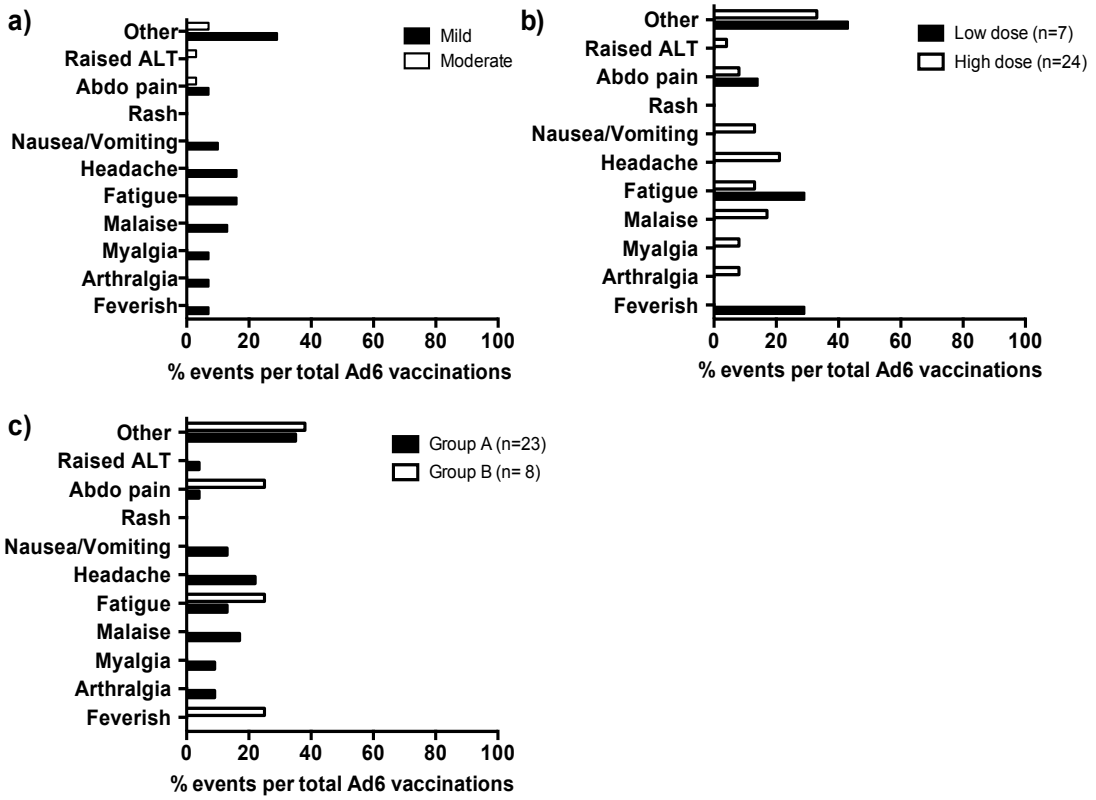
There were no serious adverse events associated with vaccination.

Figure 3-5 Systemic adverse events (AEs) possibly, probably or definitely associated with vaccination following ChAd3-NSmut



Total number of vaccines given in each group is in brackets. **a)** systemic AEs by severity. **b)** AEs after low dose (5×10^8vp, medium 5×10^9 vp) and high dose (2.5×10^{10}vp). **c)** comparison of patients receiving concurrent IFN/RBV therapy (Group A) with those receiving vaccination alone (Group B). Other AEs following ChAd3 vaccination were: cough, dry skin, itchy eyes, non-specifically unwell and abnormal clotting parameters.

Figure 3-6 Systemic adverse events (AEs) possibly, probably or definitely associated with vaccination following Ad6-NSmut



Total number of vaccines given in each group is in brackets. **a)** systemic AEs by severity. **b)** AEs after low dose (5×10^8vp, medium 5×10^9 vp) and high dose (2.5×10^{10}vp). **c)** comparison of patients receiving concurrent IFN/RBV therapy (Group A) with those receiving vaccination alone (Group B).. Other AEs following Ad6 vaccination were: small area of facial hair loss, hypothyroidism, monoarthritis, feeling shaky, mouth ulcers, raised blood sugars, sore throat, constipation and abnormal Alkaline Phosphatase.

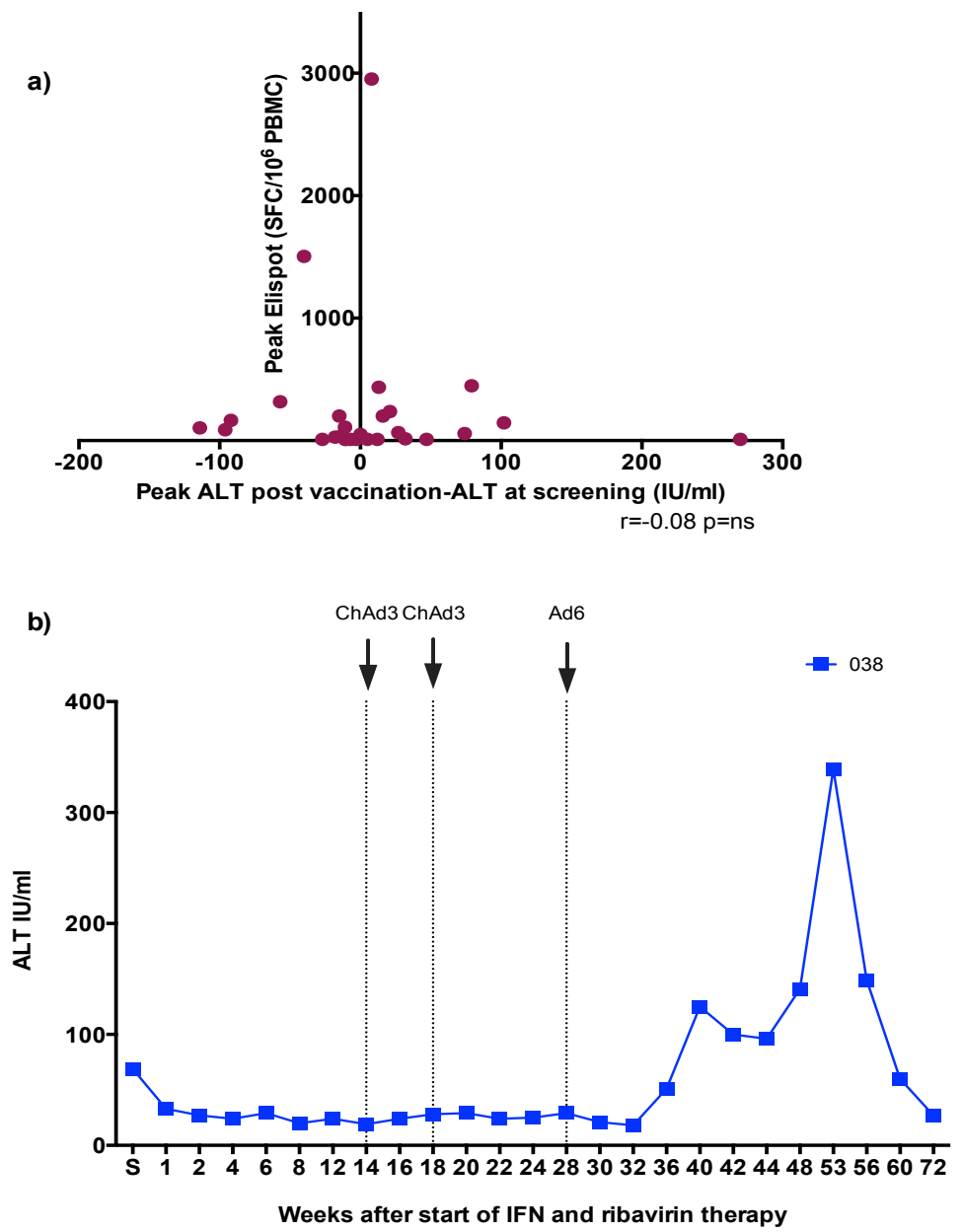
3.5.1 Changes in ALT following vaccination

I closely monitored ALT levels, as a surrogate marker of liver inflammation, in all patients. In the group receiving vaccination alone (Arm B) there were no new elevations in ALT possibly or probably related to vaccination. This was defined as any elevation in ALT above the upper limited of normal, which occurred within 8 weeks of vaccination and was ≥30% higher than a pre-vaccine ALT level. This definition was chosen as one which would capture the largest number of potentially related changes in ALT whilst taking into account normal

fluctuations in ALT levels. In the group receiving concurrent IFN/RBV therapy there was one transient flare in ALT in patient 002-038 where ALT peaked at 339 IU/ml, 11 weeks after boosting vaccination (Figure 3-7b). This was not associated with the reappearance of a positive HCV viral load (virological breakthrough). A liver biopsy at the end of treatment (23/5/11) revealed non-specific inflammation and ALT returned to baseline levels within 6 weeks.

I therefore examined the relationship between ALT and immunogenicity and found no association with change in ALT and immune responses (Figure 3-7a) r=-0.08 p=ns). Indeed, ALTs in the two individuals with the strongest immune responses (002-027 and 002-039) either decreased or stayed the same following vaccination (data not shown).

Figure 3-7 Changes in ALT in relation to immune responses and over time in two individuals



a) Change in ALT from post vaccination to screening is shown in patients receiving high dose vaccination in Arms A and B. This is correlated with immunogenicity represented by peak IFNγ-ELISpot. **b)** The kinetics of ALT over time in patient 038 in relation to vaccination is shown.

3.6 Kinetics of T cell responses in patients receiving low (5×10^8vp) and medium (5×10^9vp) dose vaccination

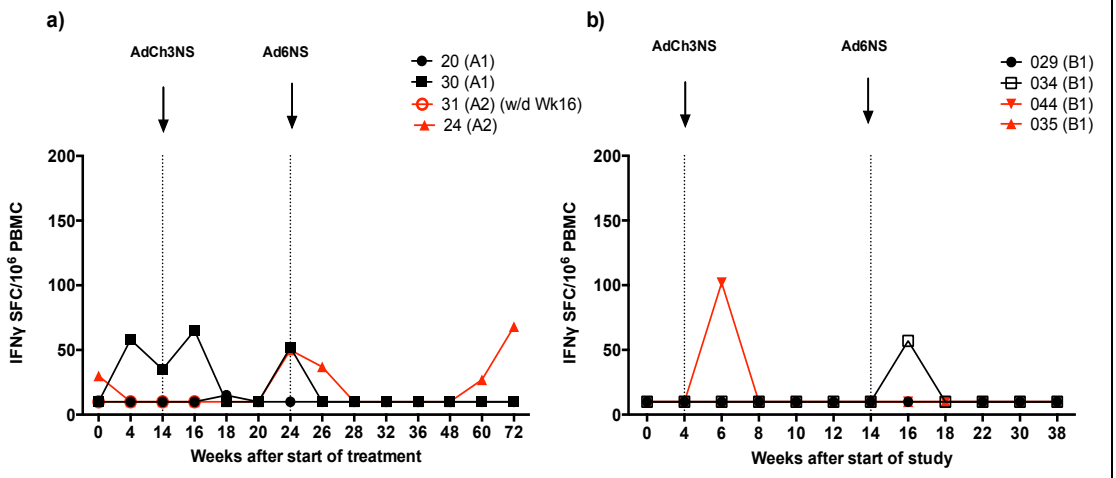
First the immunogenicity of heterologous adenoviral vaccination was assessed using escalating doses of ChAd3 (priming vector) and Ad6 (boosting vector) in

patients receiving concurrent treatment with IFN/RBV (Arm A) and in those receiving vaccination alone (Arm B). In the low and medium dose groups in Arm A, vaccines were administered 10 weeks apart following a 2 or 14 week lead in with IFN/RBV at 5×10^8vp (A1) and 5×10^9vp (A2). In the low and medium dose groups in Arm B, patients received prime/boost vaccines 10 weeks apart, at 5×10^8vp (B1) and 5×10^9vp (B2) (see *Materials and Methods*).

IFN-γ ELISpot studies demonstrated that low and medium dose vaccinations were poorly immunogenic (Figure 3-8a). In the 3 patients who completed the vaccination protocol, only 2 had detectable positive T cell responses, however these were weak (0-68 SFC/10^6PBMC) and did not show any temporal relationship with vaccination.

In patients receiving vaccination alone (Arms B1 and B2), 2 individuals (patients 034 and 044) made small non-sustained T cell responses to the NS proteins contained within the HCV vaccine immunogen, 2 weeks following vaccination (range 57-102 SFC/10^6PBMC) (Figure 3-8b). However overall, responses were reduced compared with those induced in healthy volunteers primed with this regimen in a previous study (HCV001), where 1 out of 4 individuals responded to low dose priming and 3 out of four individuals responded to medium dose priming (mean, 905.5; range 10 to 3227) (Barnes et al. 2012).

Figure 3-8 Magnitude and kinetics of T cell responses after low and medium dose vaccination with ChAd3-NSmut (prime) and Ad6-NSmut (boost).



Kinetics of ex vivo IFN-γ ELISpot responses over time by individual, from patients vaccinated with ChAd3-NSmut prime and Ad6-NSmut (dose escalation $5X10^8$ to $5X10^9$ vp/ml) in patients receiving **a**) concurrent IFN/RBV therapy (Arms A1, A2) (patients 20, 30, 31, 24) and **b)** vaccination alone (Arms B1, B2) (patients 029, 034, 044, 035). Patient 31 withdrew after week 16 of the trial. T cell responses represent summed responses to peptide pools containing the NS proteins. The minimum value shown is $10SFC/10^6PBMC$.

3.7 T cell responses in patients receiving high dose vaccination (2.5×10^{10}vp) and concurrent IFN/RBV therapy

Next, patients were vaccinated with a high dose heterologous prime/boost regimen (2.5×10^{10}vp) with (Arm A3-A6) or without (Arm B3) concurrent PEG-IFNα/RBV. Arms A3, A4 and B3 received a single priming vaccination with ChAd3-NSmut followed by boosting vaccination with Ad6-NSmut 10 weeks later. Arms A5 and A6 received two priming vaccinations 4 weeks apart, followed by boosting vaccination 10 weeks later.

In Arm A, T cells specific for HCV antigens were detected by IFN-γ ELISpot in 11/20 patients who received high dose vaccination, of whom 6 developed new responses not detected prior to vaccination, 3 made new T cell responses as well as augmenting pre-existing responses and 2 increased pre-existing responses only. A T cell response to vaccination was defined (as described in *Materials and*

Methods) by:

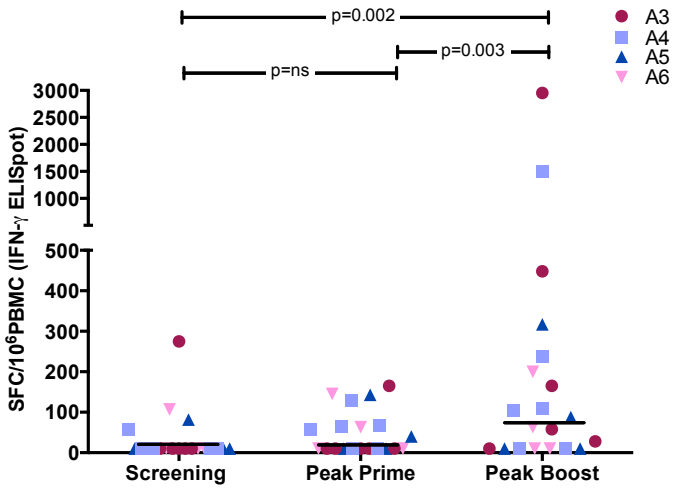
an IFN-γ ELISpot response ≥ 39 SFU/10^6 PBMC which was either:

(i) an increase in a pre-existing response by >30% **or**

(ii) a new T cell response not detected prior to vaccination.

Overall, after boosting there was a statistically significant induction of T cell responses after boosting compared with baseline (median peak boost 76 SFC/10^6 PBMC; range 0-2953) (p=0.002) (Figure 3-9).

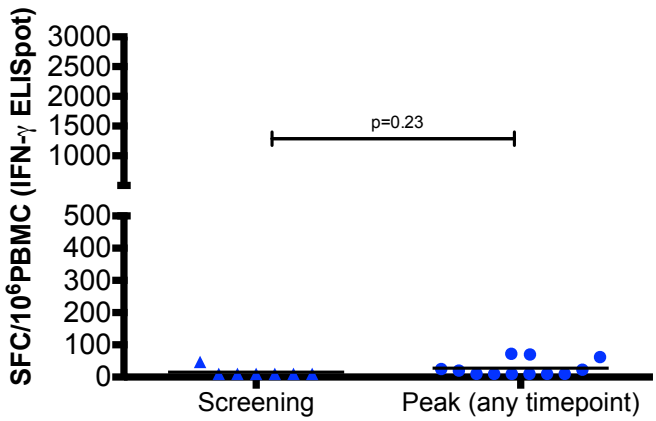
Figure 3-9 Increase in magnitude of T cell responses measured by IFNγ-ELISpot in patients vaccinated with ChAd3-NSmut (prime), Ad6-NSmut (boost), while on concurrent IFNα/RBV therapy.



Peak IFNγ–ELISpot responses following priming and boosting vaccination are shown. Responses on the y axis represent summed responses to positive pools representing the NS proteins in the vaccine immunogen. Individual patients are represented. Median values= horizontal bar. Arm A3: Prime wk 14/boost Wk 24; Arm A4: Prime Wk 2/ Boost Wk 12; Arm A5: Prime Wk 14 and Wk 18/ boost Wk 28; Arm A6: Prime Wk 2, Wk 6/ boost Wk 16 of treatment. p values determined by Wilcoxon matched-pairs signed rank test.

Next T cell responses in patients receiving IFN/RBV treatment alone, without vaccination, were assessed. There was no statistically significant increase in peak IFN-γ ELISpot response from screening (p=ns Figure 3-10), with the highest response recorded at any time point during treatment being 72 SFC/10^6 PBMC with a median of 15 SFC/10^6 PBMC (range 0-72).

Figure 3-10 T cell responses to HCV Non-structural proteins, during treatment with IFN/RBV in control arm of HCV002TV



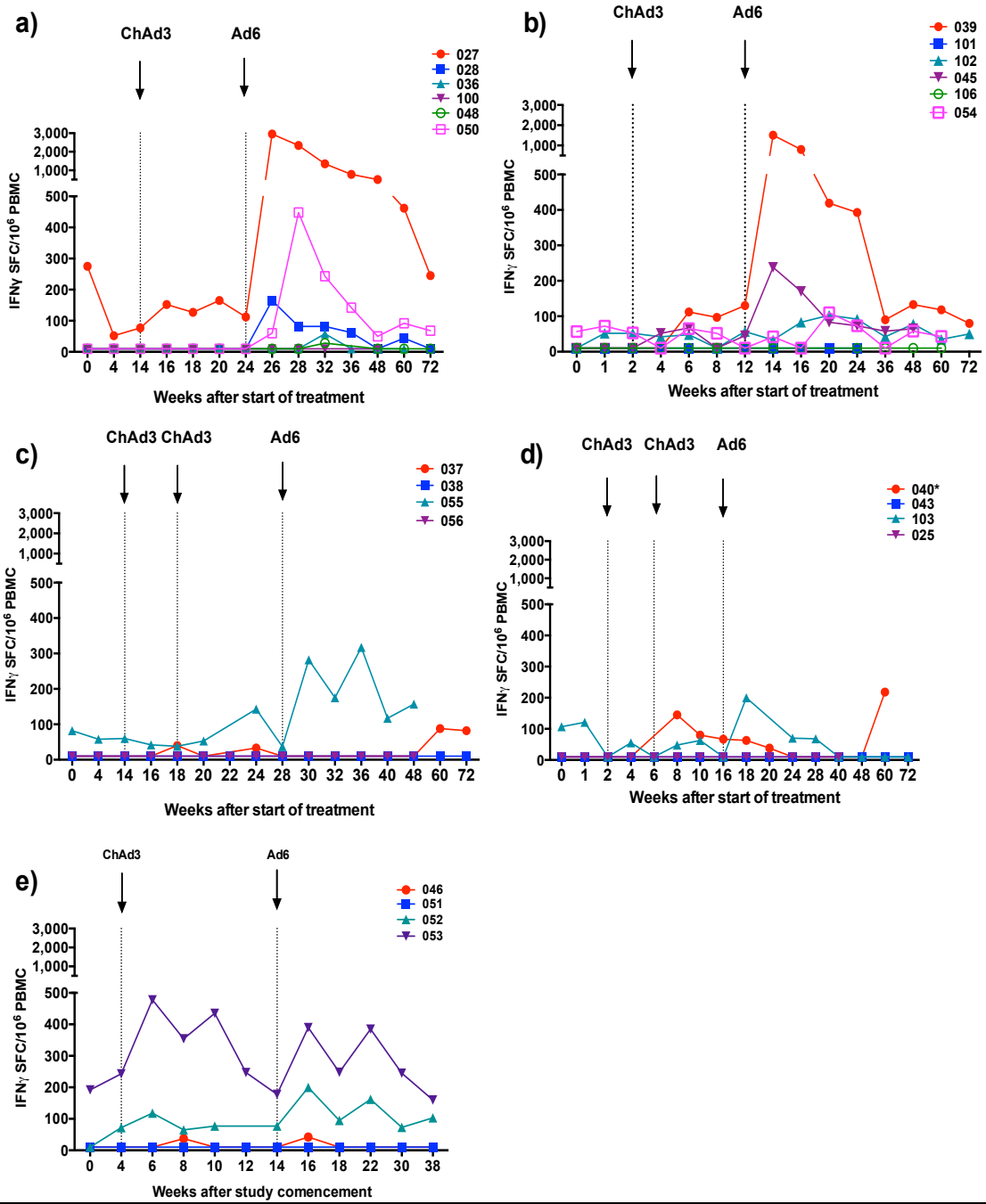
Peak IFNγ–ELISpot responses at any timepoint during treatment with IFN/RBV in patients not receiving vaccination. Responses on the y axis represent summed responses to positive pools representing the NS proteins in the vaccine immunogen. Individual patients are represented. Median values= horizontal bar. p values determined by Wilcoxon matched-pairs signed rank test, based on 6 patients for whom screening samples (pre-treatment) were available. (patients 001, 006. 008, 009, 010, 011, 012)

The kinetics of T cell responses in vaccinated patients was next assessed. There was a temporal relationship between vaccination and development of T cell responses, with peak IFNγ production detected 2 to 4 weeks following vaccination in the majority of patients (Figure 3-11). There was no statistically significant difference in SVR rates in arms A3-A6 between vaccine responders and non-responders (4/9 vaccine responders achieved SVR compared with 6/10 non-responders Fisher's exact test p=ns), although the study was not powered to address this.

Amongst patients not on treatment (Arm B3) 2/4 patients made T cell responses following vaccination, however responses were lower than in Arm A (Figure 3-11e). The median post prime response was 76 SFC/10^6PBMC; range 0-435, and median post boost response 121 SFC/10^6PBMC; range 0-390. These were not statistically significantly increased from a baseline median of 0

SFC/10^6PBMC; range 0-192. The kinetics of T cell responses by individual patients is shown in Figure 3-11.

Figure 3-11 Magnitude and kinetics of HCV specific T cell responses after high dose vaccination with ChAd3-NSmut (prime) and Ad6-NSmut (boost).



Kinetics of ex-vivo IFN-γ ELISpot responses over time, by individual, in patients receiving a)-d) concurrent PEG-IFNα/RBV therapy and e) vaccination alone. **a)** Arm A3 **b)** Arm A4 **c)** Arm A5 **d)** Arm A6 **e)** Arm B3. Responses shown are summed responses to NS peptide pools (see Materials and Methods). *patient relapsed and developed a new IFN-γ ELISpot response not attributed to vaccination.

3.7.1 What is the optimal vaccine regimen?

There were three different vaccination strategies assessed in this study; vaccination with or without concurrent antiviral therapy, vaccination in patients with a low viral load (on IFN/RBV) versus those with a high viral load and finally, single prime versus double prime vaccination.

3.7.1.1 Does concurrent IFN/RBV therapy impact on vaccine responses?

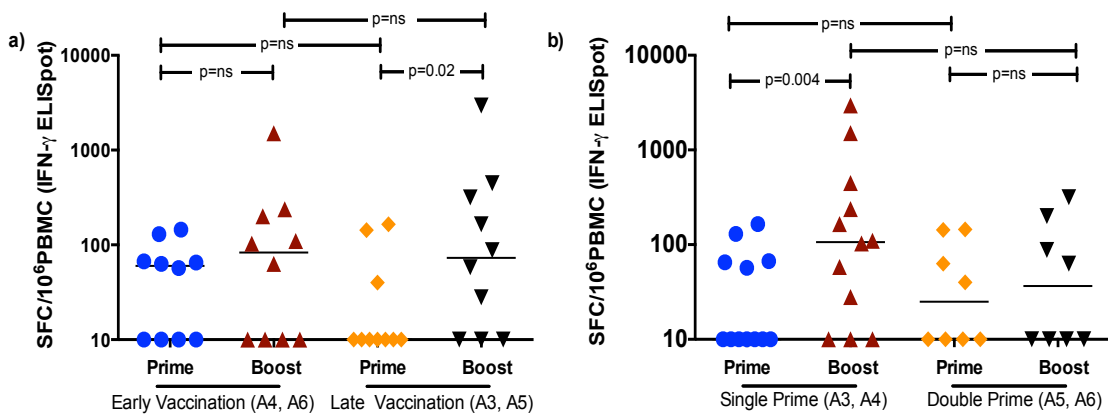
It is possible that IFN therapy could inhibit T cell responses and therefore impair vaccine immunogenicity. To explore this, a small group of 4 patients (Arm B3) received high dose vaccination without concurrent IFN/RBV. As demonstrated in Figure 3-11e, there were only 2 patients who mounted a vaccine response and this did not have a clear kinetic relationship to vaccination. One of these, (patient 002-053) also had an unusually high baseline T cell response, however did make a response to new peptide pool after vaccination (see Table 3-5 p121). Although it is difficult to directly compare T cell responses in Arm A and Arm B statistically due to the small sample size in Arm B, there was no indication that an IFN free vaccination regime would boost T cell responses. The median peak boost responses were similar between the Arms (121 SFC/10^6 PBMC in Arm B compared with 76 SFC/10^6 PBMC in Arm A) but there were no high magnitude responses as seen in certain individuals in Arm A (for example patients 027 and 039).

The other variable present in Arm B was that of ongoing viraemia at the time of vaccination: at priming the median viral load was 1.22×10^6 copies/ml and at boosting 2.06×10^6copies/ml. The effect of vaccination in the context of viraemia is explored further in the following analysis.

3.7.1.2 Is vaccinating in the context of a low viral load better than vaccinating with a high viral load?

Mouse models of LCMV infection have shown that therapeutic vaccination after a period of viral suppression is more efficacious than vaccinating when viral load is high (Wherry, Blattman and Ahmed, 2005). Therefore I compared vaccination with a low viral load after 14 weeks of IFN/RBV therapy (Arms A3 and A5) with vaccination given after 2 weeks of IFN/RBV treatment (Arms A4 and A6). For clarity I will refer to these two groups as the late and early vaccination groups from this point onwards.

Figure 3-12 Comparison of T cell responses to early versus late vaccination and single versus double priming vaccination with ChAd3-NSmut/NSmut



Peak magnitude IFNγ-ELISpot responses to summed NS pools (see Materials and Methods) are shown, by individual. Group medians are shown (horizontal bars). **a**) T cell responses in groups receiving early vaccination at wk 2 of IFN/RBV treatment (A4 and A6) and late vaccination at wk 14 of IFN/RBV treatment (A3 and A5). **b**) T cell responses in groups receiving a single priming vaccination (A3 and A4) compared with double priming vaccination (A5 and A6). p values were determined by Mann-Witney (unpaired data) and Wilcoxon matched-pairs signed rank test (paired data).

Viral loads were effectively suppressed in the late vaccination group. At the time of priming, 9/10 patients had an undetectable viral load and all had a negative viral load by the time of boosting. In the early vaccination group, median viral load at the time of first prime was 11,441 IU/ml (range 0-241,115), and 1/10

patients had a negative viral load. By the time of boosting (at week 12 in Arm A4 and week 16 in Arm A6), 5/10 patients had an undetectable viral load, 3/10 had a detectable but unquantifiable viral load (<15 IU/ml) and one patient had a low level of 998 IU/ml. Data was unavailable for one individual due to technical difficulties.

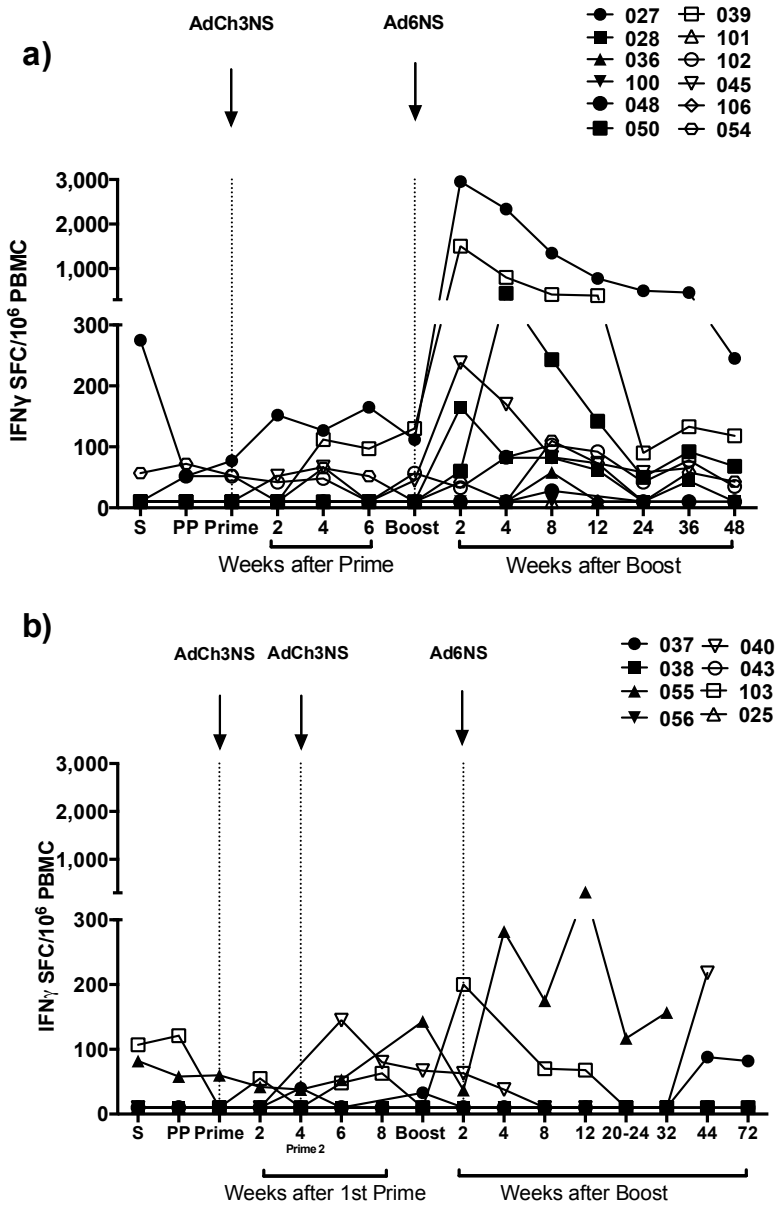
Similar numbers of patients mounted a vaccine-induced T cell response in the early and late vaccination groups: 6/10 in the early vaccination arms, compared with 7/10 in the late vaccination arms (p=ns) (Figure 3-12a). The highest response was seen in the late vaccination arm (2953 SFC/10^6 PBMC), however overall there was no difference in magnitude of responses between these two groups (Figure 3-12a). Median peak boost response was 63 SFC/10^6PBMC (range 0-1505) in the early vaccination arms, compared with 73 SFC/10^6PBMC (range 0-2953) in the late vaccination arms. Neither group showed a significant increase between screening and T cell responses after priming (data not shown) however only the late vaccination arms showed a significant increase in T cell responses between priming and boosting. Aside from this, there was no demonstrable benefit to vaccination following a prolonged period of viral suppression.

3.7.1.3 Is double priming more effective than single priming?

I also assessed the effect of administering a double prime vaccination 4 weeks apart (in Arms A5 and A6) compared with a single prime (Arms A3 and A4). 4/8 patients had positive T cell responses to vaccination in the double prime arms (median 36.5 SFC/10^6 PBMC; range 0-317) compared with 7/12 individuals receiving single prime (median 107 SFC/10^6 PBMC; range 10-2953), which was

not statistically significant (Fisher's exact test) (Figure 3-12b). There was no difference in magnitude of responses to priming between the two groups, however the single prime arms did show a significant increase in peak T cell responses between priming and boosting, unlike the double prime arms. There was also a trend towards higher responses in the single prime arms as illustrated in Figure 3-13 p107. The two highest responses were seen in the single prime arms (2953 and 1505 SFC/10^6 PBMC in Arms A3 and A4) whereas the highest response in a single individual in the double prime arms was 317 SFC/10^6 PBMC (Arm A6).

Figure 3-13 Magnitude and kinetics of T cell responses to HCV antigens after single and double priming vaccination with ChAd3-NSmut/Ad6-NSmut.



Kinetics of ex-vivo IFN-γ ELISpot responses over time, by individual patients receiving concurrent IFN/RBV therapy. Responses shown are summed responses to positive NS peptide pools (see Materials and Methods). S= screening. PP= pre-priming vaccination. **a)** Single Prime Arms A3 closed symbols, A4 open symbols **b)** Double Prime Arms A5 closed symbols, A6 open symbols.

3.8 Antivector Immunity

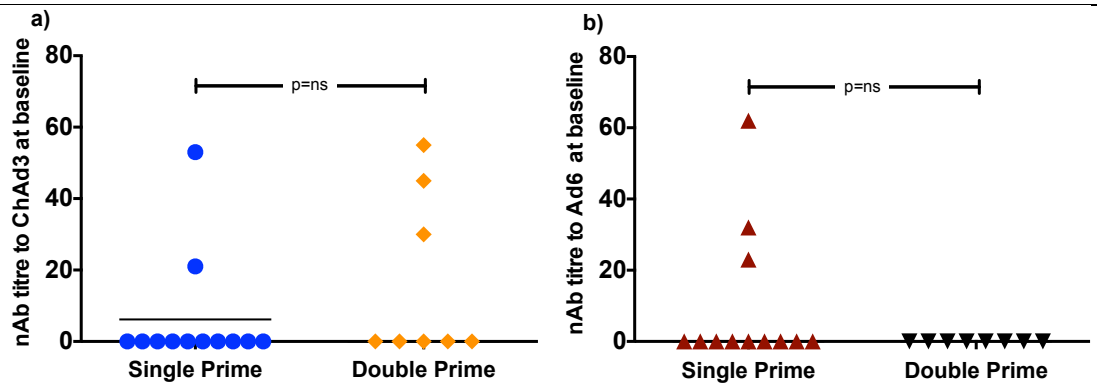
One explanation for the trend towards lower T cell responses in the double prime arms (A5 and A6) is that repeat exposure to adenoviral vectors induces antivector immunity. This was assessed through measuring neutralising

antibody (nAb) titres as well as T cell responses to Ad6-NSmut and ChAd3-NSmut.

3.8.1 Antivector Antibodies at baseline

At baseline, there was no statistically significant difference in either anti-Ad6 or anti-ChAd3 antibodies in the single prime arms compared with double prime arms (Figure 3-14). Patients with a nAb titre >200 were excluded from study enrollment (See *Materials and Methods*). nAbs were detected using an *in vitro* infectivity assay, performed by Okairos, which measures the ability of adenoviruses to infected a cell line after being incubated with serum from vaccinated patients.

Figure 3-14 Levels of antivector antibodies prior to vaccination in single prime compared with double prime arms in patients receiving concurrent treatment with PEGα-IFN/RBV



Baseline neutralising antibody titres (nAbs) to the priming vector ChAd3 (**a**) and to the boosting vector Ad6 (**b**) are shown by individual. Single Prime Arms are A3 and A4, Double Prime Arms are A5 and A6. Group medians are shown by a horizontal bar. p values were determined using a Mann-Witney unpaired t-test.

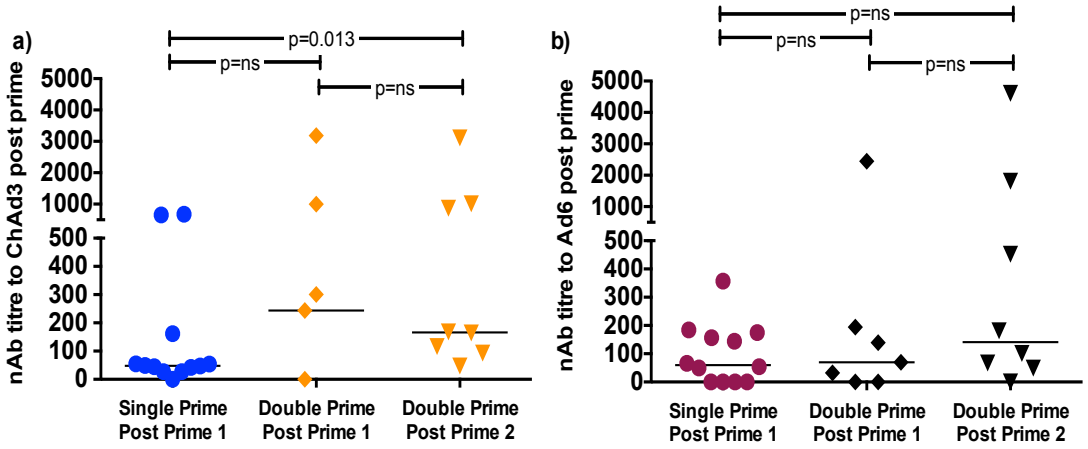
3.8.2 Antivector antibodies following vaccination with ChAd3/Ad6

3.8.2.1 Antibodies induced by priming with ChAd3

Following priming vaccination, nAbs were readily detectable not only to the homologous vector ChAd3, but also to the heterologous vector Ad6 (Figure 3-15). After the first priming vaccination, there was no difference in induction of antivector antibodies between the single and double prime arms. However, following the second priming vaccination, levels of anti-ChAd3 nAbs were higher in the double prime compared with single prime arms (p=0.013 Figure 3-15a). This difference was maintained at the time of boosting (Figure 3-16).

Antibodies that were cross-reactive with the heterologous vector (Ad6) also increased in some individuals following priming with ChAd3 (Figure 3-15b). In the double prime arms, 3 out of 8 patients had positive anti-Ad6 nAbs (titre >200) following a second priming vaccination (median 141; range 0-4608), compared with 1 out of 8 after the first priming vaccination (median 70; range 0-2445) (p=ns). This was similar to the single prime arms where only 1 out of 12 patients developed post prime anti-Ad6 nAbs (median 60; range 0 to 357).

Figure 3-15 Induction of antivector antibodies post priming vaccination with ChAd3-NSmut

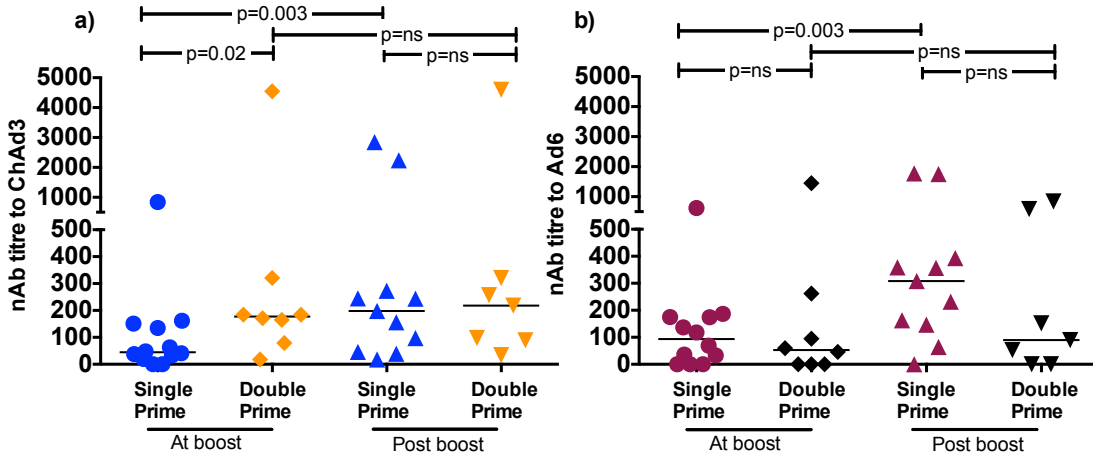


Induction of neutralising anti-vector antibodies following priming with ChAd3-NSmut (2.5 X10^{10}vp) in patients receiving concurrent treatment with IFN/RBV. **a)** nAbs to the homologous priming vector (ChAd3), 4 weeks post single and double prime. **b)** nAbs to the heterologous boosting vector (Ad6), 4 weeks post single and double prime. Median=horizontal line

3.8.2.2 Antibodies at and after boosting

Cross-reactive antibodies between heterologous adenoviruses, generated during a prime/boost strategy may significantly inhibit the T cell responses generated by the boosting vector. I evaluated the magnitude of cross-reactive antibodies generated in the single and double prime arms at the time of boosting and also the level of nAbs generated after boosting. Although there were more anti-ChAd3 antibodies in the double prime arms than in the single prime arms at the time of boosting (p=0.003) (Figure 3-16a), there was no significant difference in levels of cross-reactive anti-Ad6 nAbs (Figure 3-16b). After boosting, nAbs to ChAd3 and Ad6 both increased in the single prime arms (p=0.003), however not in the double prime arms.

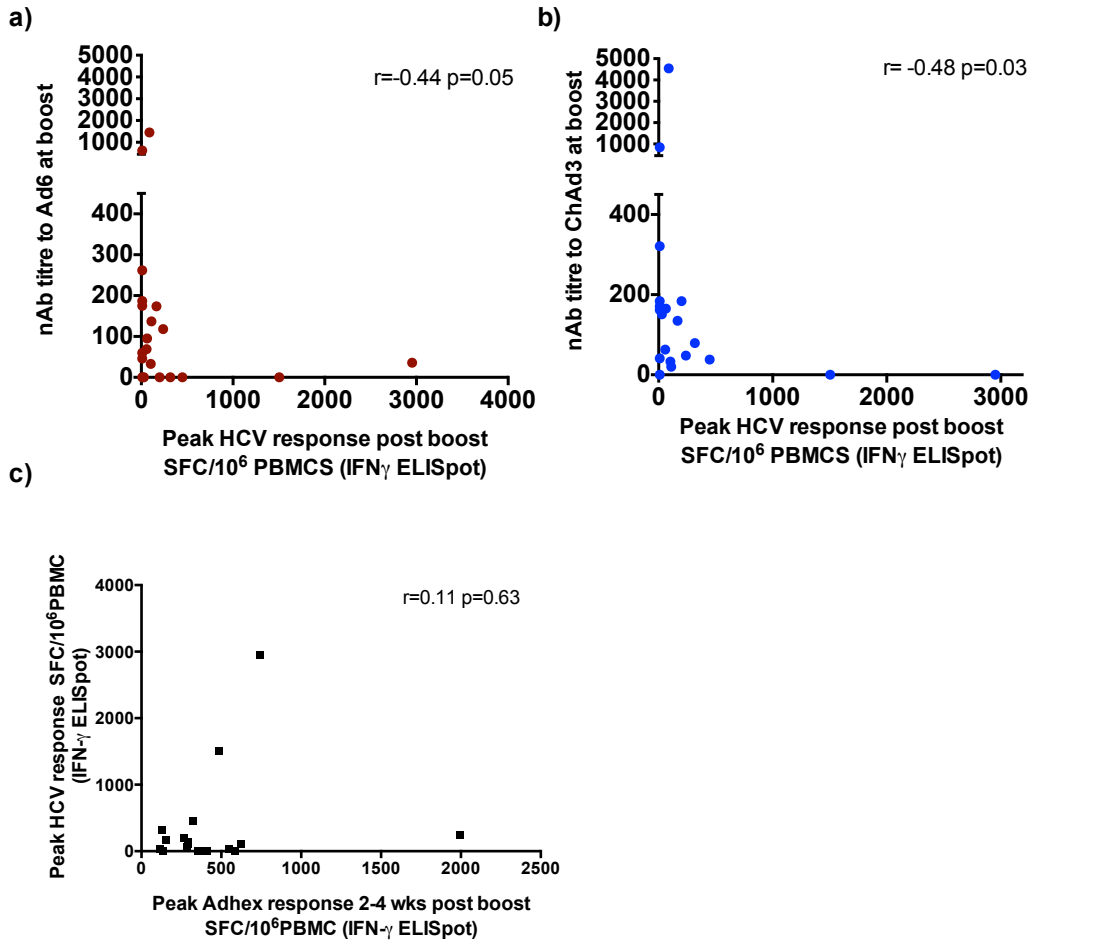
Figure 3-16 Induction of antivector antibodies post boosting vaccination with Ad6-NSmut



Induction of neutralising anti-vector antibodies following boosting with Ad6-NSmut (2.5 $\times 10^{10}$vp) in patients receiving concurrent treatment with IFN/RBV. **a)** nAbs to the priming vector (ChAd3) at the time of boosting, and 4 weeks post boosting in single prime and double prime arms **b)** nAbs to the boosting vector (Ad6) at the time of boosting, and 4 weeks post boosting in single prime and double prime arms. Median=horizontal line

It is possible that the higher induction of antivector antibodies amongst the single prime arms could reflect better immunogenicity of vaccination overall. However anti-ChAd3 nAbs correlated negatively with peak IFN-γ ELISpot responses (r=-0.48, p=0.03) and anti-Ad6 nAbs also trended in this direction although did not reach significance (r=-0.44, p=0.05) (Figure 3-17a and b). In contrast, anti-vector T cells correlated strongly with a more robust T cell response to the vaccine insert (r=0.9, p=0.0001(Figure 3-17c), reflecting stronger vaccine immunogenicity in those patients.

Figure 3-17 Correlation between antivector immunity and immunogenicity of the HCV immunogen



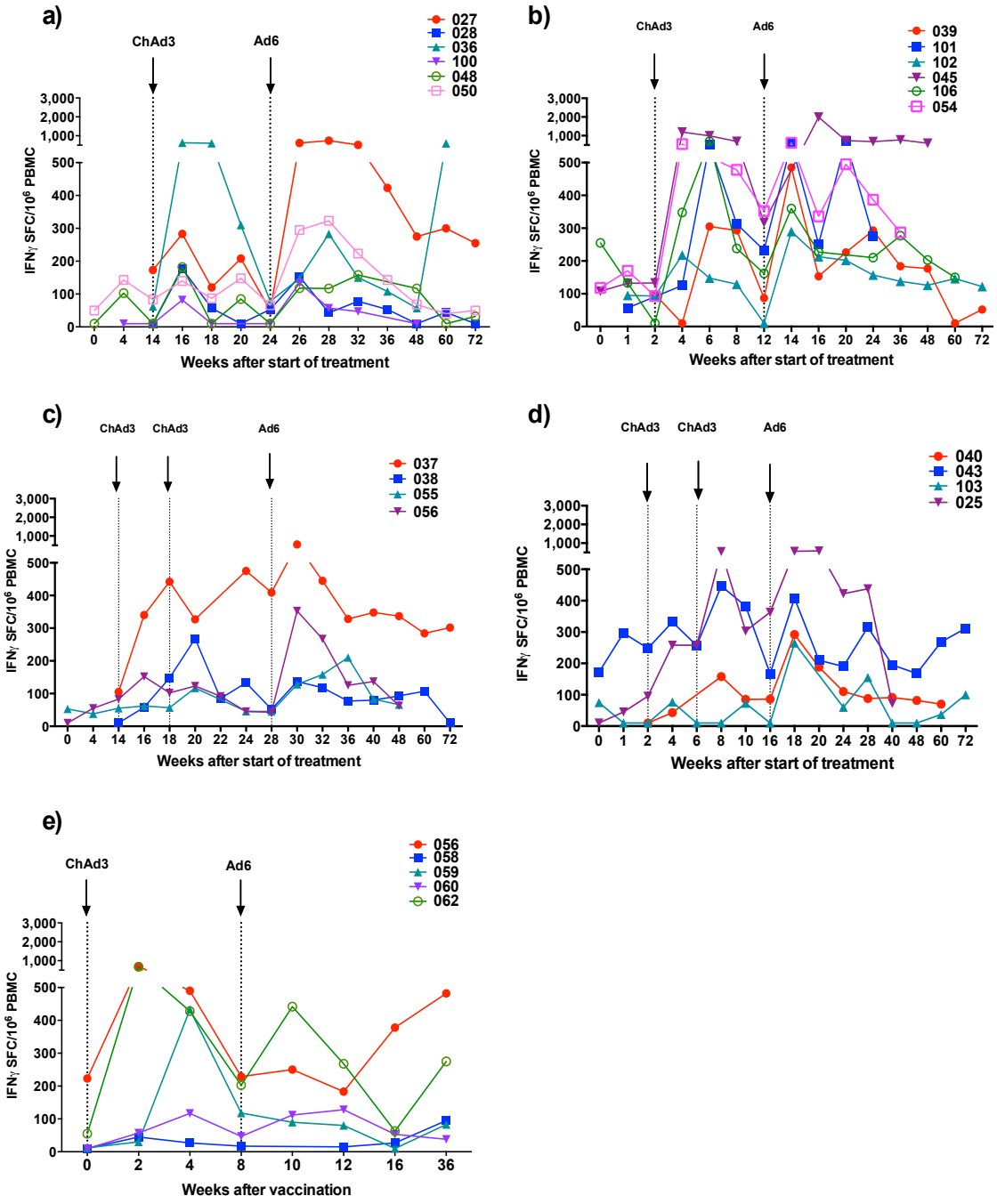
Correlation between nAbs to ChAd3 (**a**) or Ad6 (**b**) with peak post boost vaccine responses (measured by IFN-γ ELISpot). **c)** Correlation between peak anti-vector T cells and vaccine responses via IFN-γ ELISpot at any time point by individual in patients receiving high dose (2.5 $\times 10^{10}$vp) vaccination and concurrent IFN/RBVtherapy (Arms A3-A6).

3.8.3 Antivector T cell responses

There was no significant correlation between antivector T cell responses (to adenoviral hexon proteins) and anti-HCV T cell responses induced by vaccination (Figure 3-17c), as illustrated by the T cell responses charted by individual in Arm A in Figure 3-18. For example in Arm A3, 002-036 was a very strong responder to the vector (peak 600 SFC/10^6 PBMC) however made almost no response to the insert (peak 58 SFC/10^6 PBMC) (Figure 3-18a). Similarly in Arm A4, patient

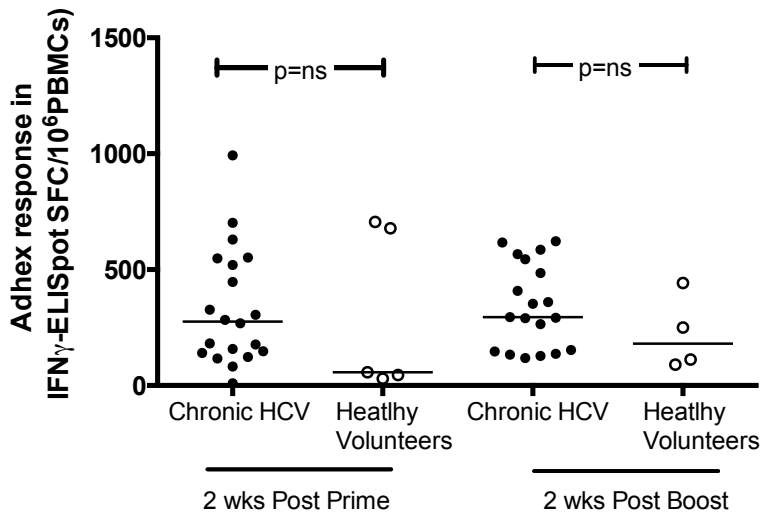
002-101 was one of the strongest T cell responders to the vector, however made no HCV specific response (shown previously in Figure 3-11a p102). This suggests that in some individuals it was possible to prime T cells to one antigen (the hexon) but not another (the HCV immunogen). This is supported by the larger magnitude responses overall to the hexon; the median peak post prime and peak post boost T cell responses in patients were 276 SFC/10^6 PBMC (range 77-1193) and 356 SFC/10^6 PBMC (range 133-1995) respectively. These responses were overall similar to those seen in healthy volunteers vaccinated with this regimen (Figure 3-18e). Although data is only available in 5 volunteers, there was no significant difference in healthy volunteers' T cell responses to the hexon measured 2 weeks after priming and boosting (median post prime 57 SFC/10^6 range 30-706, median post boost 181 SFC/10^6 range 90-442) and that of patients with chronic HCV (median post prime 275 range 10-995 SFC/10^6, median post boost 295 SFC/10^6 range 118-622 (Figure 3-19).

Figure 3-18 Anti-vector T cells following vaccination in Arm A and in healthy volunteers



Magnitude and kinetics of T cell responses to the hexon protein contained within the adenoviral vectors ChAd3 and Ad6. **a)-d)** Patients were vaccinated with 2.5 X 1010vp ChAd3 prime followed by Ad6 boost in conjunction with IFN/RBV therapy. Arms A3 (a) A4 (b) A5 (c) A6 (d) are shown. **e)** Healthy volunteers from group 10 in study HCV001 primed with ChAd3-NSmut and boosted with Ad6-NSmut (2.5 X10^{10}vp) are shown (data provided courtesy of Dr. E. Barnes)

Figure 3-19 T cell responses to the adenoviral hexon in patients with chronic HCV compared with healthy volunteers, vaccinated with ChAd3-NSmut and Ad6-NSmut.

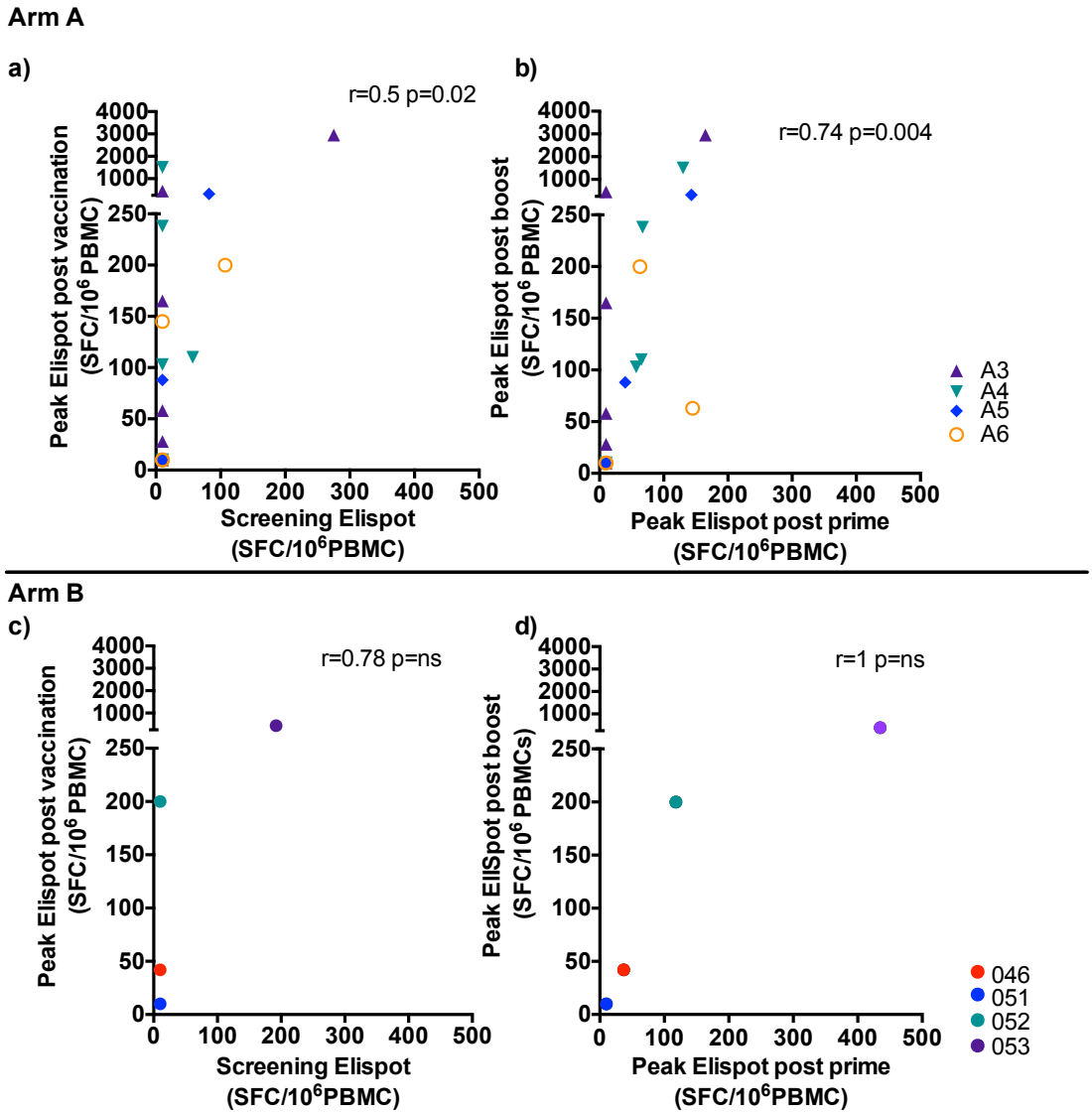


Magnitude of T cell responses to the hexon protein contained within the adenoviral vectors ChAd3 and Ad6, measured 2 weeks after priming and boosting vaccinations. **Chronic HCV** refers to patients vaccinated with 2.5 × 10^{10}vp ChAd3 prime followed by Ad6 boost in conjunction with IFN/RBV therapy. Patients from Arms A3-A6 are included. **Healthy volunteers** from group 10 in study HCV001 were primed with ChAd3-NSmut and boosted with Ad6-NSmut (2.5 ×10^{10}vp). Post boost data from volunteer 001-058 was not available. Other volunteers were 001-056, 001-060, 001-062) (figure derived from data provided by Dr. E. Barnes)

3.8.4 Do baseline HCV specific T cells predict response to vaccination?

It is possible that individuals with pre-existing T cell responses at baseline will be more likely to respond to vaccination either due to (i) an increase in those pre-existing responses, or (ii) a predisposition to generating HCV specific T cell responses. In Arm A, baseline T cell responses were shown to correlate moderately with peak at any time point (r=0.5 p=0.02) (Figure 3-20a), as well as peak prime and peak boost responses (r= 0.6 p=0.005, r=0.5 p=0.02 respectively) (data not shown). Response to priming vaccination also predicted response to boosting vaccination r=0.74, p=0.004 (Figure 3-20b). In Arm B, none of these factors were statistically significant (Figure 3-20c-d).

Figure 3-20 Correlation between baseline, peak prime and peak boost T cell responses to vaccination in Arms A and B.



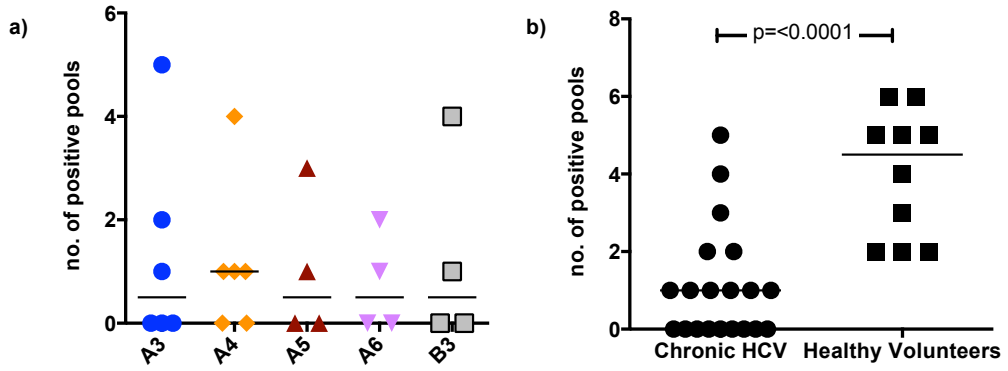
Correlation between ex vivo IFN-γ ELISpot responses by individual at baseline and post vaccination. Patients received ChAd3-NSmut prime and Ad6-NSmut (2.5 X 10^{10}vp) either with concurrent IFN/RBV therapy (Arm A) or vaccination alone (Arm B). **a) and c)** correlation between screening and peak response post vaccination. **b)** and **d)** correlation between peak response post prime and peak post boost. r= Spearman r test.

3.9 Mapping of peptide specific T cell responses

3.9.1 Breadth

The breadth of T cell responses to HCV has been correlated with clearance of acute infection in both human and animal studies (Lauer et al. 2004; Lechner, Wong, et al. 2000). The breadth of vaccine-induced responses was assessed with IFN-γ ELISpot assays using peptides grouped into six peptide pools of 73-112 peptides each, representing the NS3-NS5 immunogen (pools F/G=NS3, pool H= NS4A/B, pool I= NS5A, pools L/M= NS5B (see *Materials and Methods, p68*). 10 healthy volunteers vaccinated with these vaccines recognised a median of 4.5 pools, with 2 people recognizing all six pools (Figure 3-21b). In contrast, most patients with chronic infection responded only to a single pool, although 3 patients (1 from Arm A3, 1 from Arm A4 and 1 from Arm B3) recognised 5, 4 and 4 pools respectively (Figure 3-21a).

Figure 3-21 Breadth of T cell responses following vaccination with high dose ChAd3-NSmut/Ad6-NSmut.

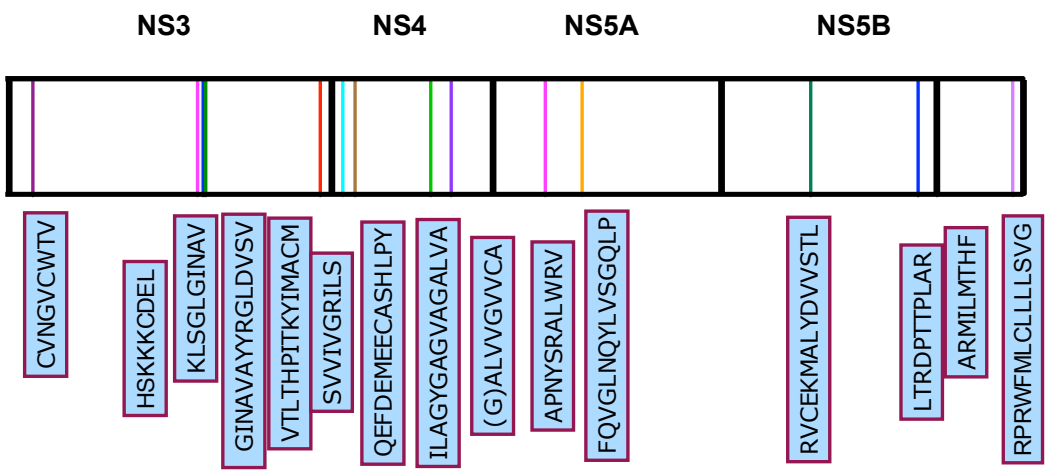


Number of vaccine related positive pools is shown (see *Materials and Methods*) measured at any timepoint following vaccination by IFN-γ ELISpot. Assays were performed using peptides grouped into six peptide pools of 73-112 peptides each, representing peptides from the NS3-NS5 immunogen (pools F/G=NS3, pool H= NS4A/B, pool I= NS5A, pools L/M= NS5B). Individual patients are represented by symbols. Group median is indicated by the horizontal bar. **a)** Study groups from HCV002TV are represented on the *x* axis. Patients in Arm A received concurrent IFN/RBV therapy, Arm B received vaccination alone. **b)** comparison between patients with chronic HCV from study HCV002TV and healthy volunteers from study HCV001. 001 volunteers are from Group 7 and Group 10. Group 7 (001-026, 001-032, 001-038, 001-039, 001-037) received double priming with ChAd3NSmut followed by Ad6-NSmut boost 2.5 X 10^{10}vp. Group 10 (001-056, 001-058, 001-059, 001-060, 001-062) received single priming with ChAd3NSmut followed by Ad6-NSmut boost 2.5 X 10^{10}vp. Figure derived from data provided by Dr. E. Barnes.

3.9.2 Identifying the optimal epitope targets of vaccine-induced T cells

Next, T cell responses were mapped to minipools and then to individual peptides using sequential IFN-γ ELISpot assays, where positive peptide pools were divided into subpools then into individual peptides. Where possible, the optimal epitope within the peptide was also identified. In 11 out of 15 patients where T cell responses could be mapped to the target peptide (Table 3-5), a total of 15 antigenic targets were identified across the non-structural immunogen (Figure 3-22). The most immunodominant epitope was $NS3_{1406\ (KLSGLGINAV),}$ where responses were identified in 5 out of the 11 mapped patients and in 25% of all HLA-A2 positive patients receiving high dose vaccination (Table 3-5). Of note, despite the inclusion of 8 HLA-A1 patients in this study, there were no responders to the HLA-A1 restricted epitope $NS3_{1446\ (ATDALMTGY)}$ which was frequently targeted in healthy volunteers vaccinated with this regimen (Barnes et al. 2012). Other responses were identified across the non-structural insert from NS3 up to the final peptide in the insert in NS5B.

Figure 3-22 Epitopes targeted by vaccine-induced T cells, in patients vaccinated with ChAd3-NSmut and Ad6-NSmut.



The black rectangle is a schematic representation of the HCV genome from NS3-NS5B. Non-structural regions of the HCV genome are separated by vertical black lines. Epitopes targeted by vaccine-induced T cells are represented by different coloured vertical lines at the corresponding position along the HCV genome, and the amino acid sequence at each epitope is shown in the ringed box below each coloured line.

Table 3-5 Mapping of T cell targets from patients in Study HCV002TV, who have made T cell responses following vaccination with ChAd3-NSmut (prime) /Ad6NS-mut (boost).

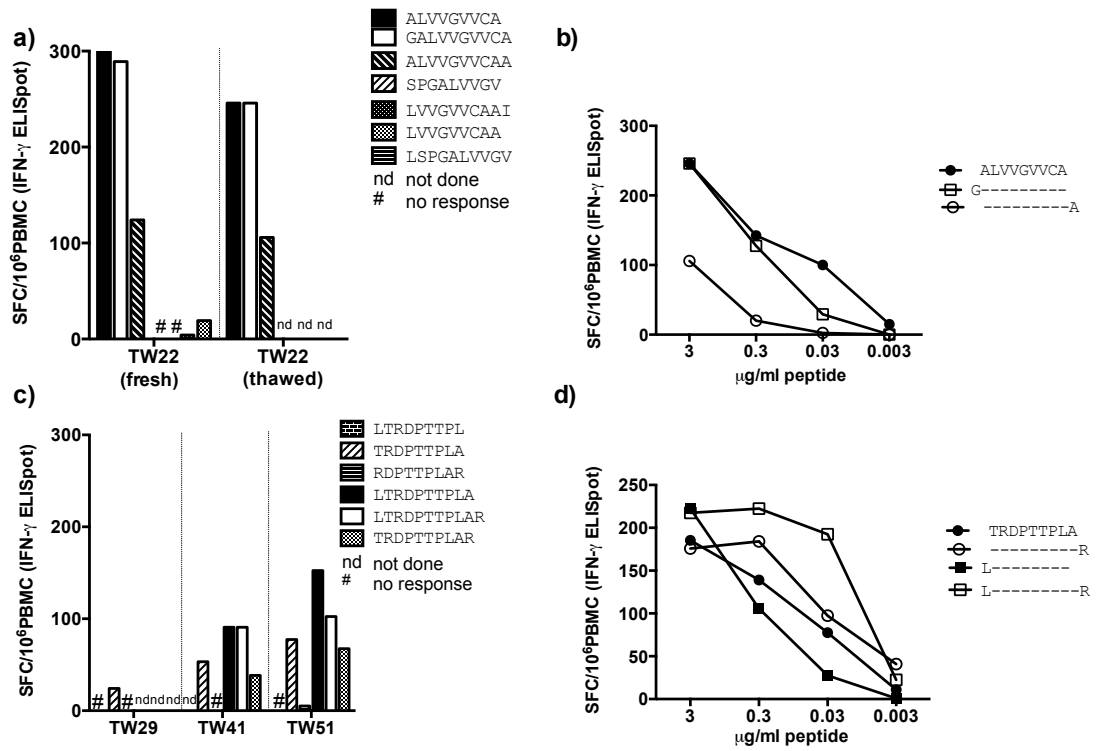
Group (vaccines)	Patient	HLA-A	HLA-B	HLA-C	pre-vaccination +ve pools (IFNγ-ELISpot SFC/10^6PBMCs)		post vaccination +ve pools (IFNγ-ELISpot SFC/10^6PBMC, week of peak response)		Mapped to peptide (parent pool)
					Non-vaccine	Vaccine	Non-vaccine	Vaccine	
A1 (Wks 14,24)	20	2 30	18 8	5 7	-	-	-	-	-
	30	68 2	53 51	6 1	A (125)	F (58)	A (135, Wk16)	-	-
A2 (Wks 14, 24)	24	2 2	44 27	5 1	A (205)	-	A (408, Wk26)	-	-
	31	29 2	44	5 16	A	-	-	-	-
A3 (Wks 14,24)	27	30 2	44	7 5	A (57)	F (40), G (45), H (170), L (120)	D (155, Wk26)	F (213, Wk26), G (748, Wk26), H (922, Wk26), L (918, Wk26), **M** (152, Wk26)	CVNGVCWTV (F) KLSGLGINAV (G) LTTGSVVIVGRIILS/ SVVIVGRIILSGRPA* (H) QEFDEMEECASHLPY* (H) ILAGYGAGVAGALVA* (H) RVCEKMALYDVVSTL* (L) RPRWFMLCLLLLSVG* (M)
	28	11 2	44 37	6 5	A (672), B (38)	-	A (553, Wk26), B (43, Wk26), C (35, Wk26)	**G** (88, Wk26) **H** (43, Wk26), **M** (35, Wk26)	KLSGLGINAV* (G)
	36	3 2	44 51	16 14	A (65)	-		-	
	100	24 2	13 60	6 10	-	-	-	-	
	48	1 3	8 35	4 7	A (72)	-	A (50, Wk16)	-	
	50	2 30	8 49	7	A (172)	-	A (102, Wk16)	**F** (50, Wk38), **G** (388, Wk28)	KLSGLGINAV (G)
A4 (Wks 2, 12)	39	24 30	13 7	6 7	-	-	-	**G** (80, Wk14), **H** (85, Wk14), **I** (794, Wk14), **L** (37, Wk14), **M** (510, Wk14)	TPSPAPNYSRALWRV/ APNYSRALWRVAAEE* (I) FQVGLNQYLVGSQLP* (I) LSRARPRWFMLCLLL/ RPRWFMLCLLLLSVG* (M)
	101	24 3	81 35	18 4	D (45)	-	B (31, Wk14)	-	-
	102	68 3	27 39	1 7	-	M (52)	-	M (104, Wk20)	-
	45	11 1	8 7	7	A (44), C (84)	-	C (103, Wk16)	**G** (238, Wk14)	HSKKKCDEL (G) VTLTHPITKYIMACM (G)
	106	3 29	35	4 8	A (158)	-	A (131, Wk16)	-	
	54	23 2	27 7	1 7	A (53)	M (72)	A (50, Wk20)	M (110, Wk20)	ARMILMTHF (M)
A5 (Wks 14,18,28)	37	1 2	49 44	5 7	A (150), C (39)	-	A (100, Wk18), C (58, Wk32)	**L** (40, Wk18)	
	38	66 2	41 18	7 17	A (148), B (38)	-	A (91, Wk18)	-	
	55	2 2	13 39	6 12	A (126)	M (81)	-	**F** (88 Wk36), **G** (112, Wk36), M (143, Wk36)	CVNGVCWTV (F) KLSGLGINAV (G) LTRDPTTPLAR* (M)
	56	2 30	18 8	6 5	-	-	-	-	
A6 (Wks 2,6, 16)	40	24 2	60 7	7 10	A (48)	-		**G** (145, Wk8),	KLSGLGINAV (G)
	43	2 1	62 7	7 10	A (132)	-	A (157, Wk8)	-	-
	103	2 2	44	5 16	A (80)	F (120)	A (92, Wk4)	F (67, Wk10), **G** (164, Wk18)	CVNGVCWTV (F) KLSGLGINAV (G)
	25	2 68	18 65	7 8	-	-	-	-	
B1 (Wks 4, 14)	29	29 1	44 57	12 8	-	-		-	
	34	24 68	65 35	6 16	A (90)	-	A (115, Wk14)	**I** (56, Wk16)	
B2 (Wks 4, 14)	35	2 23	44 62	4 10	-	-	-	-	
	44	2 1	8 44	4 7	A (45)	-	A (37, Wk6)	**G** (102, Wk6)	
B3 (Wks 4, 14)	46	1 2	8 44	4 7	A (43)	-	A (79, Wk18)	-	
	51	11 32	18 51	2 5	A (78)	-	A (88, Wk18)	-	
	52	1 2	7 65	7 8	A (39)	G	A (48, Wk6)	G (200, Wk16)	GINAVAYYRGLDVSV (G)
	53	2 26	64	8	A (108)	F (52), H(165), I (43)	A (109, Wk6)	F (92, Wk8), **G** (50, Wk6), H (270, Wk6), I (81, Wk6)	CVNGVCWTV (F) Fh (minpool only) ALVVGVVCA (G) Hf (minipool only)

* CD8 depletion IFNγ-ELISpot performed. New positive pools after vaccination are shown in **bold** and **red** type.

3.9.3 2 novel epitopes are identified

Many of the epitopes identified in Figure 3-22 are well described in the setting of natural infection, suggesting that HCV specific T cells relevant to natural *in vivo* immune responses are generated. Where epitopes were not well described, I aimed to identify the optimal (shortest most immunogenic) epitope within the peptide by performing IFN-γ ELISpot assays using peptides of different lengths. Two novel epitopes were identified using this method: $NS4B_{1891\text{-}1899}$ (ALVVGVVCA) in patient 053 and $NS5B_{2804\text{-}2814}$, (LTRDPTTPLAR) in patient 055.

Figure 3-23 Identification of novel epitopes $NS4B_{1891\text{-}1899}$ (ALVVGVVCA) and $NS5B_{2804\text{-}2814}$, (LTRDPTTPLAR).



Comparison of IFNγ-ELISpot responses to optimal length peptides in 2 patients 002-053 (a-b) and 002-055 (c-d), following vaccination with ChAd3-NSmut/Ad6-NSmut. **a**) magnitude of response at 3μg/ml to possible optimal peptides in patient 002-053 **b**) IFNγ-ELISpot responses to serially diluted peptides on thawed cells at week 22 in patient 002-053 **c**) magnitude of response at 3μg/ml to possible optimal peptides in patient 002-055 **d**) IFNγ-ELISpot responses to serially diluted peptides on thawed cells at week 37 in patient 002-055.

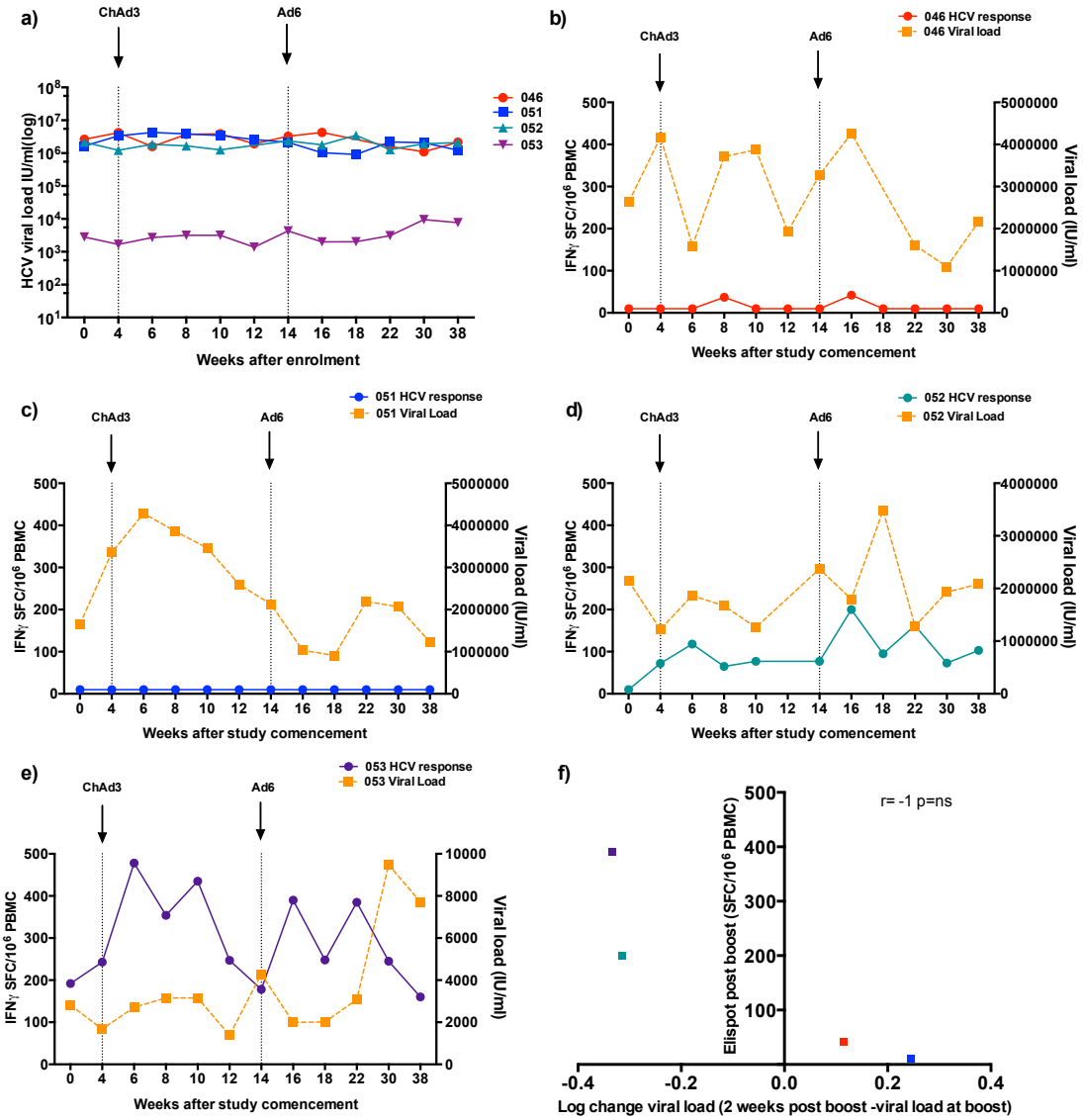
In patients 053, responses were mapped to the peptide NS4B$_{1897\text{-}1901}$, LSPGALVVGVVCAAI. To define the optimal epitope, seven different peptides were trialed (Figure 3-23a). It was not possible to differentiate between the immunogenicity of two versions GALVVGVVCA and ALVVGVVCA until peptide titration was performed, where it became clear that at lower, more physiological concentrations, ALVVGVVCA was the most likely epitope (Figure 3-23b). Since neither this epitope nor this 15 amino acid long peptide have been described in the context of HCV infection previously, I tested this peptide in 2 other HLA-A2 positive volunteers who had a T cell response to pool containing this peptide (pool H) however neither made a T cell response to this optimal (data not shown).

When the T cell response in patient 002-055 was mapped to peptide NS5B$_{2800\text{-}2814}$ RVYYLTRDPTTPLAR, I first tested the optimal LTRDPTTPL, which has previously been described in the context of chronic HCV infection (Day et al. 2002). This however was negative on two occasions (Figure 3-23c) so I preceded to trial 5 other versions. LTRDPTTPLA appeared to generate the strongest T cell responses (153 SFC/10^6PBMC compared with the next strongest version LTRDPTTPLA**R** (103 SFC/10^6PBMC). However, peptide titration assays revealed that the R in the terminal position was important, with higher responses across the diluted peptide range with the longer optimal LTRDPTTPLAR (Figure 3-23d).

3.10 What is the effect of vaccination on viral load?

I next assessed the effect of vaccination on viral load in patients receiving high dose vaccination without concurrent IFN/RBV therapy (Arm B3). In clinical practice viral load is frequently measured as $\log_{10}$ fold change. Using this, I tracked viral load over time. Overall, there was no significant change between viral load measured at boosting and 24 weeks later (range -log0.09 to -log 0.29 p=ns) (Figure 3-24a). I also examined viral loads measured in IU/ml to detect small variations in relation to T cell responses. Patients 046 and 051 had no T cell response to vaccination and no change in viral load. However the two individuals with IFN-γ ELISpot responses to vaccination (052 and 053) both had a small decrease in viral load post vaccination, although this was in the context of an oscillating viral load throughout the study (Figure 3-24d-e). Although the correlation between post boost change in viral load (2 weeks after boost) and post boost IFNγ-ELISpot response was not significant, there was a trend between T cell response and decrease in viral load (r=-1 p=ns).

Figure 3-24 Effect of vaccination with ChAd3-NSmut/Ad6-NSmut on viral loads in Arm B3



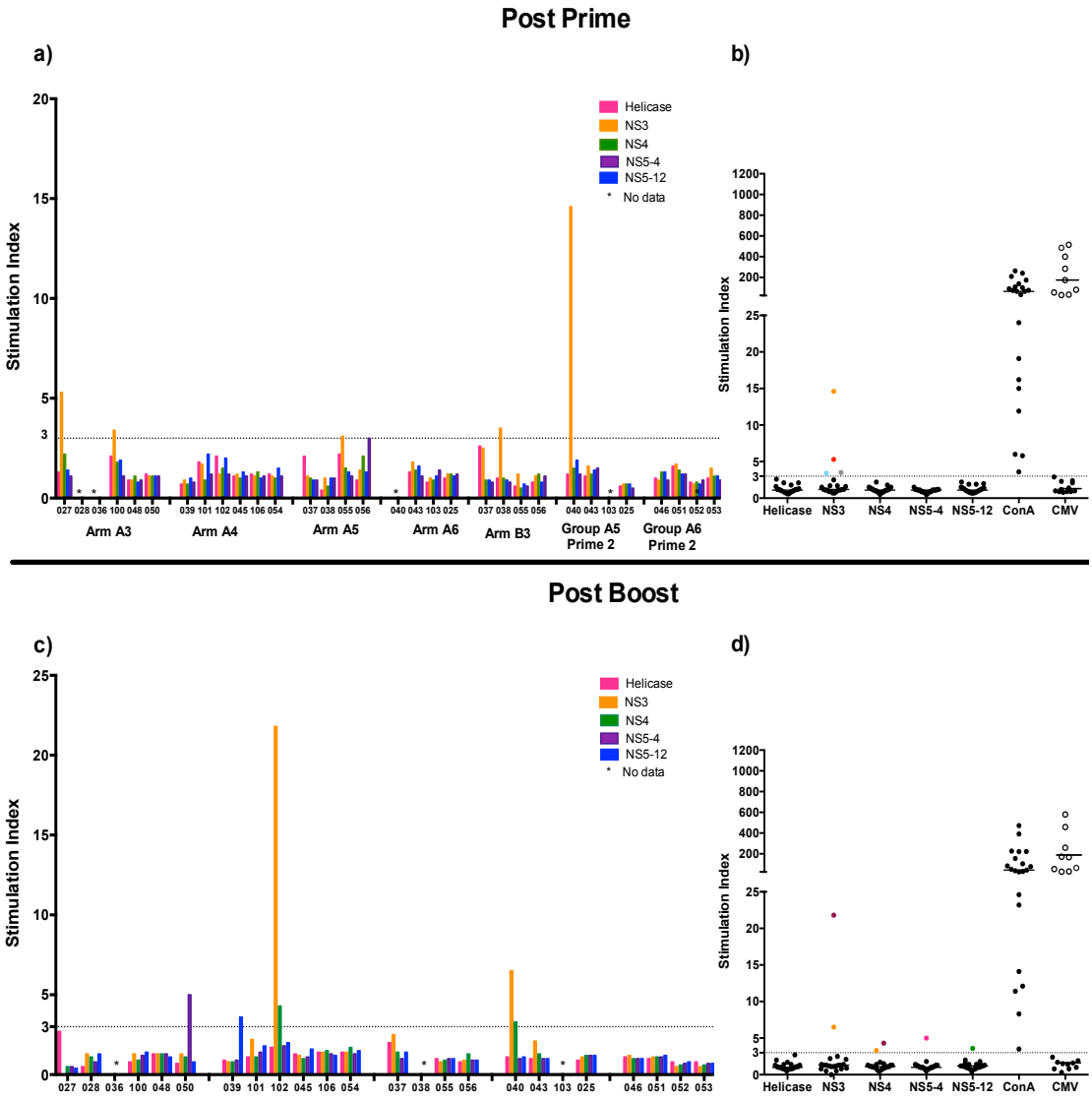
a) HCV viral load (log_{10}) over time by individual (046, 051, 052, 053) in Arm B3, receiving vaccination alone (2.5×10^{10}vp) **b)–e)** HCV viral load (IU/ml) compared with HCV specific IFNγ-ELISpot responses by individual **f)** correlation between change in viral load and IFNγ-ELISpot responses 2 weeks after boosting vaccination in Arm B3 using Spearman r test and 2 tailed p value.

3.11 What is the quality of T cells produced by vaccination?

3.11.1 Proliferative Capacity

The ability of T cells to proliferate in response to re-exposure to antigen is critical to the success of an immune response and is a key marker of T cell functional capacity. Using thymidine proliferation assays, proliferative responses were detected after priming vaccination in only 4/21 patients with samples available for analysis compared with 8/10 healthy volunteers in study HCV001 (p=0.002 Fisher's exact test) (Barnes et al. 2012). The median stimulation index (SI) in these 4 patients was 4.4 (range 3-14.6) (Figure 3-25). Proliferative responses after boosting were only slightly stronger than after priming, with a median SI of 7.1 in the 4 patients who responded to boosting (range 3.6-26.1). Only 2 of these responded to more than one pool. In contrast, there were robust proliferative responses to CMV in CMV positive patients (median post prime SI 173.6; range 30.2-1104.0) (Figure 3-25d).

Figure 3-25 Proliferative response to high dose vaccination with ChAd3-NSmut/Ad6-NSmut



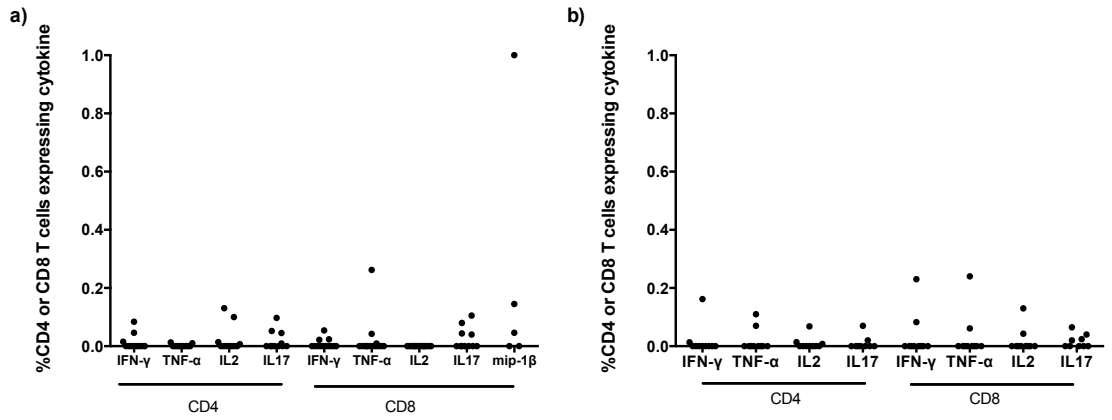
Proliferative responses to HCV antigens, in patients 4 weeks post prime with ChAd3-NSmut (**a-b**) and 4 weeks post boost with Ad6-NSmut (**c-d**) (2.5×10^{10}vp) plotted as Stimulation Index (SI). SI ≥ 3 was defined as positive. **a**) and **c**) individual patients are shown on the x axis. Responses to peptide pools are shown by different coloured columns. **b**) and **d**) SI to HCV peptide pools was compared with responses to a positive control (concanavilin A), and to CMV peptides (**o**= CMV positive patients, •= CMV negative patients defined by IFN-γ ELISpot are shown). Patients with positive SIs to HCV peptides are coloured individually (post prime: 040, 027, 100, 038) (post boost: 102, 040, 027, 039) bar=median

3.11.2 Intracellular Cytokine Staining

I next assessed cytokine production in both $CD4^+$ and $CD8^+$ T cells using intracellular cytokine staining (ICS). Initially ICS was performed on all *ex-vivo*

samples, then, due to the lack of detectable responses, was only performed where IFN-γ ELISpot responses were ≥ 150 SFC/10^6 PBMC post prime. Using ICS, cytokine secretion by CD4+ and or CD8+ T cells could be detected in 0/10 patients post prime vaccination (data not shown), and only 6/13 post boost. Most produced only a single cytokine and were at low frequency (0.01% - 0.28% of $CD4^+$ or $CD8^+$ T cells) with the exception of one patient who responded to stimulation with a single peptide with a very strong production of mip1β (1% of $CD8^+$ T cells) (Figure 3-26).

Figure 3-26 ICS responses after high dose boosting vaccination with Ad6-NSmut in patients in Arm A and Arm B



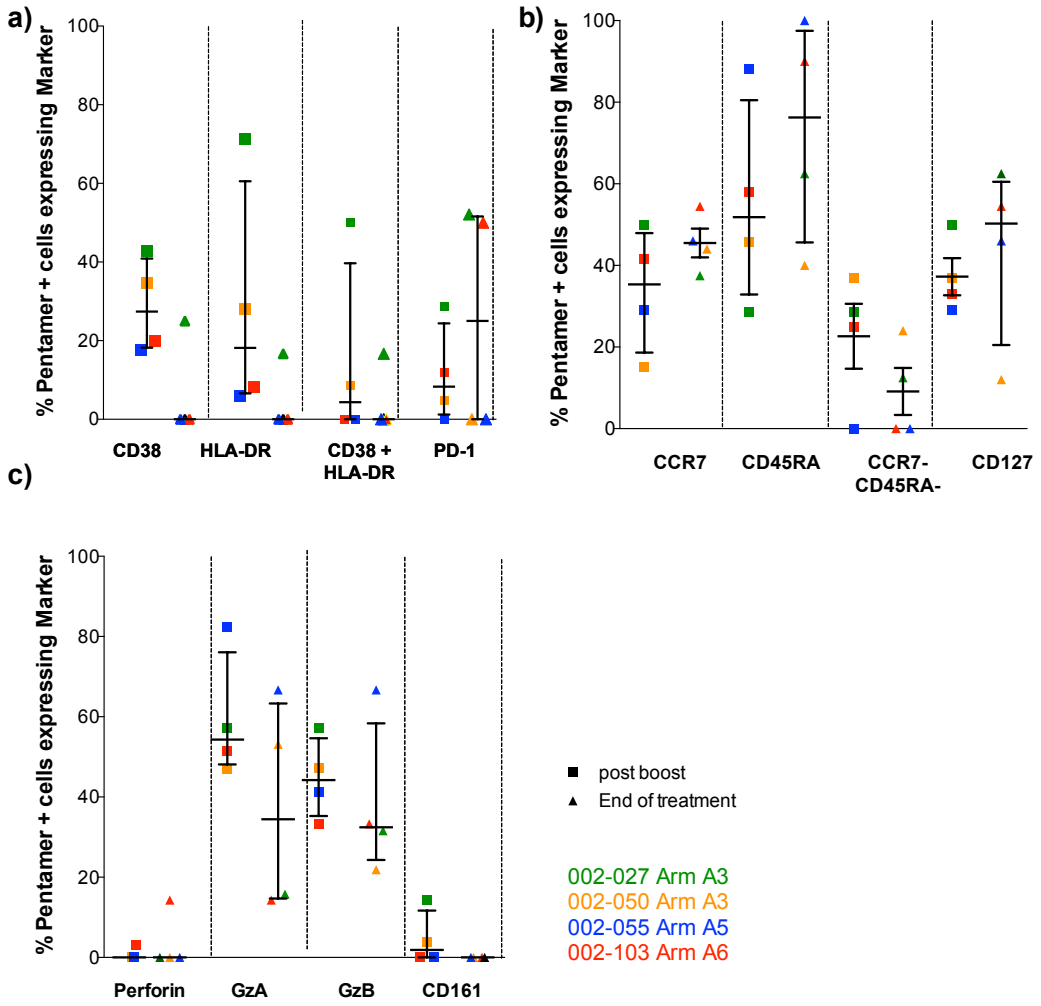
ICS responses (with background subtracted) after boosting vaccination with ChAd3-NSmut/Ad6-NSmut in patients in Arms A and B. **a**) Responses to stimulation with pooled peptides representing the NS3-4 region. Single peptide stimulation was also performed in 5 individuals (050, 052, 053, 054, 055) and mip1β was assessed in these patients. **b**) Responses to stimulation with pooled peptides representing the NS5 region.

3.11.3 Phenotyping vaccine-induced T cells

Next I used pentamers to examine the phenotype of vaccine specific T cells in 4 individuals at two timepoints: after boost vaccination and at the end of treatment (Figure 3-27). This revealed a vaccine specific $CD8^+$ T cell population which was low in perforin, with intermediate expression of granzyme A and B

after boosting (median 0%, 54.3% and 44.2% $CD8^+$ cells respectively). Levels of activation markers were also low, however were similar to those seen in healthy volunteers who received these vaccinations, with median post boost $pentamer^+/CD38^+$ cells 27.4% (range 17.6-42.9) (Figure 3-27a) compared with 25% in healthy volunteers (see Barnes et al. 2012). $Pentamer^+/HLA\text{-}DR^+$ cells were 18.17% (range 6-71.4) compared with 17.55% in healthy individuals (Barnes et al. 2012). Only a median of 26.8% (range 0-37%) of vaccine-induced cells displayed an effector memory phenotype (CCR7-/CD45RA-) after boosting (Figure 3-27b), although many cells displayed the marker of long term memory CD127 at the end of treatment (median 50% range 20.5-60.5), suggesting in some individuals the generation of a long term memory population.

Figure 3-27 Phenotype of pentamer positive HCV specific T cells



Activation status (**a**), memory differentiation (**b**) and cytotoxicity (**c**) of pentamer$^+$ cells (KLSGLGINAV $NS3_{1406}$) expressing the characteristic on x axis (gated on live $CD8^+$ cells). 4 patients (027, 050, 055, 103) receiving concurrent PEG-IFNα/ribavirin therapy, are shown post Ad6-NSmut boost 2.5×10^{10}vp (■) and at end of treatment (▲). All represent new responses post vaccination except for patient 002-027 who had an increase in a pre-existing IFNγ-ELISpot response (45 SFC/10^6PBMC to 748 SFC/10^6PBMC).

3.12 Discussion of Safety and Immunogenicity.

This is the first reported trial of therapeutic vaccination using adenoviral vectors in patients with chronic HCV and the only in depth characterisation of therapeutically induced T cell responses in chronically infected individuals. The key correlates of vaccine efficacy assessed here are the magnitude and breadth of T cell responses induced by vaccination and the functional capacity of these cells as shown by ICS, proliferative capacity and T cell phenotype.

Vaccination is safe

This study shows that T cells can be safely induced in patients with chronic HCV, to levels not previously reported in other therapeutic HCV vaccine trials, where IFNγ-ELISpot responses are typically under 200 SFC/10^6PBMC (Alvarez-Lajonchere et al. 2009; Klade et al. 2012; Habersetzer et al. 2011).

Adverse events were generally mild and did not affect the administration of further vaccinations, with the exception of one patient who developed a rash and was withdrawn from the study. Importantly liver immunopathology did not occur in 23/24 patients who received high dose vaccination. There was a significant ALT flare in one patient, although given the lack of detectable T cell responses in this patient at any stage it is unclear whether vaccine-induced immunopathology played a role in this. Nonetheless a contribution from vaccination cannot be ruled out and monitoring LFTs in future patients will remain an important part of ongoing studies.

An important confounder in the analysis of adverse events was the co-administration of IFN/RBV in Arm A, which both carry a significant side effect

profile. This means that adverse events were likely to have been overestimated in this study, as it was difficult to distinguish between AEs caused by IFN/RBV and those caused by vaccination. The true prevalence of AEs related to these vaccines is likely to be lower than that reported here.

Antivector antibodies may affect immunogenicity

This study explored the immunogenicity of different therapeutic vaccination regimens, considering the effect of single versus double priming, concurrent IFN/RBV therapy and vaccination after a period of viral suppression.

A number of trends were apparent, although few reached statistical significance, likely in part due to sample size. There was a trend towards weaker responses in the double prime arms compared with single prime arms, with fewer vaccine responders overall and no high magnitude responders. Although this could have been due to the smaller numbers of patients in the double prime Arms (8 compared with 12 in the single prime arms), antivector immunity could have also played a role. In this study it was thought that using heterologous viral vectors, including a rare human adenovirus Ad6 could circumvent this problem. There is a lower community prevalence of anti-Ad6 antibodies than other commonly used adenoviruses like Ad5 (12% compared with 35% respectively) and they appear not to be cross-reactive with Ad5 (Fattori et al. 2006; Capone et al. 2006). Similarly, anti-ChAd3 antibodies are uncommon in humans and were thought not to be cross-reactive with Ad6, making them ideal for a heterologous vaccination regimen. However, the problem of cross-reactive immunity between these vectors was initially suggested in healthy volunteers given the same vaccines as patients in this study. These volunteers had strong priming

responses with ChdAd3-NSmut, but weak boosting with Ad6-NSmut (Barnes et al. 2012). In chronically infected patients, double priming vaccination with ChAd3 induced significantly higher levels of anti-ChAd3 antibodies than single priming and was also able to induce cross-reactive anti-Ad6 antibodies, suggesting a degree of cross-reactivity between the two viruses. This was not seen in monkeys vaccinated with ChAd3 (S. Capone, personal communication) where boosting with heterologous Ad6 was robust, and anti-Ad6 antibodies were not detected after priming.

One explanation for the difference between human and animal antibody production is that, unlike animals in an isolated laboratory environment, humans are likely to have been infected with previous adenoviral infections from multiple serotypes. This could result in a multiple cross-reactive antibodies being produced upon vaccination with ChAd3, some of which will neutralise Ad6.

Furthermore, whilst these two adenoviruses are distinct serotypes, they are both within the group C subtype of adenoviruses and are fairly closely related when compared on a phylogenetic tree (Colloca et al. 2012). It is therefore possible that the presence of antibodies, to one or the other of these adenoviruses could neutralise viral particles and effectively result in a lower dose of vaccination. This is clearly significant given the poor immunogenicity demonstrated in the low and medium vaccination dose groups in this study. Further studies are ongoing within the Klenerman/Barnes group assessing the use of alternative boosting vectors, such as MVA-NSmut, which could overcome the problem of potential cross-reactivity.

In contrast, T cell responses to the hexon component of the vector correlated positively with immunogenicity of the insert. Whilst antibodies might neutralise the vector, cell mediated immunity appears to be a biomarker of effective transduction (ability to transfer foreign material into host cells) of the vector and expression of both HCV and vector proteins.

IFN therapy did not inhibit T cell responses to vaccination

The effect of co-administration of IFN was also explored in this study. There was no suggestion in this study that IFN interfered with vaccination; indeed the individuals with the highest T cell responses to vaccination were those in Arm A of the study, receiving concurrent IFN/RBV. Although only four patients received high dose vaccination without IFN/RBV therapy (Arm B3), there was no significant difference in median T cell responses between Arm A and Arm B. This is consistent with the limited data available in the literature regarding the effect of IFN on vaccination.

In mice it has been shown that endogenous Type I IFN production from adenoviral vectors themselves does not impact on adenoviral transduction (Huarte et al. 2006). Human *in vivo* data comes from a small study of 30 HCV infected individuals which showed that there was no difference in seroconversion rates to Hepatitis B virus (HBV) vaccination between those receiving concurrent IFN/RBV therapy and those receiving vaccination alone (Elefsiniotis et al. 2006). Indeed, it may be possible that by supressing viral load, IFN may indirectly have a beneficial effect on vaccine responses.

To investigate this, I compared vaccination after a long period of viral suppression with IFN/RBV (Arms A3 and A5) "late vaccination group" with vaccination administered early in treatment with a higher viral load (Arms A4 and A6) "early vaccination group". Contrary to work in murine models (Wherry, Ahmed and Blattman, 2005) there was no significant difference in the magnitude of T cell responses or the number of patients who made a T cell response between the early and late vaccination groups.

T cells induced by ChAd3-NSmut/Ad6-NSmut are of greater magnitude than in other therapeutic vaccine trials

Overall the HCV002TV vaccination strategy induced T cells at levels never before described in patients with chronic HCV infection, although it is difficult to compare results from trials using different ELISpot methodology and peptides. The most similar vaccine regimen trialled to date is the TG4040 trial, using the same proteins derived from NS3, NS4 and NS5B from a 1b HCV isolate in an MVA vector (Habsertzer et al. 2011). In the TG4040 study, the peak IFNγ-ELISpot response was <300 SFC/10^6 PBMC, compared with 2953 SFC/10^6 PBMC in HCV002TV. 5/15 (33%) of patients made a T cell response in TG4040, compared with 55% in Arm A and 50% in Arm B of HCV002TV. The TG4040 group has since presented data suggesting improved viral kinetics with vaccination, although this was not statistically significant (Wedemeyer et al. EASL 2013 oral presentation).

T cell responses in both these studies were predominantly to NS3. Further definition of antigenic targets was not performed by the TG4040 group, however it is possible that the predominant epitope was the same immunodominant NS3

epitope ($NS3_{1406\ (KLSGLGINAV)}$) identified in HCV002TV and explored further in the following two chapters.

Peptide mapping in HCV002TV also allowed me to identify two novel epitopes and demonstrated that all identified epitopes were $CD8^+$, suggesting a relative defect in the production of $CD4^+$ help. $CD4^+$ T cells have been shown to be important in the clearance of acute HCV (Thimme et al. 2001) and loss of proliferative capacity has been associated with HCV persistence (Gerlach et al. 1999). The lack of $CD4^+$ T cells detected in HCV002TV could also explain the differences in immune responses observed between healthy volunteers and patients who were vaccinated in this study.

Patients responded less well to vaccination than healthy volunteers

Although the immunogenicity of these vaccines compares favourably with other therapeutic vaccines, the magnitude and breadth of T cell responses in patients with chronic HCV were considerably lower than those observed in healthy volunteers from study HCV001, where 10/10 individuals responded to vaccination with the same regime (Barnes et al. 2012). Median peak responses were also 10 times weaker (76 SFC/10^6PBMC and 121 SFC/10^6PBMC in HCV patients from Arms A and B respectively compared with 1292 SFC/10^6PBMC in healthy volunteers).

There were however some similarities in T cell phenotype between healthy and chronically infected patients. HCV specific T cells increased in CD127 over time in healthy volunteers and in all patients analysed except one, suggesting the development of a long term memory population (Wherry et al. 2006). Both

CCR7 and CD45RA increased over time, suggesting a shift from a small proportion of effector memory cells (CCR7-/CD45RA-) to a predominantly central memory (CCR7+) or terminally differentiated phenotype (T_{EMRA})(CCR7+/CD45RA+). The significance of this as a correlate for HCV protection is not clear, however it has been shown that T_{EMRA} cells are associated with protection in acute HIV infection (Northfield et al. 2007). Whether the same is true in HCV remains to be seen.

T cells detected after vaccination in patients are exhausted

HCV specific T cells detected after vaccination demonstrated features of exhaustion including low magnitude IFNγ responses, low levels of cytokine secretion detected via ICS and poor proliferative capacity. Cytotoxicity, measured by levels of granzyme A and granzyme B detected by pentamer staining was far lower than that observed in healthy volunteers (median Granzyme A 54.3% and Granzyme B 44.2% in patients, compared with 93.6% and 90.35% respectively in healthy volunteers). Overall, despite some similarities to healthy volunteers in phenotypic markers, pentamer functional analysis clearly demonstrated a unique profile of T cells detected after vaccination in chronically infected patients where T cells display many of the features of exhausted cells. In exhausted T cells, a hierarchy of cytokine loss has been described where cytokines such as IL2 are lost first and finally IFNγ secretion is lost, consistent with the pattern observed in this study (Wherry, 2011).

Why might the T cells detected display features of exhaustion?

The reasons for T cell exhaustion are undoubtedly complex and are discussed in the section *Introduction*. They include the interplay between intercurrent Hepatitis C Virus infection and the host immune system, as well as the effect of any longstanding chronic viraemia. It has been extensively described in the literature that T cells in chronic HCV are weak and narrowly focussed and are unable to respond to further antigenic stimulus.

The phenomenon of T cell exhaustion in the context of chronic viraemia was first described in mouse models of LCMV two decades ago (Moskophidis et al. 1993) and has since been observed in many studies of chronically infected HCV patients, where HCV specific $CD8^+$ cells show impaired proliferative capacity and cytokine secretion (Gruener et al. 2001; Wedemeyer et al. 2002; Bengsh et al. 2010). These cells express multiple inhibitory molecules such as TIM-3, PD-1, 2B4 and CTLA-4 and blockade of one, or several of these molecules has been shown to restore T cell functionality *in vitro* (Nakamoto et al. 2009).

It is less clear why such a profile should also be the case in response to peripherally administered HCV antigens in the form of a vaccine. There are several broad possibilities: (i) Patients with chronic HCV have a general relative immune deficient state in comparison with healthy volunteers (ii) There exists an HCV specific defect in immune responses and/or (iii) the T cells detected after vaccination are in fact pre-existing exhausted T cells.

(i) Are patients with HCV generally immunodeficient

Patients with chronic HCV could theoretically have a generally decreased response to infection with other pathogens due to factors such as high T regulatory cells which might inhibit immune responses. As a population group in the UK they are also more likely to be socio-economically disadvantaged and may be malnourished or homeless with the poor health outcomes associated with this group; indeed studies have shown that sleep deprivation is associated with lower seroconversion (sAb) rates after HBV vaccination and lower antibody titres after Hepatitis A vaccination (Prather et al. 2012; Lange et al. 2003). Patients with HCV are also more susceptible to complications from other hepatotropic viruses, being more likely to develop, for example a fulminant hepatitis from intercurrent Hepatitis A vaccination than other individuals, however this may be the result of a robust immune response not being tolerated by a compromised liver rather than the consequences of immunosuppression.

In the HIV field, it has been shown that HIV infected individuals are less likely to respond to vaccination for HBV or HAV (Tseng et al. 2013). In the case of HCV, the data is more limited, however studies suggest that HCV infected patients are less likely to respond to HBV vaccination than healthy controls (Wiedmann et al. 2000). Although this is an antibody-mediated vaccine rather than a T cell vaccine, the results could nonetheless be extrapolated to suggest that a general 'immune defect' exists in the HCV population. Nonetheless, this was not supported by the responses to non-HCV antigens in this trial, where robust proliferative responses to CMV antigens were observed and T cell responses to the adenoviral vector were similar to those seen in healthy controls.

(ii) Patients with HCV may display an HCV specific deficit in immune responses

The discrepancy between T cell responses to non-HCV antigens (such as CMV and adenoviral vectors) and anti-HCV T cell responses in certain individuals seems to suggest there may indeed be an HCV specific defect in immunity in some people.

The patients studied have, by definition, failed to clear virus spontaneously, therefore may perhaps intrinsically lack the host immune characteristics (for example HLA types) which would enable a vigorous anti-HCV response. It has also been suggested that even after viral clearance with exogenous IFN/RBV treatment, T cells from HCV patients may remain dysfunctional, implying some kind of long term immunological reprogramming of cell mediated immunity (Missale et al. 2012).

(iii) T cells detected after vaccination could represent pre-existing T cells

Finally there exists the possibility that T cells detected after vaccination represent pre-existing responses, undetected by screening ELISpot assays. The correlation between baseline T cell responses and post vaccine responses supports this, although this could simply reflect a propensity for vigorous cell mediated immunity in these individuals. Such T cells could either be (i) pre-existing memory T cells from which circulating virus has hitherto escaped (due to immune pressure) or (ii) low frequency effector T cells which target the current circulating virus but cannot be detected at baseline due to the low sensitivity of the IFNγ-ELISpot assay.

There are arguments against both of these possibilities. If T cells detected after vaccination represent pre-existing T cells, should they no longer be exhausted (since they are no longer subject to chronic antigen stimulation)? Furthermore this scenario does not explain why the kinetics of T cell responses did not more closely mirror those of healthy volunteers, where priming induced vigorous T cell responses and boosting was weak- in vaccinated patients the opposite scenario was observed where priming was weaker than boosting. If on the other hand, detected T cells are low frequency T cells against current circulating viral strains, should they not have exerted a more significant effect on viral load or SVR rates? This begs the question of whether T cells detected are able to target circulating autologous virus and requires a closer examination of the epitopes they target. This is the subject of the following chapter.

3.13 Key Points Learned/Summary results

- Therapeutic vaccination with ChAd3-NSmut and Ad6-NSmut is safe and well tolerated.
- T cells detected after vaccination in patients with HCV are lower in magnitude and have poor functionality in comparison to those induced in healthy volunteers. They also have a different kinetic profile: patients displayed minimal response to priming, but stronger T cell responses to boosting, unlike healthy volunteers who showed the reverse pattern.
- T cell responses can be mapped to single peptides and are generally narrowly focused, with a clear immunodominant epitope $NS3_{1406}$.
- Reasons for the relatively poor immunogenicity in patients compared with healthy volunteers remain unclear. Possibilities include
  - a non-specific immunosuppressive effect in chronically infected patients (due to T regulatory cells, circulating cytokines or host factors such HLA type) or
  - HCV specific immune deficits (such as lack of $CD4^+$ help or T cell exhaustion).

4 What is the impact of circulating viral sequence on vaccine-induced T cell responses in HCV?

4.1 Aim

To explore the relationship between viral sequence and T cell responses to vaccination, both in the context of therapeutic vaccination and at a broader population level.

4.2 Background

Two major issues for a therapeutic HCV vaccine are that of viral variation and T cell exhaustion. The high level of viral variability means immune responses either from natural infection or from vaccination must be able to target a broad range of viral variants in order to achieve viral control. At the same time, chronic HCV also leads to an immunological milieu where T cell responses are frequently low in magnitude with poor proliferative capacity. This has been attributed to constant antigen exposure; therefore we might anticipate that vaccination with a novel antigen could lead to more vigorous T cell responses than that observed in chronic infection. This would also mean that T cells would be more commonly detected in patients whose viral sequence is different to the immunogen sequence (virus-immunogen mismatch).

Even if virus-immunogen mismatch is observed however, it is necessary to evaluate the variability of epitopes at a broader population level to determine the significance and likelihood of such a mismatch due to chance rather than immunological design. This can be done using the Los Alamos Database (http://hcv.lanl.gov/) which is a repository of HCV sequences worldwide. It

shows the variability of different epitopes as well as giving an indication of the likelihood that certain sequences will be found in the HCV002TV population due to chance.

The assessment of epitope variability also makes it possible to determine whether T cells detected after vaccination are likely to target common HCV sequence variants through cross-reactivity, as well as target autologous circulating virus in HCV002TV patients.

4.3 Questions

(i) Does mismatch between virus and immunogen (i.e. vaccination with a novel antigen) predict T cell response to vaccination?

(ii) Do T cell responses generated by vaccination target circulating host virus?

(iii) Do T cell responses generated by vaccination target common HCV viral variants?

4.4 Does mismatch between virus and immunogen (i.e. vaccination with a novel antigen) predict T cell response to vaccination?

I first sequenced circulating virus in patients in HCV002TV, at sites in the viral genome corresponding to T cell epitopes targeted by vaccination. I assessed autologous virus at baseline and at any point in time where viral relapse occurred in patients with a T cell response to vaccination.

4.4.1 What is the viral sequence of patients who respond to vaccination with ChAd3-NSmut and Ad6-NSmut?

Sequences were obtained at 14/15 epitopes in 11 patients prior to treatment (in

Arm A) or vaccination (in Arm B). Baseline circulating viral sequence differed from the immunogen sequence by 1-4 amino acids in all of these patients except one, patient 27 at epitope NS4B $_{1851(ILAGYGAGVAGALVA)}$ (Table 4-1).

In 4/6 of the patients who relapsed (Table 4-1), viral sequence was also obtained at the region targeted by T cells, after viral relapse. There were no amino acid mutations in 3/4 of these patients suggesting a lack of immune pressure from vaccine generated T cells. In one patient (28) there were two viral strains sequenced at baseline (KLVALG**V**NAV AND KLALG**I**NAV), one of which emerged as the predominant viral sequence after relapse (KLVAG**V**NAV), which could suggest some immune pressure at this site. As observed with the baseline viral sequences, no relapse sequences matched the vaccine immunogen.

The observation that viral and vaccine immunogen sequence do not match in patients who make a T cell response to vaccination (with the exception of one epitope) could be a matter of chance, related to the underlying variability of the viral sequence at different sites in the HCV genome. If T cell epitopes targeted by vaccine-induced cells are highly variable, or the vaccine immunogen sequence is rare, then a mismatch between patient viral sequence and immunogen sequence might be anticipated. Alternatively, viral-immunogen sequence mismatch might be a pre-requisite for a vaccine response. In this scenario, T cells presented with a 'new' antigen in the form of a vaccine immunogen might be more likely to respond that those who are constantly stimulated by the same antigens in circulating virus. To explore these two hypotheses, I compared the sequence of vaccine responders (patients who made a T cell response following vaccination) and non-responders in study HCV002TV and analysed the variability of HCV

sequences at a global population level using the Los Alamos database (see *Materials and Methods, 2.8 "*Population variation at key epitopes*", p71 above)*.

Table 4-1 HCV sequence at mapped T cell epitopes in patients who have responded to vaccination with ChAd3-NSmut/Ad6-NSmut.

Pt No.	Epitope/15mer identified (HLA restriction where known)	Viral sequence at epitope
27	CVNGVCWTV (A2) KLSGLGINAV (A2) LTTGSVVIVGRIILS/SVVIVGRIILSGRPA QEFDEMEECASHLPY ILAGYGAGVAGALVA RVCEKMALYDVVSTL (A2) RPRWFMLCLLLLSVG	CINGVCWTV KLVSLGLNAV CVVIVGRVVL REFDEMEECSQHLPY ILAGYGAGVAGALVA RVCEKMALYDVVSKL RPRWFWFCLLLLAAG
28 28B	KLSGLGINAV (A2) no detectable response	KLVALGI/VNAV KLVALGVNAV
50 50B	KLSGLGINAV (A2) no detectable response	KLVALGVNAV
39	TPSPAPNYSRALWRV/APNYSRALWRVAAEE FQVGLNQYLVGSQLP LSRARPRWFMLCLLL/RPRWFMLCLLLLSVG	APNYTFALWRVSAEEY FRVGLHDYPVGSQLP nd*
45 45B	HSKKKCDEL (B8) VTLTHPITKYIMACM no detectable response	HSKRKCDEL IILTHPITKYIMACM HSKRKCDEL IIL/VTHPITKYIMACM
54 54B	ARMILMTHF (B27) no detectable response	VRMILLTHF nd*
055	CVNGVCWTV (A2) KLSGLGINAV (A2) LTRDPTTPLAR	CINGVCWTV KLVALGVNAV LTRDPITPLAR
103	KLSGLGINAV (A2)	KLVALGINAV
40 40B	KLSGLGINAV (A2) No response to KLSGLGINAV CVNGVCWTV (A2)	KLVAMGVNAV KLVAMGVNAV CINGVCWTV[1]
53	CVNGVCWTV (A2) ALVVGVVCA	CINGVCWTV nd*
52	GINAVAYYRGLDVSV	GVNAVAYYRGLDVSV

nd*=not determined. 1=not a vaccine-induced response (T cells detected after viral relapse). B=viral relapse

4.4.2 What is the viral sequence in patients who do not respond to therapeutic T cell vaccination?

First, viral sequence in responders to vaccination was compared to that of HLA-matched non-responders at epitopes with a known HLA restriction (Table 4-2). There was no specific viral sequence which predicted response to vaccination. For example, at the HLA-A2 epitope NS5B$_{2594\ (ALYDVVSTL)}$, 11 out of 18 HLA matched patients shared the same viral sequence (ALYDDVS**K**L), however only one of these (patient 27) made a T cell response to this epitope (Table 4-2c). Even where there was a high degree of viral variability such as at NS3$_{1406\ (KLSGLGINAV)}$, 2 out of the 4 sequences identified in patients whose T cells recognised this epitope, were also found in HLA matched non-responders. At the HLA-B8 epitope NS3$_{1395\ (HSKKKCDEL)}$, 2 patients were infected with the variant (HSK**R**KCDEL), however only 1 of these made a T cell response to vaccination.

When viral sequences from vaccine responders and non-responders at 14 epitopes were compared (Table 4-2 and Table 4-3) the overarching pattern was not that a specific sequence predicted a T cell response to vaccination, but rather, that a mismatch between viral sequence and immunogen sequence was associated with a T cell response. No patient whose viral sequence matched that of the vaccine immunogen made a vaccine response except for, as previously described, the highly conserved NS4B $_{1851(ILAGYGAGVAGALVA)}$. This was particularly striking at 3 sites in the HCV genome where there were a large number of patients with viral sequences that matched the vaccine immunogen. At the HLA-B8 epitope NS3$_{1395\ (HSKKKCDEL)}$, the 4 HLA-B8 patients with an identical virus-immunogen sequence made no response to vaccination, whereas virus-immunogen mismatch was detected in the vaccine responder (HSK**R**KCDEL).

Second, at the HLA-2 epitope NS5B$_{2594\ (ALYDVVSTL)}$ there were 6 HLA-A2 matched patients whose viral sequence matched the vaccine immunogen. None of these made a vaccine response. Third, at the newly described epitope NS5B$_{2804\ LTRDPTTPLAR}$ 23/24 viral sequences matched the vaccine immunogen and were found in non-vaccine responders- the only variant sequence (LTRDP**I**TPLAR) was found in the vaccine responder (Table 4-2).

There were 3 immunogenic sites in the HCV genome (KLSGLGINAV, SVVIVGRIIL and FQVGLNQYLVGSQLP) where the vaccine immunogen sequence was not identified in any patients. Virus-immunogen mismatch at these sites could therefore be partly attributed to the rarity of the vaccine immunogen sequence. The variability of these epitopes amongst HCV sequences worldwide was therefore assessed.

Table 4-2 Circulating HCV sequence at immunogenic T cell epitope by HLA type, in patients vaccinated with ChAd3-NSmut (prime) Ad6-NSmut (boost). Viral sequence was determined at baseline and at any point of viral relapse (designated by 'B' after patient no.). The HCV vaccine immunogen sequence is represented in the top line of each table. Individual patient sequences are recorded below, with a dashed line indicating an amino acid is identical to that in the vaccine immunogen. The epitopes to which individual patients have made a T cell response (measured by ex-vivo IFNγ-ELISpot assay) are shown in red type.a) Viral sequences at an HLA-B8 epitope (HSKKKCDEL) b) viral sequence at an HLA-B27 epitope ARMILMTHF c) viral sequences at three HLA-A2 epitopes. nd= not determined ?=unclear amino acid sequence.

a)

	Pt No.	H	S	K	K	K	C	D	E	L
	45	-	-	-	**R**	-	-	-	-	-
HLA-B8 +ve pts	45B	-	-	-	R	-	-	-	-	-
	44	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-
	46	-	-	-	-	-	-	-	-	-
	53	-	-	R	-	-	-	-	-	-
	40	-	-	-	-	-	-	-	-	-
HLA-B8 neg pts	40B	-	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-
	51	-	-	-	-	-	-	-	-	-
	52	-	-	-	-	-	-	-	-	-
	39	-	-	-	-	-	-	-	-	-
	103	-	-	-	-	-	-	-	-	-
	27	-	-	-	-	-	-	-	-	-
	28	-	-	-	-	-	-	-	-	-
	28B	-	-	-	-	-	-	-	-	-
	38	-	-	-	-	-	-	-	-	-
	43	-	-	-	-	-	-	-	-	-
	55	-	-	-	-	-	-	-	-	-
	54	-	-	-	-	-	-	-	-	-
	37	-	-	-	R	-	-	-	-	-
	100	-	-	-	-	-	-	-	-	-
	102	-	-	-	-	-	-	-	-	-
	56	nd								
	101	nd								
	36	nd								

b)

	Pt No.	A	R	M	I	L	M	T	H	F
HLA-B27 +ve pts	54	**V**	-	-	-	-	**L**	-	-	-
	102	-	-	-	V	-	-	-	-	-
HLA B27 neg pts	28	-	-	-	-	-	-	-	-	-
	28B	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-
	39	-	-	?	-	-	-	-	-	-
	101	V	-	-	-	-	L	-	-	-
	45	-	-	-	-	-	-	-	-	-
	37	-	-	-	-	-	-	-	-	-
	38	-	-	-	-	-	-	-	-	-
	55	-	-	-	-	-	-	-	?	-
	40	-	-	-	-	-	-	-	-	-
	43	-	-	-	-	-	-	-	-	-
	103	-	-	-	V	-	-	-	-	-
	46	-	-	-	-	-	-	-	-	-
	52	-	-	-	-	-	-	-	-	-
	53	-	-	-	-	-	-	-	-	-
	51	-	-	-	-	-	?	-	-	-
	27	nd								
	25	nd								
	36	nd								
	100	nd								
	106	nd								
	56	nd								

c)

	Pt No.	C	V	N	G	V	C	W	T	V	K	L	S	G	L	G	I	N	A	V	A	L	Y	D	V	V	S	T	L
	27	-	**I**	-	-	-	-	-	-	-	-	-	**V**	**S**	-	-	**L**	-	-	-	-	-	-	-	-	-	-	**K**	-
HLA-A2 +ve pts	28	-	I	-	-	-	-	-	-	-	-	-	**V**	**A**	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-
	28B	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	V	-	-	-	-	-	-	-	-	-	-	K	-
	40	-	I	-	-	-	-	-	-	-	-	-	**V**	**A**	**M**	-	**V**	-	-	-	-	-	-	-	-	-	-	K	-
	40B	-	**I**	-	-	-	-	-	-	-	-	-	V	A	M	-	V	-	-	-	-	-	-	-	-	-	-	K	-
	103	-	I	-	-	-	-	-	-	-	-	-	**V**	**A**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50	-	I	-	-	-	-	-	-	-	-	-	**V**	**A**	-	-	**V**	-	-	-	-	-	-	-	-	-	-	K	-
	50B										nd																		
	52	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	V	-	-	-	-	-	-	-	-	-	-	K	-
	44	-	I	S	G	A	-	-	-	-	-	-	V	A	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-
	46	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-
	38	-	I	-	-	-	-	-	-	-	Q	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-
	36	-	I	-	-	-	-	-	-	-	nd																		
	43	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	V	-	-	-	-	-	-	-	-	-	-	K	-
	25	-	I/V	-	-	-	-	-	-	-	-	-	V	V	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-
	55	-	I	-	-	-	-	-	-	-	-	-	**V**	**A**	-	-	**V**	-	-	-	-	-	-	E	-	-	-	K	-
	37	-	I	-	-	-	?	?	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	D	-	-	-	-
	53	-	I	-	-	-	-	-	-	-	-	-	V	T	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-
	100	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-
	54	-	I	-	-	-	-	-	-	-	-	-	V	A	M	-	V	-	-	-	-	-	-	-	-	-	-	K	-
	56	n	d								nd										nd								
HLA A2 neg pts	48	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	V	-	-	-	-	-	-	-	-	-	-	K	-
	39	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-
	45	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-
	45B	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	I/V	-	-	-	-	-	-	-	-	-	-	K	-
	51	-	I	-	-	-	-	-	-	-	-	-	V	A	-	-	V	-	-	-	-	-	-	-	-	-	-	K	-
	101	-	I	-	-	-	-	-	-	-	nd										nd								
	102	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	nd								

Table 4-3 Circulating HCV sequence at immunogenic T cell epitopes, non-HLA matched, in patients vaccinated with ChAd3-NSmut (prime) Ad6-NSmut (boost). HLA restriction of these epitopes is less robustly defined, so patients divided into HLA groups. Viral sequence was determined prior to vaccination and at any point of viral relapse (designated by 'B' after patient no.) The HCV vaccine immunogen sequence is represented in the top line of each table. Individual patient sequences are recorded below, with a dashed line indicating an amino acid is identical to that in the vaccine immunogen. The epitopes to which individual patients have made a T cell response (measured by ex-vivo IFNg-ELISpot assay) are shown in red type. nd= not determined ?=unclear amino acid sequence. Epitopes are presented according to their order in the HCV genome in part a) and b) of this table

a)

Pt No.	G I N A V A Y Y R G L D V S V	V T L T H P I T K Y	S V V I V G R I I L	Q E F D E M E E C A S H L P Y	I L A G Y G A G V A G A L V A
43	- V - - - - - - - - - - - - -	- - - - - - V - - -	C - - - - - - V V -	R - - - - - - - - - S Q - - - -	- - - - - - - - ? - - - - - -
55	- V - - - - - - - - - - - - -	I - - - - - V - - -	C - - - - - - - V -	R - - - - - - - - - S Q - - - -	n d
103	- - - - - - - - - - - - - - -	- - - - - - - - - -	C - - - - - - V V -	- - - - - - - - - S Q - - - -	- - - - - - - - - - - - - - -
28	- - - - - - - - - - - - - - -	- - - - - - - - - -	C - - - - - - V V -	R - - - - - - - - - S Q - - - -	- - - - - - - - - - - - - - -
28B	- V - - - - - - - - - - - - -	- - - - - - - - - -	C - - - - - - V V -	R - - - - - - - - - S Q - - - -	- - - - - - - - - - - - - - -
52	- **V** - - - - - - - - - - - - -	- - - - - - - - - -	C - - - - - - V V -	- - - - - - - - - S Q - - - -	- - - - - - - - - - - - - - -
106	- V - - - - - - - - - - - - -	- - - - - v - - -	C - - - - - - - V -	R - - - - - - - - - S Q - - - -	- - - - - - - - - - - - - - -
25	- - - - - - - - - - - - - - -	- - - - - I/V - - -	C - - - - - - V V -	R - - - - - - - - - S Q - - - -	- - - - - - - - - - - - - - -
45	- I - - - - - - - - - - - - -	**I I** - - - - - - - -	C - - - - - - - V -	R - - - - - - - - - S Q - - - -	- - - - - - - - - - - - - - -
045B	- I/V - - - - - - - - - - - - -	I I/L - - - - - - - -	C - - ? - - - - V -	R - - - - - - - - - S Q - - - ?	n d
51	- V - - - - - - - - - - - - -	I - - - - - V - - -	C - - - I - - V V -	- - - - - - - K - S Q - - - -	n d
54	- V - - - - - - - - - - - - -	- - - - - - - - - -	C - - - - ? - - V -	R - - - - - - - - - S Q - - - -	n d
27	- L - - - - - - - - - - - - -	- - - - - - V - -	**C - - - - - - V V -**	**R - - - - - - - - - S Q** - - - -	- - - - - - - - - - - - - - -
38	- L - - - - - - - - - - - - -	- V - - - - - - - -	C - - - - - - V V -	- - - - - - - M - del - - - - -	- - - - - - - - - - - - - - -
53	n d	? ? ? - - - - - - - -	C - - - - - ? V V -	R - - - - - - - - - S Q - - - -	n d
101	n d	- - - - - - - - - -	C - - - - - - - - -	R - - - - - - - - - S Q - - - -	- - - - - - - - - - - - - - -
102	n d	- - - - - - V - - -	C - - - - - - - V -	R - - - - - - - - - S Q -	- - - - - - - - - - - - - - -
40	- V - - - - - - - - - - - - -	- - - - - V - - -	C - - - - - - - V -	R - - - - - - - - - S Q - - - -	n d
040B	- V - - - - - - - - - - - - -	? - - - - - V - - -	C - - - - - - - V -	R - - - - - - - - - S Q - - - -	n d
48	- V - - - - - - - - - - - - -	n d	n d	n d	n d
39	- - - - - - - - - - - - - - -	n d	n d	n d	- - - - - - - - - - - - - - -
50	- V - - - - - - - - - - - - -	n d	n d	n d	- - - - - - - - - - - - - - -
46	- - - - - - - - - - - - - - -	n d	n d	n d	- - - - - - - - - - - - - - -
36	n d	n d	n d	n d	- - - - - - - - - - - - - - -
37	n d	n d	n d	n d	- - - - - - - - - - - - - - -
56	n d	n d	n d	n d	- - - - - - - - - - - - - - -
100	n d	n d	n d	n d	- - - - - - - - - - - - - - -

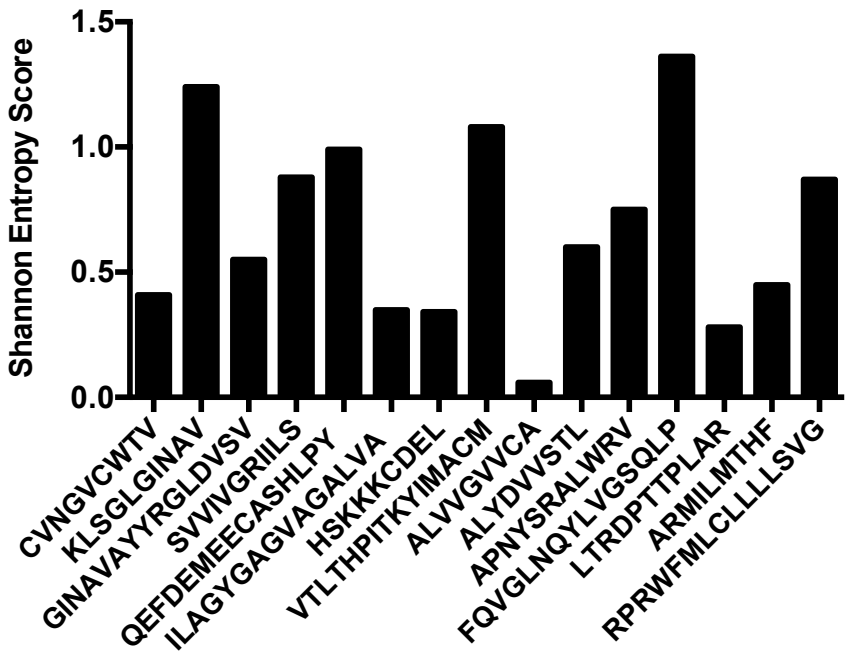
b)

Pt No.	A	L	V	V	G	V	V	C	A	A	P	N	Y	S	R	A	L	W	R	V	A	A	E	E	F	Q	V	G	L	N	Q	Y	L	V	G	S	Q	L	P	L	T	R	D	P	T	T	P	L	A	R
28	-	-	-	-	-	-	-	-	-	-	-	-/Y	-	K	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	E	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
028B	-	-	-	-	-	-	-	-	-	S	-	-	-	K	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	E	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-	-	-	-	-	K	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	E	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
045B	n	d								n	d														n	d																-	-	-	-	-	-	-	-	-	-	-	-	-
103	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-	-	-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	-	Y	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	D	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-	-	-	-	-	T	F	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55	n	d								-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	D	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
56	-	-	-	-	-	-	-	-	-	n	d														n	d															-	-	-	-	-	-	-	-	-	-	-
43	-	-	-	-	-	-	-	-	-	-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	S	-	-	-	H	D	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
51	n	d								-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	-	Y	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	n	d								-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
52	-	-	-	-	-	-	-	-	-	n	d														n	d															-	-	-	-	-	-	-	-	-	-	-
53	**n**	**d**								-	-	-	-	K	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	E	-	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
54	n	d								-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	D	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	D	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
106	-	-	-	-	-	-	-	-	-	-	-	-	-	T	F	-	-	-	-	-	S	-	-	-	-	R	-	-	-	H	D	-	P	-	-	-	-	-	-	-	n	d								
102	-	-	-	-	-	-	-	-	-	n	d														n	d															-	-	-	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-	-	n	d														n	d															-	-	-	-	-	-	-	-	-	-	-
36	-	-	-	-	G/R	-	-	-	-	n	d														n	d															n	d									
100	-	-	-	-	-	-	-	-	-	n	d														n	d															n	d									
37	-	-	-	-	-	-	-	-	-	n	d														n	d															-	-	-	-	-	-	-	-	-	-	-
40	n	d								n	d														n	d															-	-	-	-	-	-	-	-	-	-	-
040B	n	d								n	d														n	d															-	-	-	-	-	-	-	-	-	-	-
27	n	d								n	d														n	d															-	-	-	-	-	-	-	-	-	-	-
50	n	d								n	d														n	d															-	-	-	-	-	-	-	-	-	-	-

4.5 How variable is the HCV genome at sites corresponding to immunodominant T cell epitopes?

In order to get an overview of variability at different immunogenic sites in the HCV genome, I calculated the Shannon Entropy Score for each epitope identified in HCV002TV, using a median of 2149 sequences (range 1131-3896) from the Los Alamos database (www.hcv.lanl.gov/). This score is a quantitative measure of uncertainty within a set of data. In this case, the higher the score, the greater the degree of variability or number of mutations at an epitope. The Shannon Entropy score showed that the level of viral sequence diversity varies widely between epitopes, with some epitopes being highly conserved (such as ALVVGVVCA) and others (such as the highly immunogenic KLSGLGINAV) being very variable (Figure 4-1).

Figure 4-1 The degree of variability in genotype 1 HCV sequences at a population level



Epitopes identified in patients vaccinated with ChAd3-NSmut (prime)/Ad6-NSmut (boost) are shown on the *x* axis. The lower the Shannon entropy score (*y* axis) the more conserved the epitope is between genotype 1 sequences.

To get a more complete picture of variability, I then used these sequences to assess the frequency of the vaccine immunogen sequence at each epitope, and compare it to the most common viral variants at a population level. This confirmed three patterns of variability:

(i) A high degree of variability with no predominant sequence population wide

(ii) A distinct genotype 1a version and 1b version

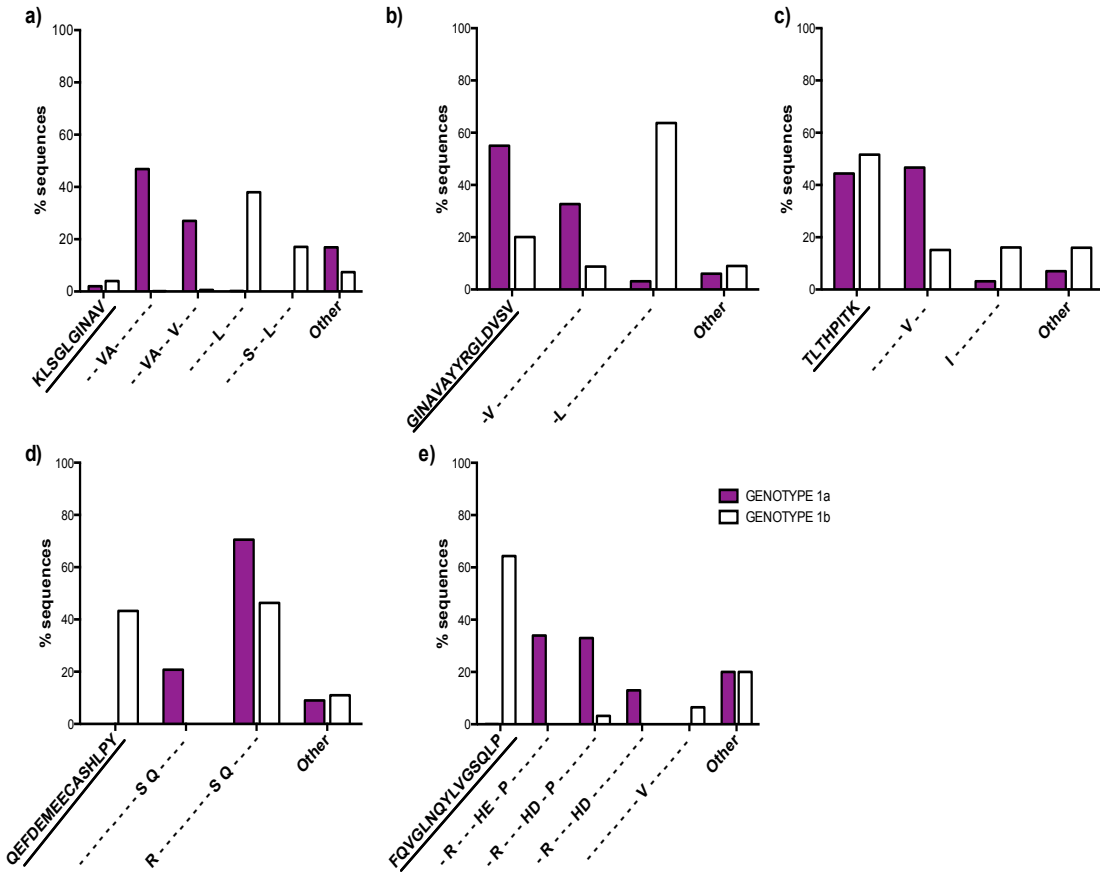
(iii) Conserved epitopes

4.5.1 Variable T cell Epitopes Identified in HCV002TV

10 of the 15 identified T cell epitopes are variable epitopes, where multiple common HCV sequence variants exist. 5 of these epitopes are highly variable both within and between subtypes (Figure 4-2). At 5 other epitopes there was a predominant genotype 1a or 1b version which represented >50% circulating sequences (Figure 4-3). At such variable sites, or sites where the immunogen sequence is rare in a patient's genotype subtype, it is possible that the mismatch described between viral and vaccine immunogen sequence (shown in Table 4-1) could be due to chance. For example at the most common epitope recognised by T cells induced by therapeutic vaccination, $NS3_{1406\ (KLSGLGINAV)}$, there was no single variant present in more than 30% of sequences at a population level (Figure 4-2a). Of note, the sequence contained within the vaccine immunogen is rare and has been identified in only 0.4% of genotype 1a sequences and 4.0% of genotype 1b sequences. The rarity of the immunogen sequence means that T cells primed with this immunogen must be able to recognise other more commonly encountered

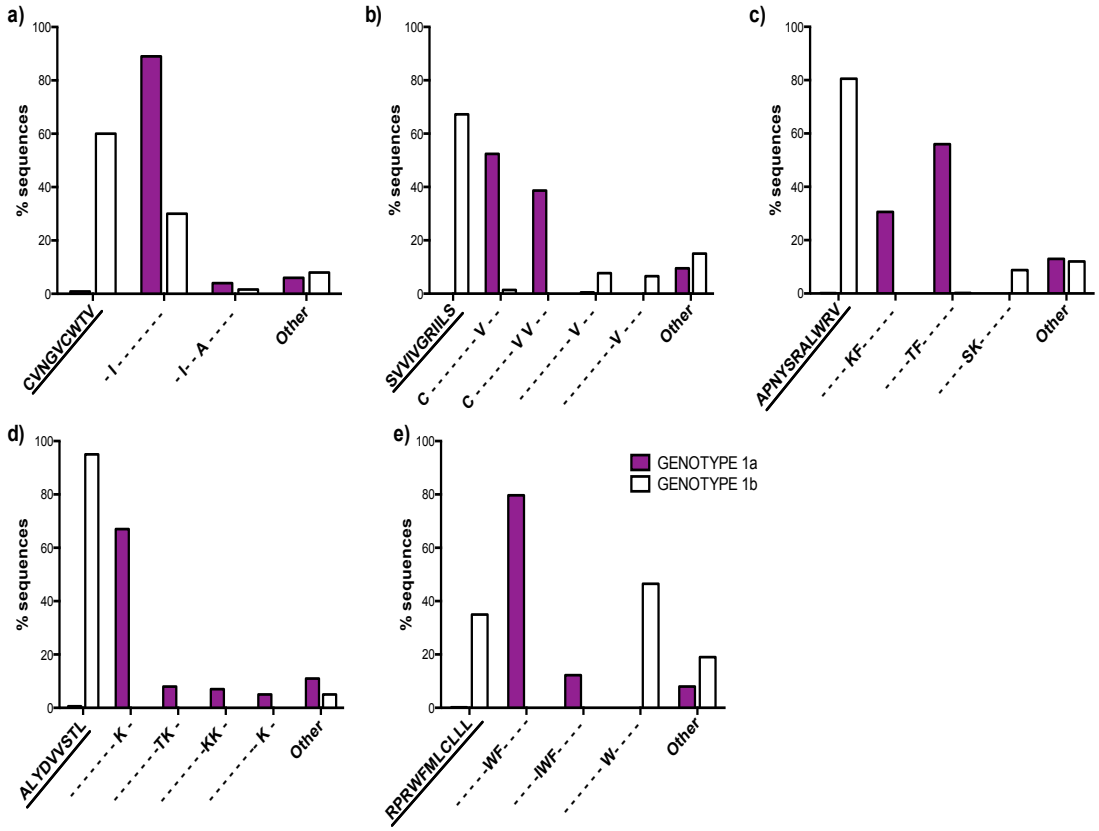
viral sequences at this site in the viral genome for effective immunogenicity. This issue is explored further in Section 4.7, "Do T cells induced by vaccination recognise common viral variants at T cell epitope sites?", p161 and Section 5 "Identifying the ideal antigen: Epitope $NS3_{1406}$ in depth" p183.

Figure 4-2 Variable T cell epitopes identified in study HCV002TV



Each panel represents a different T cell epitope. The amino acid sequence contained within the vaccine immunogen (underlined) and sequence variants present in >5% genotype 1 sequences are listed on the *x* axis. "-" indicates amino acid homology with the immunogen. % frequency at a population level (from Los Alamos Database) is indicated on the *y* axis. Shaded column= genotype 1a , unshaded column=genotype 1b.

Figure 4-3 T cell epitopes identified in Study HCV002TV with predominant genotype 1a or 1b sequence variants



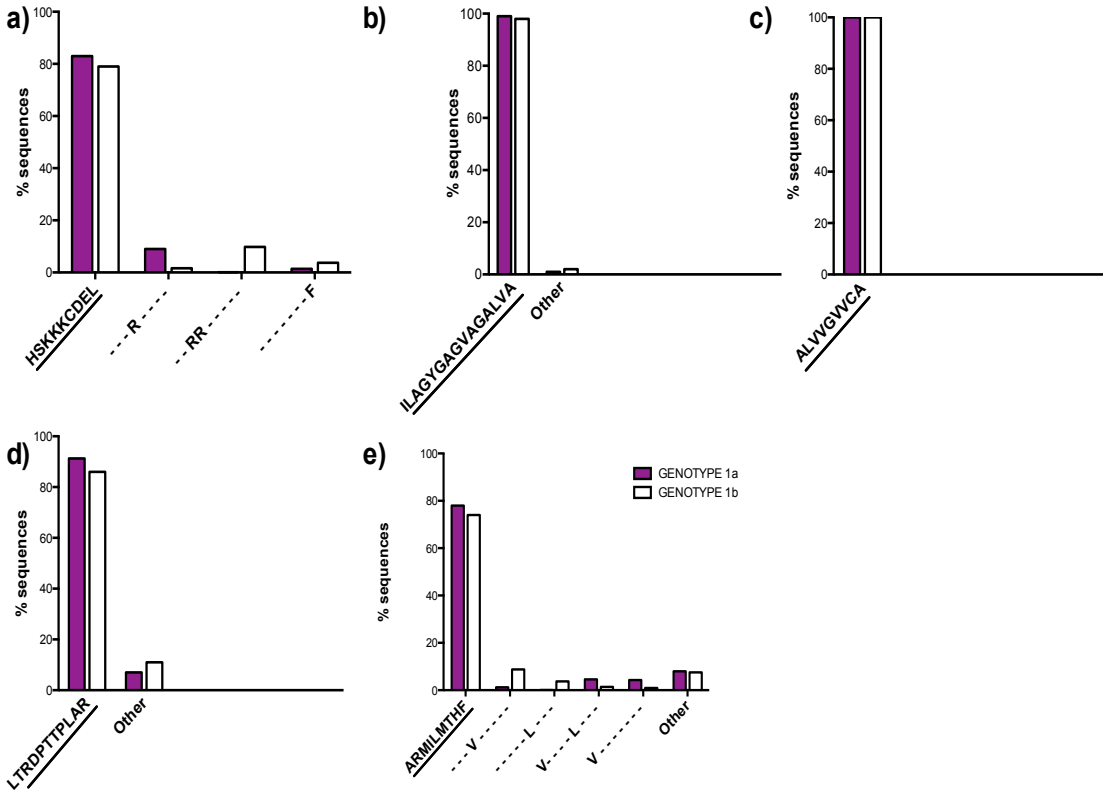
Each panel represents a different T cell epitope. The amino acid sequence contained within the vaccine immunogen (underlined) and sequence variants present in >5% genotype 1 sequences are listed on the *x* axis. "-" indicates amino acid homology with the immunogen. % frequency at a population level (from Los Alamos Database) is indicated on the *y* axis. Shaded column= genotype 1a , unshaded column=genotype 1b.

4.5.2 Conserved T cell Epitopes identified in Study HCV002TV

In contrast, there are 5 epitopes from Table 4-1 where over 75% of genotype 1a/1b sequences are identical, shown in Figure 4-4. This group of epitopes contains the only site where the vaccine immunogen and viral sequence matched: at epitope ILAGYGAGVAGALVA, where 99% of geno1a and 98% of geno1b sequences are identical. However at the other conserved epitopes, it is striking that the viruses in patients who responded to vaccination did not match the common vaccine immunogen sequence. At epitope HSKKKCDEL, the variant

(HSK**R**KCDEL) identified in the patient who made a T cell response to this site, is present in only 9% of genotype 1a and 1.6% of genotype 1b sequences. At epitope LTRDPTTPLAR, the variant identified in the vaccine responder (LTRDP**I**TPLAR) is found in only 0.9% and 2% of genotype 1a and 1b sequences respectively. Similarly at epitope ARMILMTHF the vaccine responder had a sequence (**V**RMIL**L**THF) present in only 4.6% and 1.4% of genotype 1a and 1b sequences respectively. This suggests that it is highly unlikely that these sequences were identified in vaccine responders due to chance. More likely, T cell responses are generated preferentially in individuals where virus-immunogen mismatch is present. The mismatch between circulating virus and vaccine immunogen raises the question of whether T cells induced by vaccination target circulating virus.

Figure 4-4 Conserved T cell epitopes identified in study HCV002TV



Each panel represents a different T cell epitope. Sequence variants present in >5% genotype 1 sequences are listed on the *x* axis, The amino acid sequence contained within the vaccine immunogen is underlined. % frequency at a population level (from Los Alamos Database) is indicated on the *y* axis. Shaded column= genotype 1a , unshaded column=genotype 1b.

4.6 Do T cells induced by vaccination target circulating autologous virus in patients with chronic HCV?

Next, I assessed whether vaccine-induced T cells could target circulating autologous virus in patients, using peptides and pentamers corresponding to (i) the vaccine immunogen and (ii) autologous virus in patients.

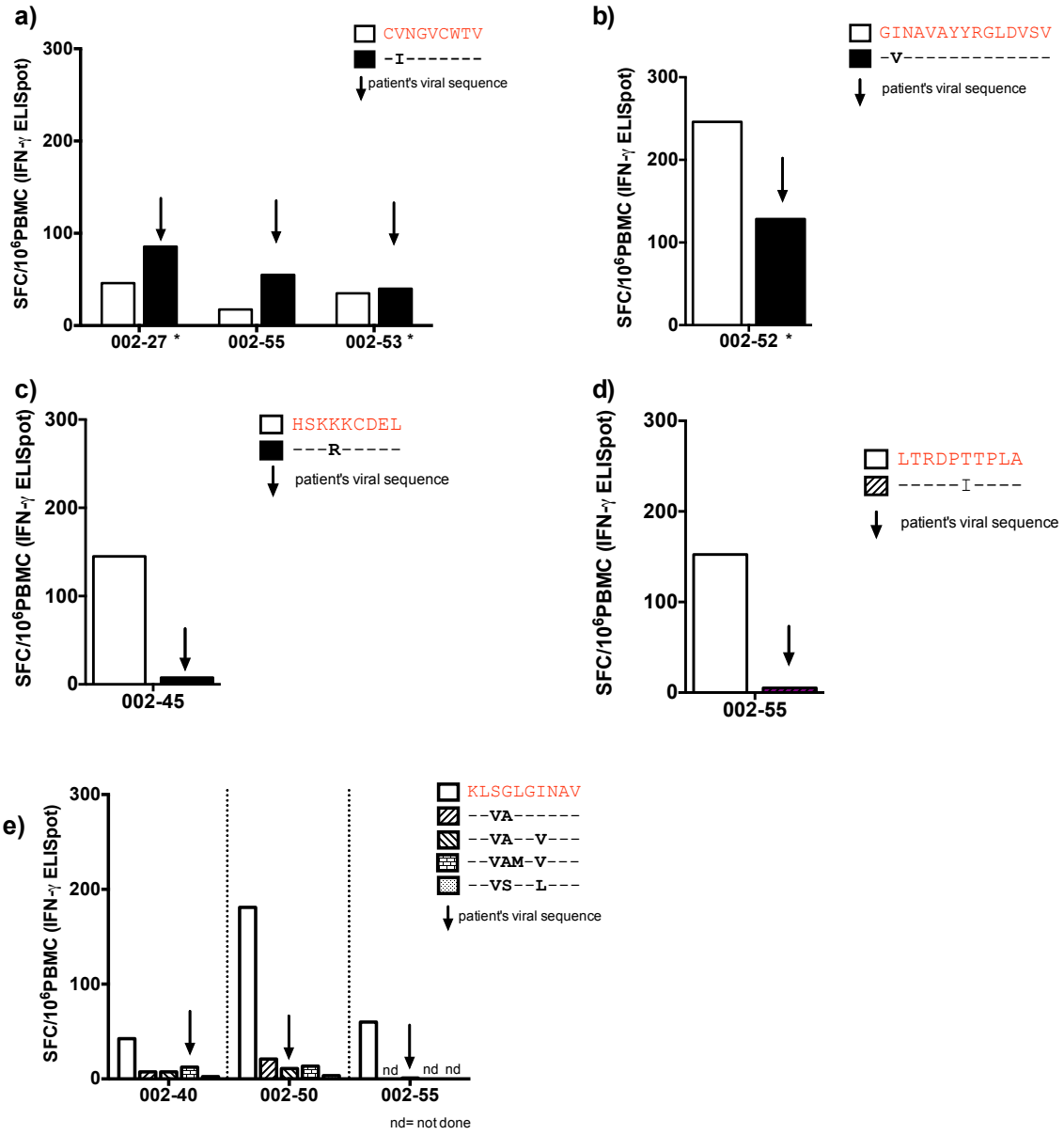
5 different epitopes from Table 4-1 were assessed in 7 patients (Figure 4-5). At 2 of these, $NS3_{1073}$ (CVNGVCWTV) and $NS3_{1411\text{-}1425}$ (GINAVAYYRGLDVSV), there was a high and moderate degree of T cell cross-reactivity respectively between peptides

representing the vaccine and viral antigens (Figure 4-5a-b). At $NS3_{1073\ (CVNGVCWTV)}$ responses to the viral sequence were stronger than those to the vaccine sequence, while at $NS3_{1411\text{-}1425\ (GINAVAYYRGLDVSV)}$ in patient 002-52, T cell responses to autologous viral sequence were 52% of the magnitude of those to the vaccine immunogen. However, T cells which recognised pools containing these peptides were detected prior to vaccination in patient 002-52, as well as in 2 of the 3 patients tested for responses at $NS3_{1073\ (CVNGVCWTV)}$ (pts 002-27 and 002-53). Therefore it is possible, that the seemingly cross-reactive T cells identified represent pre-existing T cells rather than newly primed cross-reactive responses.

At the next 3 epitopes, $NS5B_{2804}$ (LTRDPTTPLA[1]), $NS3_{1395\ (HSKKKCDEL)}$ and $NS3_{1406\ (KLSGLGINAV)}$ vaccine-induced T cells failed to recognise peptides corresponding to the circulating autologous virus in patients (Figure 4-5c-e). In fact, when multiple variants of $NS3_{1406\ (KLSGLGINAV),}$ corresponding to viral sequences from HCV002TV patients, were trialed, none were recognised by vaccine-induced T cells.

[1] The shortened version of this epitope LTRDPTTPLA instead of LTRDPTTPLAR was used in this experiment as at that time it was thought to represent the optimal length peptide

Figure 4-5 T cell responses to peptides corresponding to the vaccine sequence and host virus sequence at immunogenic epitopes



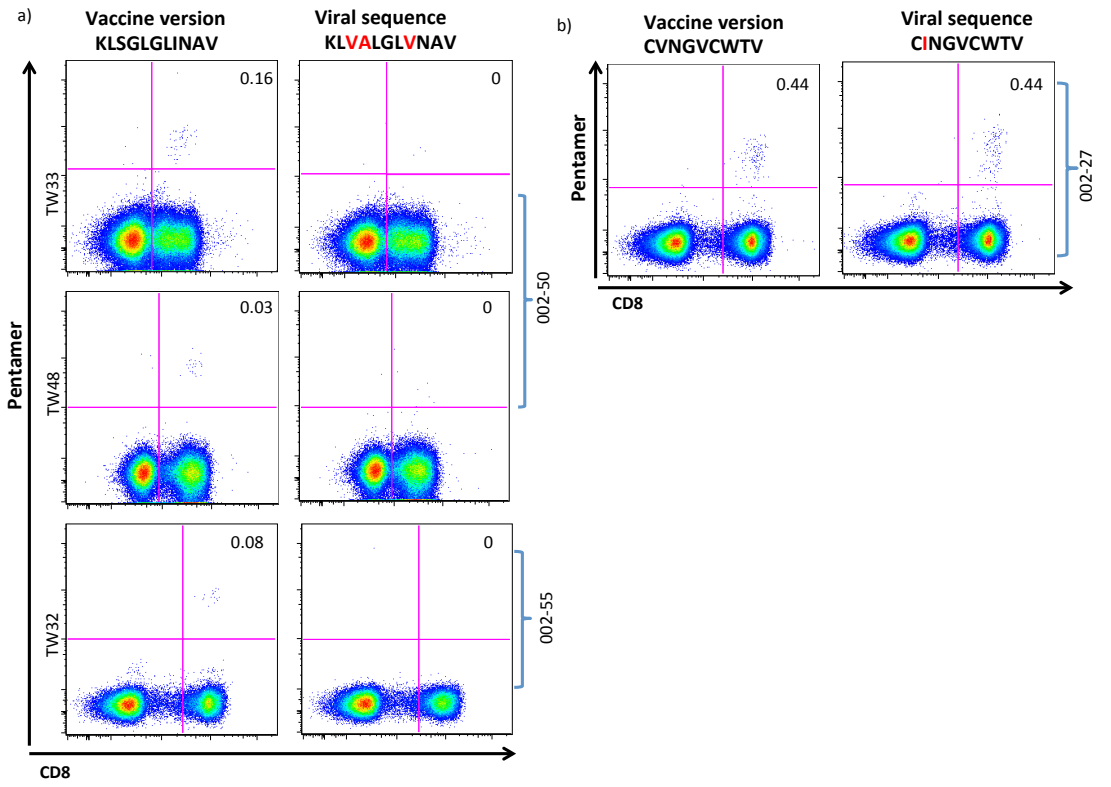
Each epitope is represented by a different graph. Patient numbers are indicated on the *x* axis and IFN-γ ELISpot response on the *y* axis. The vaccine sequence is listed in red font and the column in the graph corresponding to the patient sequence is marked by an arrow. For epitope $NS3_{1406}$ circulating viral variants found in other HCV002TV patients were included, in addition to the sequence of the autologous circulating virus. * positive IFNγ response to pool containing this peptide prior to vaccination. Response increased post vaccination

It is possible that T cells which do not produce IFNγ could be missed using an IFNγ-ELISpot assay. Therefore, a non-functional assay was performed, using pentamers matched to the patient's viral sequence and to the vaccine sequence at $NS3_{1073}$

(CVNGVCWTV) and $NS3_{1406}$ (KLSGLGINAV) (Figure 4-6). T cells specific for both the vaccine sequence and the patient's autologous viral sequence at epitope $NS3_{1073}$ (CVNGVCWTV) could be identified, consistent with the IFN-γ ELISpot results from Figure 4-5 above. Also in keeping with the previous results, T cells specific for the vaccine sequence could be identified at epitope $NS3_{1406}$ (KLSGLGINAV) in both individuals tested, however there were no T cells which recognised circulating virus in either patient (Figure 4-6a).

This raises the question of whether such a lack of T cell recognition could pose a problem more broadly, where vaccine-induced T cells might not recognise the most commonly identified HCV sequence variants. Such a possibility would mean vaccine-induced T cells would be highly specific to a single sequence variant only- that contained within the vaccine immunogen. The next section examines the cross-reactivity of vaccine-induced T cells to common HCV sequence variants at a population level.

Figure 4-6 HCV specific T cells identified by pentamer staining in patients with chronic HCV vaccinated with ChAd3-NSmut prime/Ad6-NSmut boost.



Responses to the pentamer corresponding to the vaccine sequence (left) and patient virus (right) in each panel. % pentamer positive $CD8^+$ T cells is shown in the top right of each panel. Patients received concurrent treatment with IFN/RBV. TW= treatment week **a**) Pt 002-50 9 weeks (top panels) and 24 weeks (middle panels) post boosting vaccination. Bottom panels: Patient 002-55 TW32, 4 weeks post boosting vaccination **b**) Patient 002-27, TW28, 4 weeks post boosting vaccination.

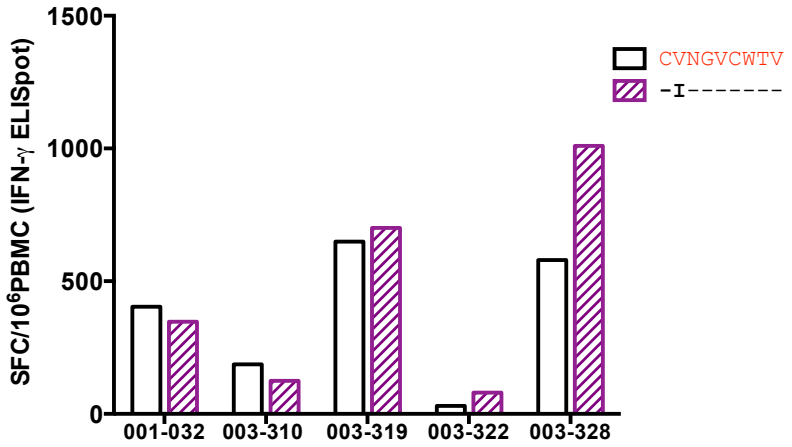
4.7 Do T cells induced by vaccination recognise common viral variants at T cell epitope sites?

To investigate the cross-reactivity of vaccine responses without the potential confounder of concurrent or previous HCV infection, T cells were assessed from healthy volunteers at three common epitopes: $NS3_{1073\ (CVNGVCWTV)}$, $NS3_{1406\ (KLSGLGINAV)}$ and $NS3_{1446\ (ATDALMTGY)}$. All volunteers were vaccinated with the same immunogen (NSmut), however different viral vectors delivered the insert. Participants from study HCV001 received the same vaccines as in HCV002TV.

Individuals from study HCV003 were vaccinated with ChAd3-NSmut/MVA-NSmut (See Materials and Methods).

T cell responses to $NS3_{1073\ (CVNGVCWTV)}$ were assessed in 5 healthy volunteers (Figure 4-7). This showed that T cells primed by the immunogen containing CVNGVCWTV recognised the variant (C**I**NGVCWTV) indeed, in 3/5 cases responses to the variant exceeded those to the vaccine immunogen. This confirms the findings of excellent cross-reactivity between these two common sequence variants suggested in patients with chronic HCV in Figure 4-5a.

Figure 4-7 Cross-reactivity of T cells to peptides corresponding to common variants at epitope $NS3_{1073}$ in healthy volunteers



The vaccine immunogen sequence is in red type. Each peptide is represented by a different column. Magnitude of T cell response is measured on the y axis by IFNγ ELISpot. Volunteers prefaced with "003-" received ChAd3 prime (Wk0) and MVA boost (Wk8). Volunteers prefaced with "001-" received ChAd3 prime (Wk0 and Wk4) followed by Ad6 boost (Wk14).

Next, 4 sequence variants (identified in patients from HCV002TV) at position $NS3_{1406\ (KLSGLGINAV)}$ were assessed in healthy volunteers (Figure 4-8a). As shown in patients with chronic HCV in Figure 4-5e, vaccine-induced T cells, primed by version KLSGLGINAV failed to recognise peptides matched to these variant HCV sequences. This demonstrates that the previous findings of poor T cell cross-

reactivity in patients from HCV002TV were not due to previous infection with different HCV variants, but rather, result from the nature of the T cells primed by this immunogen.

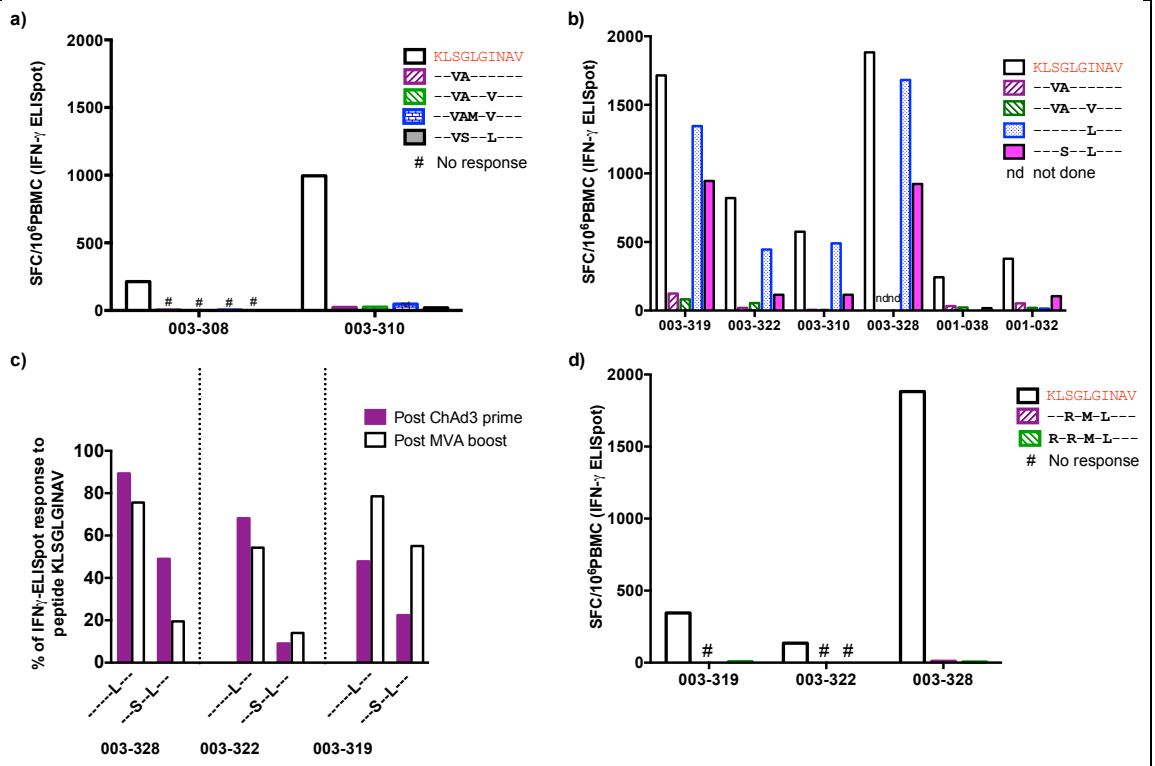
To assess the broader significance of this finding, vaccine-induced T cells were next tested against the most common $NS3_{1406}$ sequence variants at a population level. Sequences identified in >5% of genotype 1 sequences at a population level (see Figure 4-2a) were selected for further assays. In 6 healthy volunteers, vaccine-induced T cells failed to respond to 2 of the most common population variants (KL**VA**LGINAV and KL**VA**LG**V**NAV), representing 39.6% of genotype 1 sequences overall and 74% genotype 1a sequences. However, the two sequences more commonly found in genotype 1b (KLSGLG**L**NAV and KLS**S**LG**L**NAV) were sometimes recognised. All volunteers from study HCV003 responded to stimulation by the variant KLSGLG**L**NAV, with a median response 81.5% as strong as that to the vaccine immunogen (range 54%-87%). However, none of the HCV001 volunteers recognised this variant. Responses to the second common genotype 1b variant, KLS**S**LG**L**NAV, displayed more inter-individual variation. 2/6 volunteers mounted significant responses to this (003-319 and 003-328), although these were only 55% and 49% of the response to the vaccine matched peptide (KLSGLGLINAV) respectively. In 3/6 volunteers, detectable but greatly reduced responses to KLS**S**LG**L**NAV were observed at 14%, 20% and 27% of the vaccine matched response.

It is possible that the different results seen in volunteers from study HCV003 and study HCV001 might be due to the different boosting vectors used (MVA versus

Ad6). Next therefore, I compared T cell responses to the variant peptides KLSG**L**NAV and KLS**S**LG**L**NAV at two timepoints in the same patients: post priming with ChAd3 and post boosting with MVA (Figure 4-8c). In 2/3 patients there was similar or better T cell cross-reactivity following ChAd3 priming compared with MVA boost, suggesting that the vector was not responsible for the cross-reactive responses observed in volunteers from HCV003.

Finally I assessed whether vaccine-induced T cells could target genotype 3 viral sequences at this epitope site, although this is not a known epitope in genotype 3 infection. Analysis of 257 sequences showed that this is a highly conserved region in genotype 3 HCV, with only two major variants KL**R**G**M**G**L**NAV (77.8% genotype 3 sequences) and **R**L**R**G**M**G**L**NAV (15.6% genotype 3 sequences). T cells primed by the genotype 1 vaccine immunogen NSmut failed to recognise either of these variants when tested in 3 volunteers (Figure 4-8d).

Figure 4-8 Cross-reactivity of vaccine-induced T cells to peptides corresponding to different viral sequences at NS3$_{1406}$ in healthy volunteers

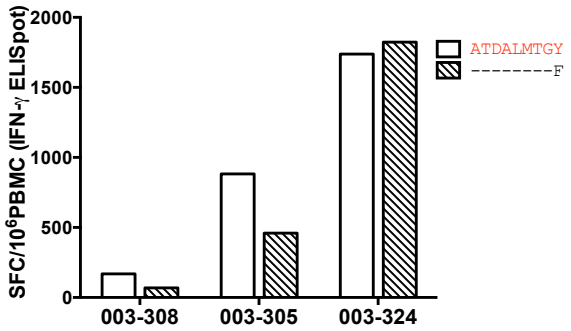


Volunteers prefaced with "003-" received ChAd3-NSmut prime (Wk0) and MVA-NSmut boost (Wk8). Volunteers prefaced with "001-" received ChAd3-NSmut prime (Wk0 and Wk4) followed by Ad6-NSmut boost (Wk14). The vaccine immunogen sequence is in red type. Peptides are represented by different columns. Individual patients are shown on the *x* axis. Magnitude of T cell response is measured by IFN-γ ELISpot, indicated on the *y* axis. # indicates no detectable T cell response. **a**) T cell responses to peptides representing viral variants identified in HCV002TV patients. **b**) T cell responses to peptides representing the most common genotype 1 sequence variants at a population level at NS3$_{1406}$. **c)** Comparison of T cell cross-reactivity following vaccination with two different vectors: ChAd3 and MVA. 3 volunteers are shown on the x axis. – indicates amino acid homology with the vaccine immunogen sequence KLSGLGINAV. Magnitude of T cell response to peptides KLSGLG**L**NAV and KLS**S**LG**L**NAV as a % of IFNγ-ELISpot response to the vaccine immunogen KLSGLGINAV is shown on the *y* axis **d)** T cell responses to peptides representing the most common genotype 3 sequence variants at NS3$_{1406}$.

I next examined T cell responses at an epitope to which healthy volunteers frequently made a vaccine related response- NS3$_{1446\ ATDALMTGY}$. Although no patients from HCV002TV made a T cell response to this epitope, it has been commonly described in chronic infection, and was targeted by all HLA-A1 volunteers in study HCV001 (Barnes et al. 2012). There are two main commonly

found sequence variants at this site: the vaccine immunogen ATDALMTGY and ATDALMTG**F** which represent 40% and 54% of genotype 1 sequences respectively. All three volunteers tested made a T cell response which was cross-reactive with the common sequence variant (ATDALMTG**F**), ranging from 40% of the response to the vaccine peptide (in patient 003-308) to slightly stronger than the vaccine peptide (Figure 4-9).

Figure 4-9 Cross-reactivity of T cells to peptides corresponding to common variants at epitope NS3$_{1446}$ (ATDALMTGY) in healthy volunteers

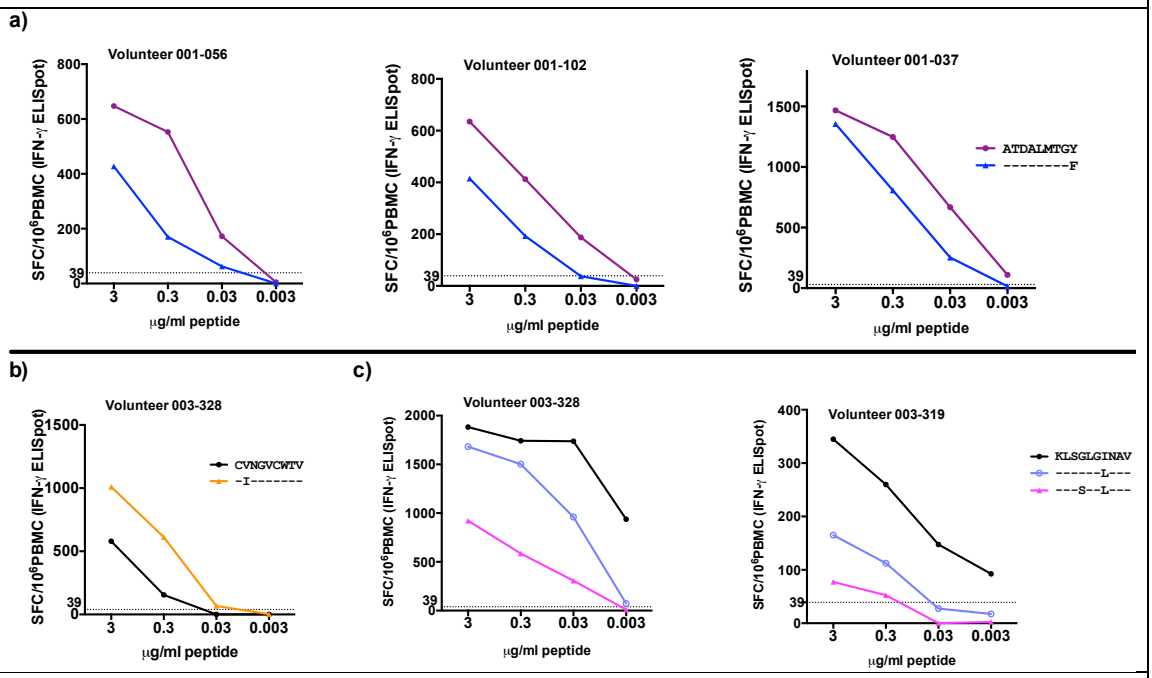


The vaccine immunogen sequence is in red type at the top of the figure legend. Sequence variants are represented by different columns. "–" represents amino acid homology with the vaccine sequence. Magnitude of T cell response is measured on the *y* axis by IFNγ ELISpot. Volunteers were vaccinated with ChAd3-NSmut (Wk0) and MVA-NSmut (Wk8) in study HCV003.

All of these experiments were conducted using supraphysiological concentrations of peptides (3μg/ml peptide, see Materials and Methods). It is possible that peptides which appear to be cross-reactive *in vitro* at high concentrations, are not cross-reactive when tested at lower doses which more closely simulate the *in vivo* environment. I therefore performed peptide dilution assays at three epitopes: NS3$_{1446}$ (ATDALMTGY), NS3$_{1073}$ (CVNGVCWTV) and NS3$_{1406}$ (KLSGLGINAV) (Figure 4-10). This showed that cross-reactivity (measured as a % of the T cell response to the vaccine immunogen peptide) decreased with lower concentrations at 2 of the 3 epitopes.

At $NS3_{1446\ (ATDALMTGY)}$, T cell responses to the variant peptide decreased from a median of 66% (range 65-92) at 3µg/ml, to 36% (range 20-38) at 0.03µg/ml. Responses were negative at the lowest concentration tested in 2/3 (0.003µg/ml) (Figure 4-10a). At epitope $NS3_{1406\ (KLSGLGINAV)}$ cross-reactivity of peptide (KLSGLG**L**NAV) decreased from 89% at the highest concentration to 7% at the lowest concentration in volunteer 003-328 and from 48% to 19% in volunteer 003-319. The second peptide (KLS**S**LG**L**NAV) was even less cross-reactive decreasing from 49% and 22.4% (in volunteers 003-328 and 003-319 respectively) to undetectable levels at the lowest concentration . Epitope $NS3_{1073\ (CVNGVCWTV)}$ presented a different scenario, because the variant peptide produced a stronger T cell response than the vaccine immunogen peptide. This was consistent across all concentrations tested.

Figure 4-10 T cell cross-reactivity at 3 epitopes in healthy volunteers, using different peptide concentrations



Each plot represents a different volunteer, vaccinated with the NSmut immunogen, containing the amino acid sequence listed in full at each epitope. "–" represents amino acid homology with the vaccine sequence. Volunteers prefaced with "003-" received ChAd3-NSmut prime (Wk0) and MVA-NSmut boost (Wk8). Volunteer 001-037 received ChAd3-NSmut prime (Wk0 and Wk4) followed by Ad6-NSmut boost (Wk24). Volunteer 001-056 received ChAd3-NSmut prime (wk0) and Ad6-NSmut boost (Wk8). Volunteer 001-102 received Ad6-NSmut prime (Wk0, Wk4) and ChAd3-NSmut boost (Wk24). Magnitude of T cell response is measured by IFNγ-ELISpot, indicated on the *y* axis. Peptide concentration is listed on the *x* axis. A dotted line shows a T cell response of 39 SFC/10^6 PBMC, defined as a positive IFNγ-ELISpot response (see Materials and Methods).

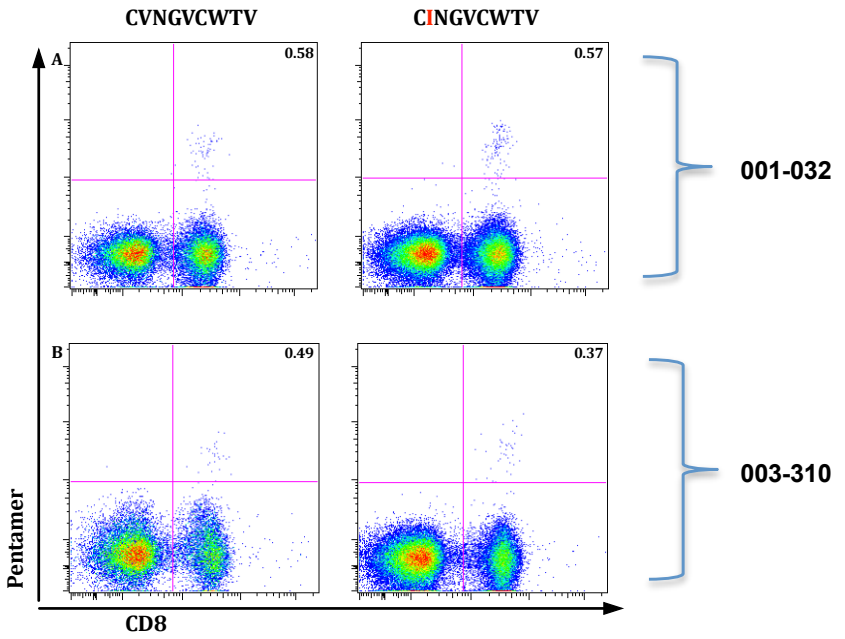
4.8 Functional analysis of T cells which recognise HCV sequence variants at epitope $NS3_{1073}$

Next I used pentamer staining in 2 healthy volunteers to confirm that vaccination induced similar proportions of T cells which recognised both the vaccine immunogen and the common HCV sequence variant at epitope $NS3_{1073}$ (Figure 4-11).

These could represent the same T cells capable of binding different variants of peptide $NS3_{1073}$, or two different naïve T cell populations primed by the one

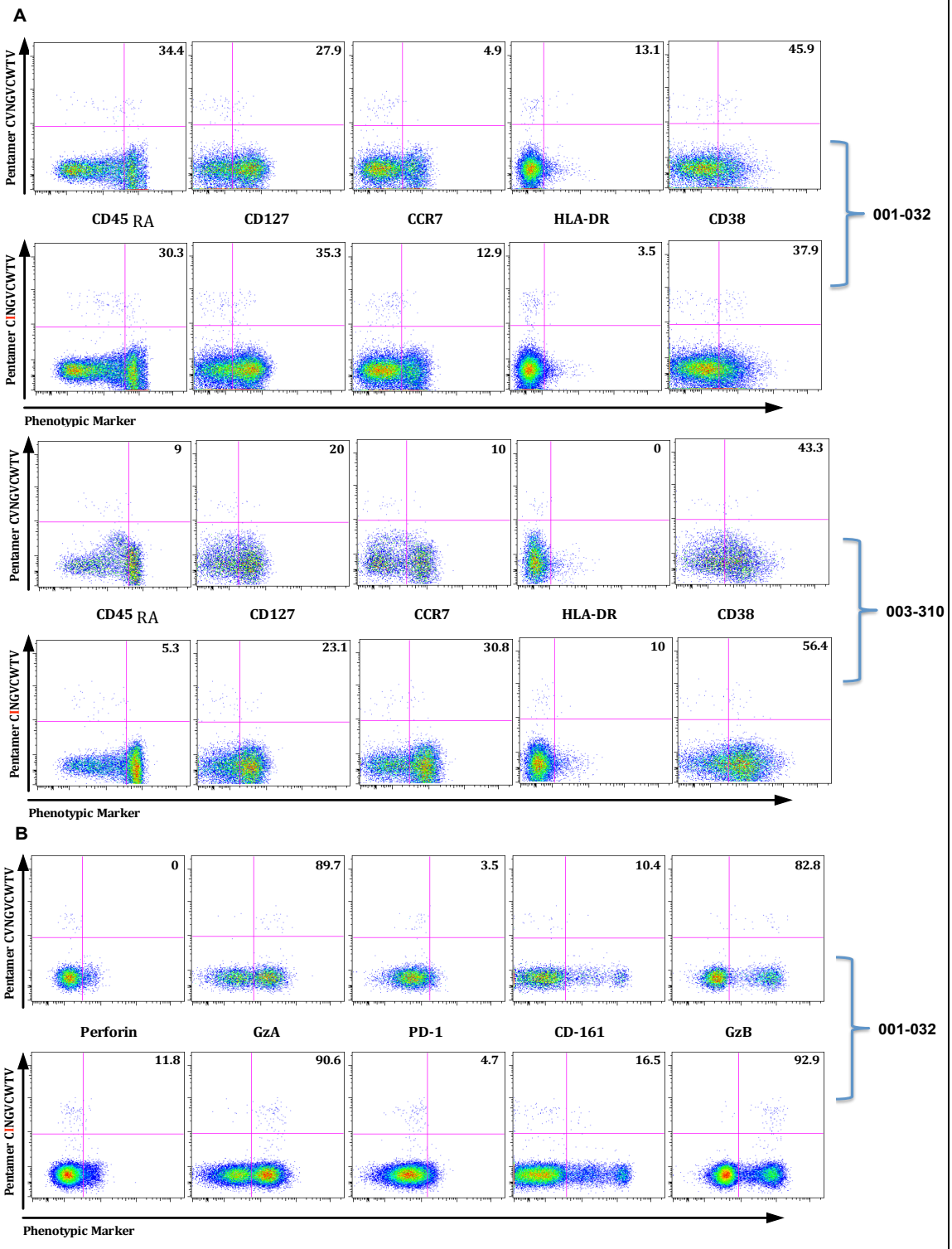
immunogen. To evaluate whether both T cell populations identified were functional, I assessed the phenotype and function of the cells with pentamer and ICS stains (Figure 4-12 and Figure 4-13).

Figure 4-11 Comparison of epitope specific T cell responses to two variants of epitope NS3$_{1073}$ following vaccination of two healthy volunteers



Staining is with pentamer NS3$_{1073\ CVNGVCWTV\ or\ CINGVCWTV}$ 4 wks post second prime (001-032) and 6 weeks post boost (003-310). Gating is on live CD3$^+$ (see Materials and Methods). Left panels represent the vaccine matched pentamer (CVNGVCWTV); right panels represent the common variant pentamer (CINGVCWTV). % pentamer$^+$/CD8$^+$ T cells is indicated in the top right of each panel. **A**) Volunteer 001-032 received ChAd3-NSmut prime (Wk0, Wk4), **B**) 003-310 received ChAd3-NSmut prime (Wk0) and MVA-Smut boost (Wk8).

Figure 4-12 Phenotypic comparison of vaccine-induced CD8⁺ cells, stained with two versions of pentamer NS3$_{1073}$ in healthy volunteers.

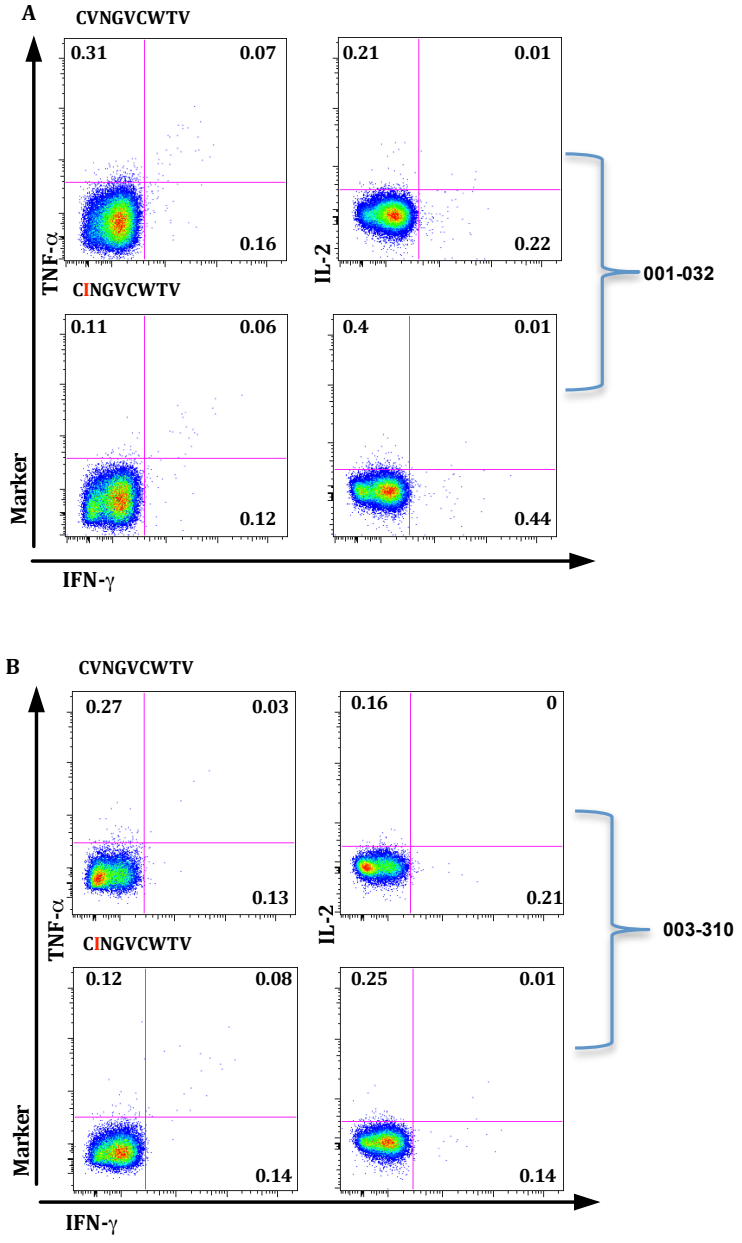


The pentamer corresponding to the vaccine sequence (CVNGVCWTV)(top row of each group) and variant pentamer (CINGVCWTV) (bottom row) is shown. Cells are gated on CD8⁺ cells (see Materials and Methods). % CD8⁺/pentamer⁺ cells is indicated in the top right corner of each plot. A) External antibody stains in volunteer 001-032 (4 weeks post second priming vaccination with ChAd3-NSmut) and 003-310 (6 weeks post boosting vaccination with MVA-NSmut). B) Internal antibody stains in volunteer 001-032.

The phenotype of T cells recognising the vaccine matched pentamer and the variant at epitope $NS3_{1073\ (CVNGVCWTV)}$ was similar in both volunteers assessed (001-032 and 003-310) (Figure 4-12). This was predominantly an effector memory population (CCR7-/CD45RA-), representing 67% and 80% of vaccine pentamer+ cells in volunteers 001-032 and 003-310 respectively, and 72% and 67% of the variant pentamer populations. Both pentamer populations had similar levels of activations markers CD38 and HLA-DR. A second staining panel was performed in volunteer 001-032 (Figure 4-12B). This showed that both populations of T cells were high in granzyme A (89.7%-90.6%) and granzyme B (82.8%-92.9%) and low in perforin (0-11.8%).

Next, ICS was performed using peptides corresponding to the vaccine immunogen and to the common population variant at epitope $NS3_{1073}$ (Figure 4-13). Both groups of cells were functionally similar, producing IFN-γ as expected from the ELISpot assays, and similar amounts of IL-2 and TNF-α, in both volunteers.

Figure 4-13. Comparative ICS of vaccine-induced cells in healthy volunteers, stimulated with peptides corresponding to (i) the vaccine immunogen (top panel) or (ii) the most common variant HCV sequence (bottom panel).



Cells are gated on $CD8^+$ cells (see Materials and Methods). % $CD8^+$ cells is indicated in each quadrant. TNF-α production (Left) and IL-2 production (Right) are shown. **A**) Volunteer 001-032, 4 weeks after second prime with ChAd3-NSmut. 001-032 received ChAd3-NSmut (Wk0), ChAd3-NSmut (Wk4) Ad6-NSmut (Wk14). **B**) Volunteer 003-310 TW 14, 6 weeks post boost with MVA-NSmut. 003-310 received ChAd3-NSmut (Wk0), MVA-NSmut (Wk8).

4.9 Discussion of viral variation and T cell responses

T cell responses to vaccination are determined by autologous viral sequence

This work demonstrates the importance of including the analysis of viral sequence alongside the assessment of immunogenicity in a new vaccine regime. Here, I have demonstrated that at sites in the HCV genome where patient viral sequence matched the vaccine immunogen, there was no T cell response detected. In contrast, at sites where there was virus-immunogen mismatch (i.e. sites in the HCV genome where autologous virus and the vaccine immunogen were different), T cells targeting those sites could be detected.

Sequence analysis showed that virus-immunogen mismatch was not simply due to the inherent variability of the HCV genome at T cell epitopes. At conserved sites in the HCV genome, it was striking that all patients who made a T cell response to vaccination demonstrated virus-immunogen mismatch.

The importance of HLA type when assessing viral variation

Measurement of viral variability may poorly reflect what is happening within a minor HLA type. If for example, there is an epitope which is restricted to an infrequently encountered HLA type, the analysis performed here may not pick pup the variability of such an epitope. In this way, an epitope, which appears conserved at a population level, may actually be highly variable in a given population. This is seen for example at the HLA-B27 epitope $NS5B_{2841(ARMILMTHF)}$ where 78% of genotype 1a sequences and 74% of genotype 1b sequences contain the consensus ARMILMTFH sequence, represented by the vaccine immunogen. However when the relevant HLA-B27 subgroup is examined, mutations are

frequent. Neumann-Haefelin and others showed in a study of chronically infected patients that 6/6 HLA-B27 patients had mutations at this epitope, as opposed to only 9/64 non-HLA-B27 patients (Neumann-Haefelin et al. 2006). Sequencing of the patients in study HCV002TV supported this with 2/2 HLA-B27 patients having mutations at this epitope but only 2/14 non-HLA B27 patients. Similarly, the HLA-A3 epitope TVHTGVTK is conserved in HLA-A3 negative individuals, but variable, with mutations linked to fitness cost, in HLA-A3 positive patients (Fitzmaurice et al. 2011).

Nonetheless, even when sequences at 5 epitopes with known HLA restriction were assessed in relevant HLA matched populations in HCV002TV, the same pattern of virus -immunogen mismatch was seen in patients who made a T cell response to those epitopes. Such a consistent pattern suggests that an antigenic specific process such as T cell suppression from T regulatory cells, tolerization or T cell exhaustion from chronic antigen exposure may play a significant role in determining therapeutic vaccine response.

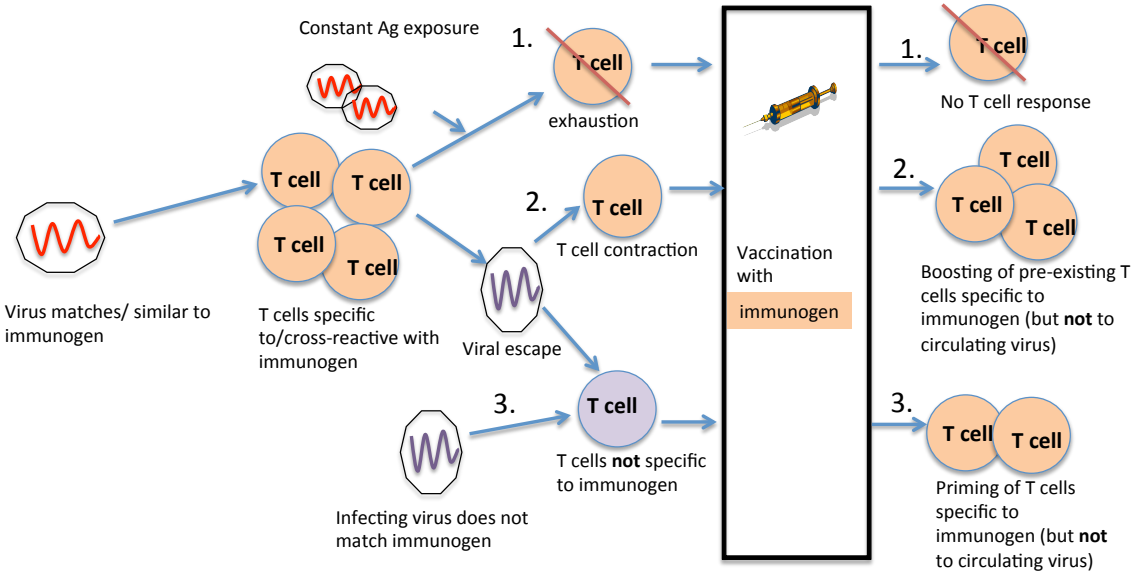
T cell exhaustion may explain virus-immunogen sequence mismatch at immunogenic epitopes

It is possible that T cells specific to epitope sequences contained within circulating autologous virus are exhausted due to constant antigenic stimulation. By presenting T cells with previously 'unseen' antigenic variants at T cell epitopes, vaccination could prime new T cell responses from naïve T cell populations. This could explain why individuals whose circulating virus matched that of the vaccine immunogen did not respond to vaccination. This also explains the pattern of virus-

immunogen mismatch in patients who made a T cell response to vaccination, where for example the only person to respond to the HLAB8 epitope $NS3_{1395}$ (HSKKKCDEL) was infected with the well-described escape variant (HSK**R**KCDEL) (Timm et al. 2004). However such a scenario also raise the possibility that the T cells detected at, for example this HLAB8 epitope, could represent rested memory T cells, from which virus has subsequently escaped, rather than naïve T cells primed by novel antigens.

If the model of exhaustion is extrapolated further, it provides one explanation for why all patients with the same viral sequence as each other at a given epitope, did not uniformly make a T cell response to that epitope. Each patient could have had previous HCV infections with different viral variants, or even concurrent infection with quasispecies not detected with the Sanger sequencing methods used here. This could lead to different populations of T cells being exhausted and thus influence the generation of vaccine responses. This is depicted in a putative model of T cell responses to vaccination in Figure 4-14.

Figure 4-14 Putative model of T cell responses to therapeutic vaccination



3 scenarios are presented. Scenarios 1 and 2: Patients are infected with a virus similar to/identical to the vaccine immunogen. **1**. Chronic antigen (Ag) exposure renders T cells exhausted and vaccination is ineffective. **2**. Virus escapes and T cells to the initial virus (matched to the immunogen) contract and recover. Vaccination boosts these pre-existing T cell responses however they fail to target current (escaped) circulating virus . **3**. Infecting virus is different to the vaccine immunogen. Vaccination primes naïve T cells specific to the vaccine immunogen but these fail to target circulating virus.

Alternatively, inter-individual variation could be due to different naïve T cell repertoires between individual patients, which limits their capacity to be primed by novel antigens (Wolfl et al. 2008). This possibility has wider implications for the success of prophylactic or therapeutic T cell vaccines, since the vast heterogeneity of T cell receptor combinations worldwide could limit the immunogenicity of any T cell vaccine, even if it includes multiple epitopes targeted by different HLA types.

It is also possible, as alluded to above, that the T cell responses identified following vaccination represent low frequency T cell populations from which virus has escaped. In this explanation (depicted as scenario 2 in Figure 4-14 above) patients infected with a virus which matches that contained within the vaccine immunogen,

make T cells specific to that virus (and thus also to the vaccine immunogen). The virus then escapes due to immune pressure and those immunogen specific T cells contract to low level populations, not detectable by *ex-vivo* assays. Such low frequency populations have been shown to exist by others who have expanded these populations in vitro (Timm et al. 2007). Vaccination then boosts those immunogen specific T cells, which no longer target circulating virus (since it has escaped). Such a scenario may also explain the poorly functional T cells detected after vaccination where the magnitude and quality of T cell responses in those who respond to vaccination is vastly different to those observed in healthy volunteers vaccinated with this regimen (see Chapter 3).

However it is difficult to explain how the scenario of rested memory T cells could hold true at sites in the vaccine immunogen where the immunogen sequence is encountered extremely rarely in the HCV population, for example epitope $NS3_{1406}$ (KLSGLGINAV) which represents fewer than 4% of genotype 1 sequences. It does not seem plausible that all 6 patients who made a T cell response to this epitope could have been previously infected with this rare sequence variant. It may be that the explanation for T cell responses is epitope specific, and that in some cases they represent boosted pre-existing T cells and in others naïve T cells. An alternative possibility at epitope $NS3_{1406}$ is that patients could have been infected with a similar, more commonly encountered, sequence variant which is cross-reactive with the immunogen sequence KLSGLGINAV. This would result in the same scenario of pre-existing T cells, which recognise the immunogen (through cross-reactivity). Such a scenario would rely on the ability of other sequence variants to

prime T cells cross-reactive to the immunogen sequence, a possibility discussed in Chapter 5 p183.

T cells do not target circulating virus

The possibility that T cells are exhausted may explain why T cells detected following vaccination did not target circulating virus in many cases and suggest that a lack of immune pressure may be why viral sequences remained largely unchanged at sites of immunological response in patients who relapsed. There was only one epitope site, $NS3_{1073\ (CVNGVCWTV)}$ where T cells recognised both circulating virus and immunogen with an equal strength response. This is consistent with work on this epitope in patients with chronic HCV from Soderholm and others (Soderholm et al. 2006). At all other epitopes, there was either a diminished or completely abrogated T cell response to the autologous virus. This was especially significant at the most immunogenic epitope ($NS3_{1406}$) where $NS3_{1406}$ specific T cells failed to recognise both circulating autologous virus as well as viral variants from other patients.

T cells are cross-reactive between common sequence variants at epitope $NS3_{1073}$ but not at other epitopes.

These observations lead to the first systematic examination of T cell cross-reactivity to common HCV variants at T cell epitopes, which carries implications not only for therapeutic vaccines but also for future work on prophylactic HCV vaccines. Previous work exploring T cell cross-reactivity has been hampered by the need to use samples from patients with chronic HCV or murine models. By

testing immunogenicity in healthy volunteers, vaccinated with a single immunogen, the possible confounding effects of previous viral infection, pre-existing T cell responses and concurrent IFNα therapy were removed.

Functional and pentamer assays confirmed that vaccine-induced $NS3_{1073\ (CVNGVCWTV)}$ specific T cells did recognise the other major sequence variant at this site, thus targeting 89% of circulating genotype 1 variants at $NS3_{1073}$. Importantly, this cross-reactivity was maintained at low peptide concentrations suggesting that this epitope is a viable component of a joint genotype 1a and 1b vaccine. Work from Fytili and others has also shown cross-reactivity with common genotype 4 and 6 variants at this epitope, however not with genotype 2 and 3, suggesting that this component of an immunogen would not be a viable candidate for the 'holy grail' of a universal cross-genotype HCV vaccine (Fytili et al. 2008).

Peptide dilution assays reveal T cells detected after vaccination are poorly cross-reactive with common viral sequences

Vaccine immunogen specific T cell responses were not cross-reactive to other common sequence variants at the other epitopes examined. Although at high concentrations there appeared to be cross-reactivity of $NS3_{1446\ (ATDALMTGY)}$ specific T cells between the two major $NS3_{1446}$ variants (representing 99% of genotype 1 viruses), peptide dilution assays demonstrated that this was not the case at lower peptide concentrations. This is in keeping with *in vivo* data from chronically infected patients which suggest that immune pressure at this site results in escape from Y to F at position 8 in this epitope in HLA-A1 patients (Timm et al. 2007). If vaccine-induced $NS3_{1446\ (ATDALMTGY)}$ specific T cells are unable to target the common

F variant (ATDALMTGF) they would be ineffective against 54% of genotype 1 variants. Given the immunodominance of this epitope in healthy HLA-A1 volunteers vaccinated in this study, it is likely that lack of cross-reactivity from vaccine-induced $NS3_{1446\ (ATDALMTGY)}$ specific T cells will be problematic in this population group.

Similarly, T cells specific for $NS3_{1406\ (KLSGLGINAV)}$ failed to recognise at least 40% of genotype 1 variant strains in all volunteers, and in some individuals this rose to least 67% of circulating strains. Analysis of population wide sequence variability showed the immunogen sequence (KLSGLGINAV) was very rare, making it critical that $NS3_{1406\ (KLSGLGINAV)}$ specific T cells can target other more commonly encountered $NS3_{1406}$ sequences. Common genotype 1a variants were not recognised by $NS3_{1406\ (KLSGLGINAV)}$ specific T cells, however in some individuals, genotype 1b variants were recognised at high peptide concentrations, albeit with a reduced magnitude response. This suggests that T cell responses at $NS3_{1406}$ may be specific to either genotype 1a or genotype 1b, making it challenging to incorporate this epitope into a generic genotype 1 vaccine. The inter-individual variability of cross-reactivity to the genotype 1b variants could be accounted for by (i) higher magnitude T cell responses in those with cross-reactive responses, allowing detection of low frequency T cells to the variant (ii) a different naïve T cell repertoire in such volunteers or less likely, (iii) previous exposure (or "priming") by another infectious agent with a similar antigen sequence to this variant.

Of course these analyses of cross-reactivity are of individual T cell responses at single epitopes. It may be that this becomes less important if multiple epitopes are targeted across the HCV genome. Although this is unlikely for the therapeutic vaccine here, where I have shown that T cell responses were narrowly focussed, it is possible that in a prophylactic vaccine given to healthy volunteers, the breadth of T cell response could overcome this problem. In such a scenario, at least some of the epitope targets may either match, or be cross-reactive with, infecting viral strains. However, it seems likely that even in this setting, cross-reactivity will remain a problem for certain immunodominant T cell responses. In healthy volunteers vaccinated with this regimen in study HCV001, all HLA-A2 patients responded to the single, poorly cross-reactive epitope $NS3_{1406\ (KLSGLGINAV)}$ or to the minipool containing this peptide. Such an immunodominant response could create a large pool of ineffective T cells, highly specific for a rare sequence variant.

It is not currently known whether it is possible to increase the degree of T cell cross-reactivity between different epitope sequences. This tantalising possibility was suggested by the inter-individual variation described at epitope $NS3_{1406\ (KLSGLGINAV)}$ raising the possibility that high magnitude T cell responses might more successfully induce cross-reactive T cells. It is also possible that priming using a different epitope variant from that used in this vaccine immunogen might increase T cell cross-reactivity. If a variant with the greatest capacity to induce cross-reactive T cell response could be identified, this would enhance immunogenicity across all viral strains, and be instrumental in the success of a universal HCV vaccine. This possibility is explored in the following chapter.

4.10 Key Points Learned/ Summary results

- At sites in the viral genome where virus and immunogen sequences match, there is no T cell response induced/recovered by vaccination.
- Mismatch between viral sequence and immunogen sequence is a pre-requisite for a T cell response to vaccination.
- Where epitopes are highly variable, or represented by a rare sequence variant in the vaccine immunogen, cross-reactivity is essential for effective immunogenicity.
- At the most immunogenic epitope $NS3_{1406}$, vaccine-induced T cells fail to target genotype 1a or genotype 3 variants and only recognise common genotype 1b variants inconsistently, at high peptide concentrations.
- T cells do not target circulating autologous virus in vaccinated patients at most epitopes, except at epitope $NS3_{1073}$.
- T cells detected after vaccination may represent naïve T cells primed by novel antigens in the immunogen or represent pre-existing rested memory T cells, from which virus has escaped.

5 Identifying the ideal antigen: Epitope NS3$_{1406}$ in depth

5.1 Aims

1. To develop an *in vitro* method for the development of potential T cell vaccine immunogen candidates
2. To identify the ideal version of epitope NS3$_{1406}$ for inclusion in a 'universal' HCV vaccine

5.2 Background

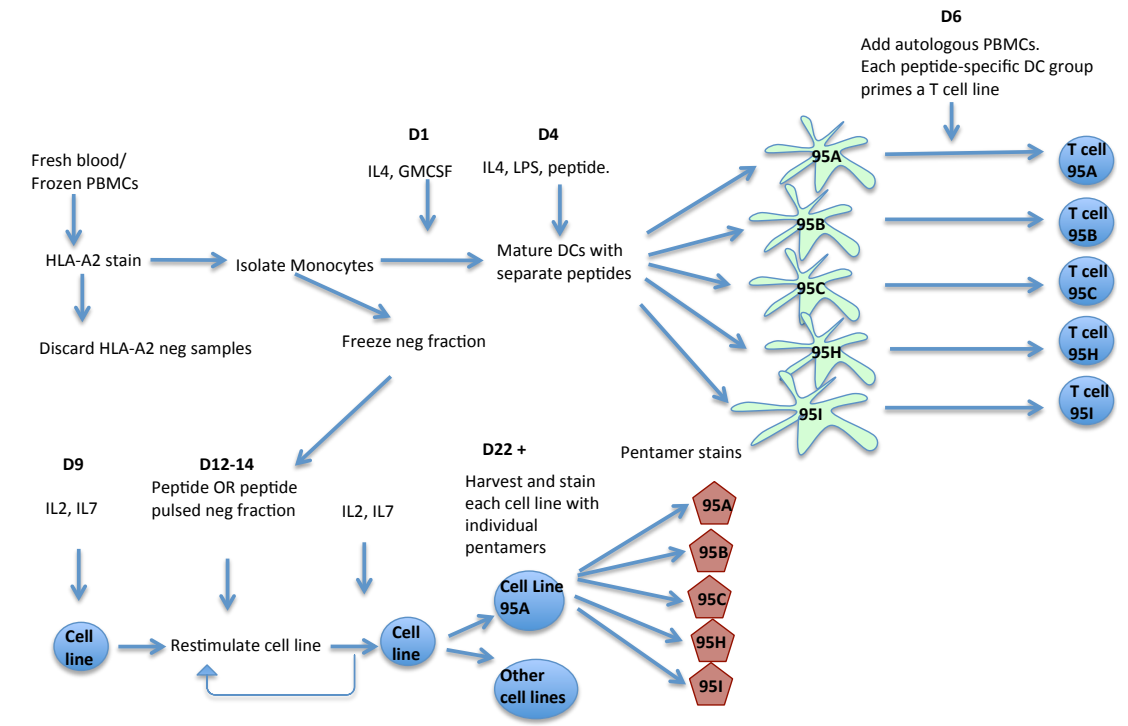
I next explored whether the existing immunogen contained in the 'NSmut' vaccine could be improved. Given the finding that T cell cross-reactivity could play a critical role in the success of a T cell vaccine and that one epitope NS3$_{1406}$ (KLSGLGINAV) was immunodominant in both healthy volunteers and patients, I assessed the cross-reactivity of this highly immunogenic HLA-A2 restricted epitope in depth.

Having identified previously that T cells primed by the vaccine immunogen at NS3$_{1406}$ demonstrated limited or no cross-reactivity to other common HCV sequences (see Section 4.7 p161), I assessed whether a different sequence variant at position NS3$_{1406}$ might generate better cross-reactivity. Such a variant would have the potential to induce T cells which recognise a large number of prevalent HCV sequences and could form part of the immunogen in a 'universal' HCV vaccine.

Ideally, HCV vaccine immunogen candidates would each be tested in animal and human safety and efficacy studies. However the lack of animal models for HCV,

and the length of time it would take to manufacture, licence and test each potential immunogen candidate *in vivo* makes this difficult for the testing of multiple immunogen candidates. Therefore I developed an *in vitro* model for testing different T cell vaccine immunogen candidates, with a view to finding either the most immunogenic (producing the biggest responses in the largest number of people) or the most cross-reactive version. This model, outlined in Figure 5-1, involved priming naive T cells with autologous dendritic cells (DCs) with different HCV sequence variants and then testing these primed T cells for cross-reactivity to other HCV variants using custom made pentamers. The HCV sequence variants chosen were those identified in the Los Alamos database which represented >5% of circulating genotype 1 sequences worldwide.

Figure 5-1 Schema for *in vitro* priming of naïve T cells using different HCV sequence variants



HLA-A2 positive samples are selected for monocyte isolation. Monocyte depleted fraction (neg fraction) is frozen and reserved for later use. DCs are matured with one of 5 individual peptides, matched to sequence variants in the HCV genome named 95A, 95B, 95C, 95H and 95I. DCs then prime naïve T cell lines which are restimulated with peptides or peptide pulsed autologous PBMC. Each T cell line is stained with 5 pentamers matched to the individual sequence variants (95A-I). neg=negative D=Day DC=dendritic cell IL= interleukin LPS= lipopolysaccharide

5.3 Questions

1. Is there an optimal version of epitope NS3$_{1406}$, which will prime T cells capable of recognising multiple common HCV sequence variants?
2. Is it possible to prime multiple different naïve T cell populations *in vitro*, which recognise common HCV sequence variants at epitope NS3$_{1406}$?

5.4 Designing a method to test candidate vaccine antigens in vitro using the Melan-A model

Priming HCV specific naïve T cells is a notoriously difficult technique (A. Cox, personal correspondence). Wolfl and others have shown that it is possible to prime naïve T cells specific for one variant of epitope NS3$_{1406}$ (KLVALGINAV) in 5 healthy individuals although attempts to prime T cells specific for a second variant (KLVAMGINAV) were largely unsuccessful, attributed to a paucity of available naïve T cells specific for this variant (Wolfl et al. 2008). It has not previously been attempted with 4 of the 5 variants I wished to use and has not been trialed on this scale or for this purpose. Therefore I first developed the technique in a robust model of DC/T cell priming- the Melan-A model.

It been shown that monocyte derived dendritic cells (MoDDC) can readily prime and expand naïve T cells specific for a melanoma antigen (Melan-A$_{27\text{-}35}$ ELAGIGILTV) (Salio et al. 2001). Melan-A specific T cells have a high naïve precursor frequency in healthy volunteers, >1:2500 CD8+ T cells and are ideal candidates for developing a protocol for an *in vitro* priming model (Pittet et al.

1999). This model could then be used for populations of T cells with much lower naïve precursor frequency, such as HCV specific T cells.

5.4.1 Sample selection and tetramer testing

First I established a method for rapidly identifying suitable samples for the experiment. These samples needed to be

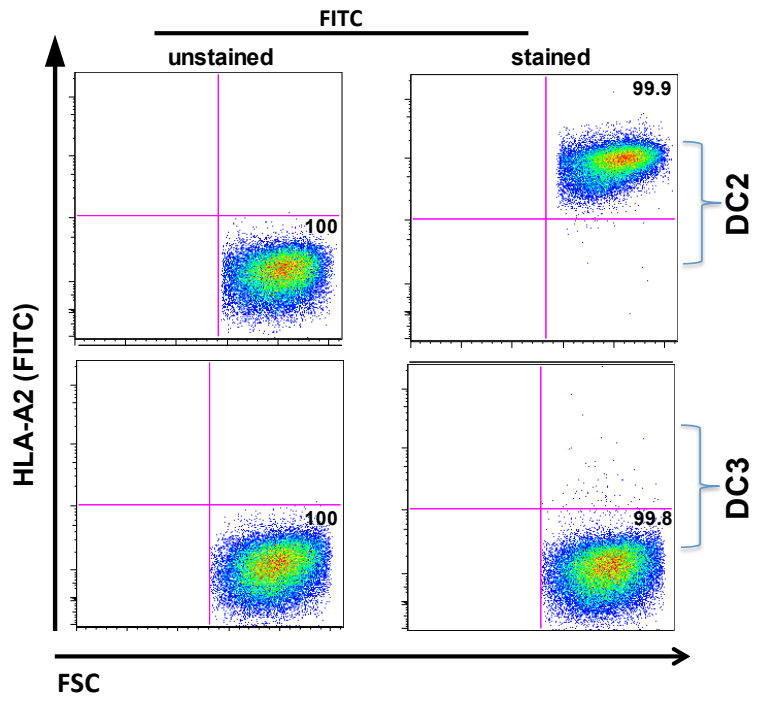
(**i**) able to yield large numbers of PBMC, to allow development of multiple cell lines and restimulation using frozen PBMC.

(**ii**) HCV negative, to ensure priming of naïve T cell populations.

(**iii**) HLA-A2 positive, for the HLA-A2 restricted epitope NS3$_{1406}$.

I used whole blood cones from blood donors, which have a high PBMC yield, are HCV Ab negative and are derived from a population with a low risk for blood borne viruses. The HLA type of these donors is unknown, so I verified a rapid whole blood staining protocol for screening samples before processing them (Figure 5-2).

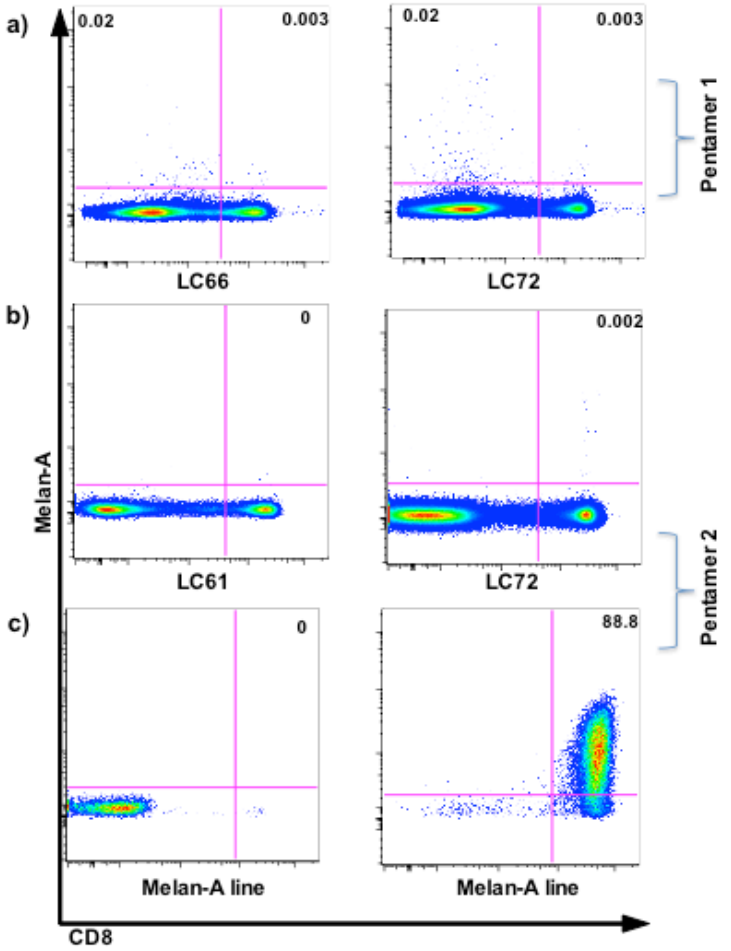
Figure 5-2 Representative FACs plots of HLA-A2 Antibody staining on whole blood



Whole blood was stained with HLA-A2 antibodies on FITC . Unstained (left) and stained (right) samples are shown. Each row represents a different volunteer: HLA-A2 positive (DC2) HLA-A2 negative (DC3). Samples are gated on single cells. % of the parent population is indicated in the FACs plots.

I next validated the Melan-A pentamer. The first version I used had a significant amount of background non-specific staining (Figure 5-3a). This was re-synthesised (ProImmune) and the second version tested against 4 frozen HLA-A2 positive samples as negative controls and a Melan-A cell line, for a positive control. With pentamer 2, there was minimal non-specific staining, (Figure 5-3b-c). There were small populations of $CD8^+$ Melan-A^+ cells at baseline (median 0.0015%. of live $CD3^+$ cells; range 0-0.001) as expected from previous studies (Pittet et al. 1999).

Figure 5-3 Representative Melan-A pentamer staining in healthy unstimulated HLA-A2 positive samples

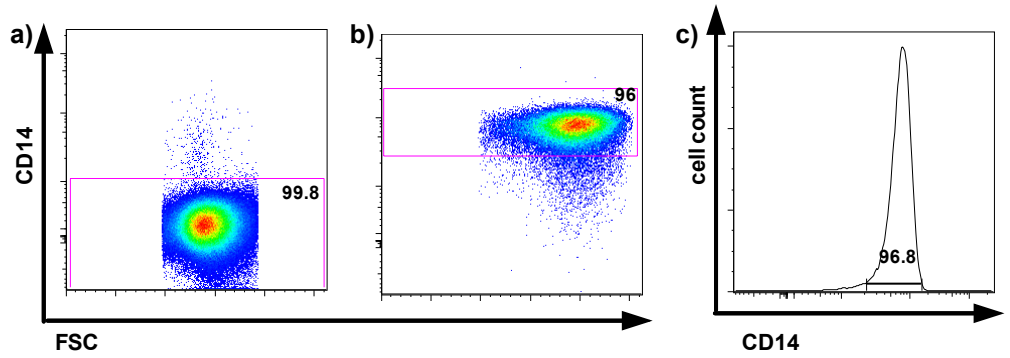


a) Background staining of Melan-A pentamer 1 in 2 HLA-A2+ve individuals (LC66 and LC72) showing high levels of non-specific staining. **b**) Staining using Melan-A pentamer 2 in 2 HLA-A2+ve individuals (LC61 and LC72), showing minimal non-specific staining. **c**) Positive control using Melan-A cell line (provided by Mariolina Salio WIMM Oxford) unstained (left column) and stained (right column) with Melan-A pentamer 2. CD8 is on the *x axis* and Melan-A on the *y axis*. % cells from the parent gate is indicated in each plots. Cells are gated on live, single, $CD3^+$ PBMC.

5.4.2 Growing Monocyte Derived Dendritic Cells

I then separated PBMC and isolated monocytes, using negative bead depletion and assessed purity of $CD14^+$ cells, which was >95% (Figure 5-4).

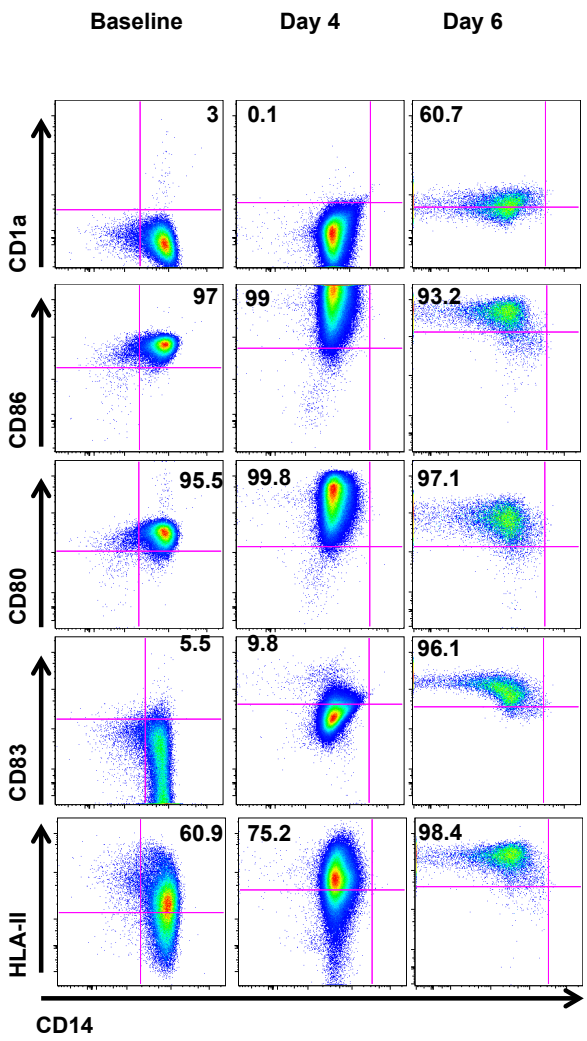
Figure 5-4 Purity of monocyte isolation from HLA-A2 individuals



Representative sample of CD14 staining, in volunteer EB2, stained after positive CD14+ bead selection. The negative (**a**) and positive (**b**) fractions are shown. **c**) Histogram of monocyte purity. Cells are gated on single live cells. % of live cells is indicated in each plot. FSC= forward scatter. Cells were stained with CD14-V500.

I then tracked the development of the DCs using surface stains at each stage of development in samples from volunteer DC4 and in 2 subsequent samples (volunteers DC2 and LC90). Phenotypic markers were assessed in 1 volunteer (DC4) at baseline and at Days 4 (immature DCs) and Day 6 (mature DCs) in DC2, DC4 and LC90 (Figure 5-5 and Figure 5-6).

Figure 5-5 Dendritic Cell development: representative FACS plots



Dendritic cell line from volunteer DC4, stained for external markers (*y axis*) at three timepoints during Dendritic Cell (DC) differentiation. % CD14+ marker+ cells is shown at baseline and at Day 4 and Day 6 post monocyte separation. Cells are gated on live cells. Gating for each marker was determined using an unstained sample at each timepoint (therefore gates are specific to each timepoint) since auto-fluorescence changed as DCs matured.

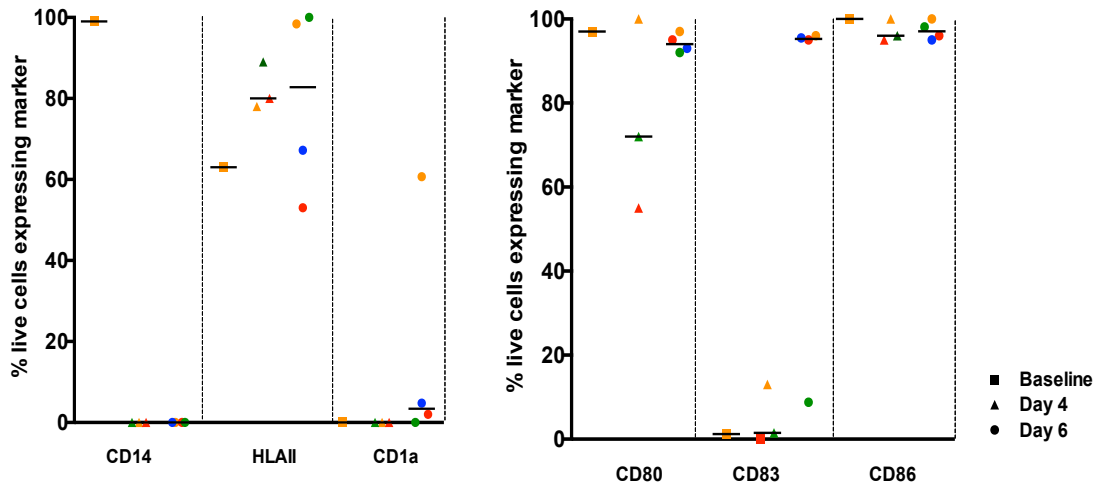
During DC maturation, there was a striking shift from a CD14 positive population (after monocyte selection) to a CD14 negative population by day 4, which remained negative in all individuals at DC maturation on day 6 (Figure 5-6). CD1a marker was not upregulated in all individuals, likely due to the presence of

human serum, which has been shown to inhibit the upregulation of CD1a, and instead cause upregulation of CD1d (Leslie et al. 2008).

CD83 is the most stringent marker of DC development. Immature DC samples at day 4 were predominantly CD83 negative, however by day 6 all fresh samples were CD83 positive. One frozen sample, LC90, failed to develop this critical differentiation marker and remained CD83 negative at Day 6 (Figure 5-6). When examined under the microscope this sample did not have a typical DC appearance, nor did cells from this line successfully prime any naïve T cells, suggesting that LC90 did not produce mature DCs.

Other markers critical for T cell priming, CD80 and CD86, were positive, however given high levels of positive staining at baseline it was not possible to show that these were upregulated over time. Ideally these stains would have been titrated to show a CD80/CD86 low population at baseline, however since other important markers displayed the expected phenotype and DCs were functional (as shown in the experiments to follow), this was not pursued further.

Figure 5-6 Dendritic Cell development over time



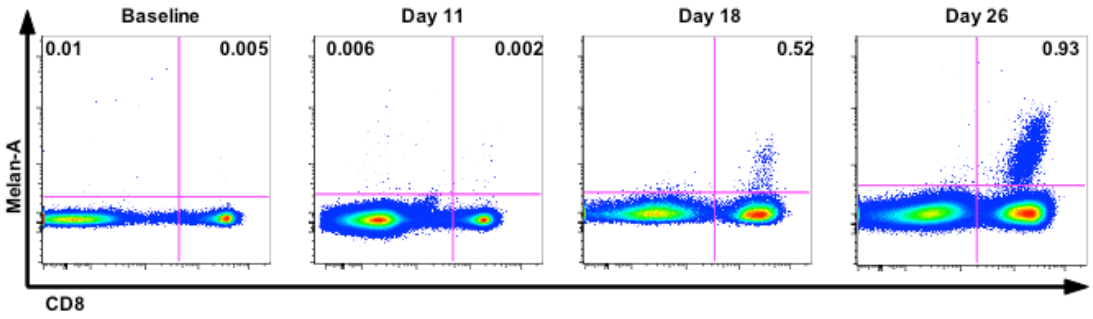
DCs were stained for markers (*x axis*) before development (DC4a), at Day 4 (DC2, LC90, DC4a) and on Day 6, at maturation (DC2, LC90, DC4a and DC4b). DC4a and DC4b are from the same donor, however different experiments. Individual volunteers are represented by different colours. DC4a=yellow, DC4b=blue, LC90= green, DC2= red. Timepoints are represented by different shapes. Cells were gated on live cells. Median (horizontal bar) is shown.

5.4.1 Monocyte derived dendritic cells can prime autologous naïve T cell populations

I next incubated the DC line, matured with Melan-A peptide, with autologous PBMC isolated at the time of monocyte separation. I then stained the sample at three consecutive timepoints for Melan-A+ cells (Figure 5-7). At baseline and at Day 11 post monocyte separation there was no identifiable Melan-A+ population. However one week later at Day 18, a large Melan-A$^+$ population could be identified (0.52% of CD3$^+$ T cells). These were re-stimulated at Day 18 after monocyte separation with more frozen autologous PBMC (8×10^6) pulsed with Melan-A peptide, and at Day 26 produced a highly expanded Melan-A specific population representing 0.93% CD3$^+$ cells or 1.76% CD8$^+$ T cells.

These results confirmed that this experimental model could produce mature DCs, which could prime naïve T cell populations.

Figure 5-7 Priming of Melan-A specific T cells from naïve precursors, using an in vitro priming model



DCs matured with Melan-A peptide were used to prime autologous naive T cells from donor DC4. Melan-A tetramer is on the y axis, CD8 on the x axis. % of CD3$^+$ cells is indicated in individual plots. Cell lines were re-stimulated with thawed autologous feeder cells pulsed with Melan-A peptide (10µg/ml) at Day 18.

5.5 Can *in vitro* priming produce HCV specific T cells which recognise the most common HCV sequence variants at epitope NS3$_{1406}$?

I next proceeded to use this *in vitro* model to prime HCV specific T cell responses as described in Figure 5-1 p184. I primed T cell lines to the vaccine immunogen sequence at NS3$_{1406}$ called 95A from here on in (as a control) and to 4 other sequence variants at this site, called 95B, 95C, 95H and 95I. These are variants present in >5% circulating genotype 1 sequences (Table 5-1), identified as described in Materials and Methods and discussed in Chapter 4.

Table 5-1 Variants of NS3$_{1406}$ used for T cell priming and their relative frequency in genotype 1.

Sequence	% 1a	% 1b	% Genotype 1 HCV overall	Peptide name
KLSGLGINAV	0	4.3	2.0	95A
--VA------	46.8	0	24.8	95B
--VA--V---	27.0	0.6	14.5	95C
------L---	0.2	38.4	18.2	95H
---S--L---	0	19.6	9.2	95I

Based on analysis of 1526 HCV sequences from Los Alamos database (hcv.lanl.gov/). "– " indicates amino acid homology with the prototype sequence in the vaccine immunogen listed in full.

In brief, peptide pulsed DCs were used to prime naive T cells and to develop T cell lines. DCs were pulsed with one of 5 peptide variants from Table 5-1 and each DC line was used to prime an autologous T cell line. These T cell lines were tested for cross-reactivity using 5 different pentamers specific for the different NS3$_{1406}$ variants (Figure 5-1 p184).

In the following sections I first describe the alterations to the methods required to successfully prime HCV specific T cells and then the results from the first pilot experiment using samples DC2 and DC4. Then I describe the subsequent experiments to confirm and expand upon these findings.

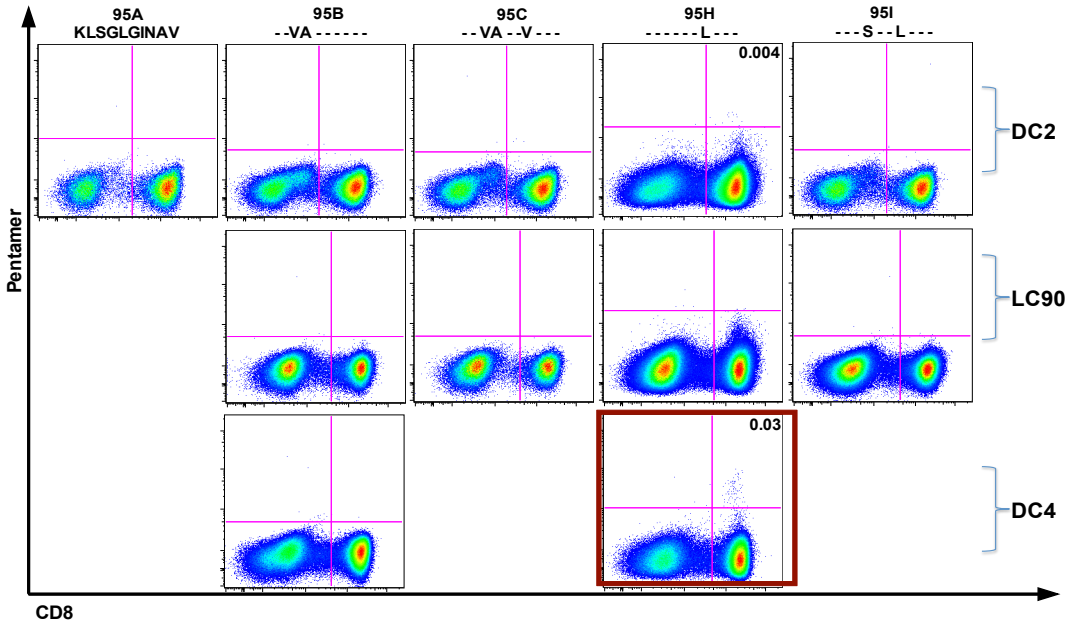
5.5.1 Refining the method for *in vitro* priming of HCV specific T cells

Modifications were necessary to the priming protocol, to account for the low pre-cursor frequency of HCV specific T cells compared with Melan-A specific T cells.

HCV specific T cell lines need longer to grow than Melan-A specific cell lines

Firstly, I needed to change the timepoints at which cell lines were tested for pentamer positive cells. Although Melan-A+ primed cell lines were detectable by Day 18 (Figure 5-7), when the first three individuals primed with HCV specific peptides (DC2, DC4, LC90) were tested at Day 20, there was only 1 positive HCV specific T cell line (DC4, primed by peptide 95H) (Figure 5-8). Furthermore, the magnitude of responses in the one positive cell line was small, with only 0.03% CD3$^+$ pentamer$^+$ T cells, in contrast to the Melan-A cell line, which had 0.52% CD3$^+$ Melan-A$^+$ T cells at this equivalent timepoint (Figure 5-7).

Figure 5-8 Day 20 pentamer staining of T cell lines primed by HCV peptides in 3 healthy individuals.



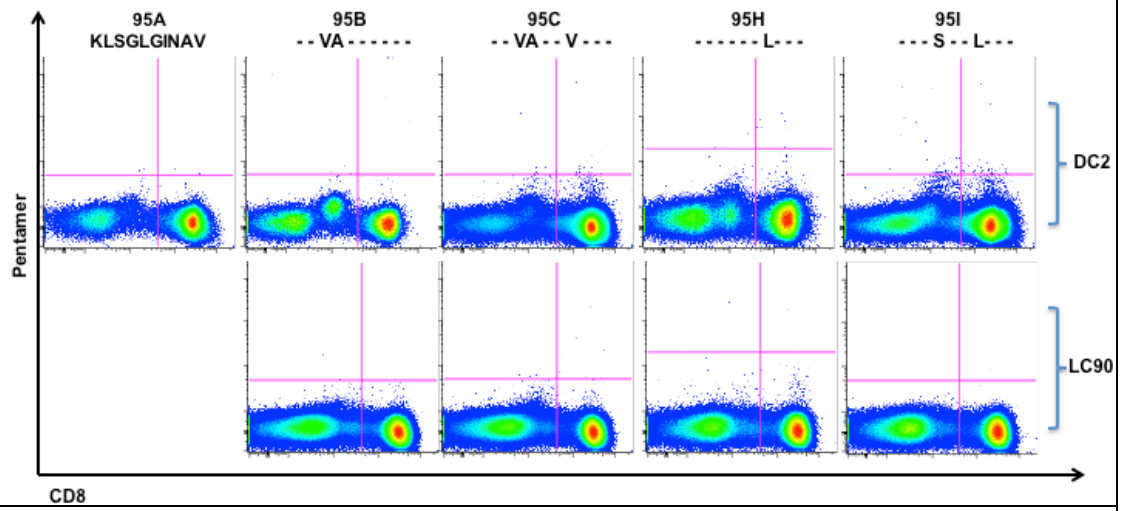
T cell lines from 3 HLA-A2 positive individuals (DC2, LC90 and DC4) were stained with pentamers matched to the peptide they were primed with, 20 days after monocyte separation at the start of the experiment. Each row represents a different volunteer. Each column represents a different cell line, primed with the peptide listed at the top of the column. The peptide contained within the vaccine immunogen is listed in full (left column), while dashed lines indicate amino acid homology with the immunogen sequence. The pentamer corresponding to the peptide used to prime the cell line is shown (y axis), with %CD3$^+$pentamer$^+$ T cells shown in the top right of the FACs plot when >0. All pentamers are on PE except for 95 H which is on APC. Cells are gated on live CD3$^+$ cells. The positive pentamer population is boxed in red.

Assessing later timepoints and restimulation with further peptide or feeder cells were clearly important for T cell priming with HCV specific peptides. At Day 20, I restimulated cell lines from DC2 and LC90 using peptide pulsed autologous frozen PBMC, in an identical manner to that used for restimulation of the Melan-A cell line. Due to a lack of available cells, I was unable to restimulate the cell lines from volunteer DC4 in this manner, so instead restimulated these lines using peptides alone (10µg/ml). When samples were restained 8 days later at Day 28, there were still no HCV specific cell lines from volunteers DC2 or LC90 (Figure 5-9). In contrast, there was significant expansion of the 95H primed cell line from DC4 (Figure 5-10). I therefore modified the protocol and restimulated

cell lines from volunteer DC2 with peptide alone at Day 28, which, at Day 35 (1 week later) successfully produced 2 HCV specific cell lines, 95C and 95H, discussed further below.

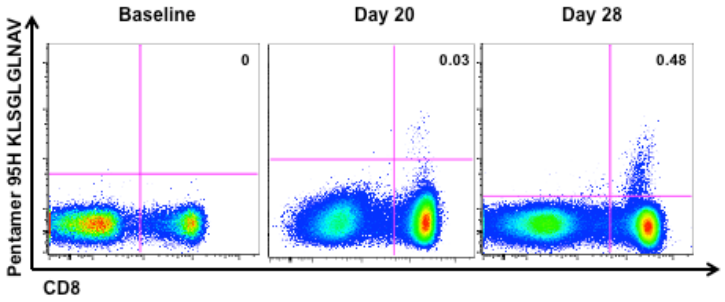
For subsequent volunteers, the timeframe for staining was thus increased up to Day 35, and peptide stimulation was chosen as the initial method of restimulation, with autologous feeder cells added at later timepoints.

Figure 5-9 Restimulation with frozen autologous feeder cells was unsuccessful at priming HCV specific T cells at Day 28.



Cell lines primed with HCV specific peptides from 2 HLA-A2 positive individuals (DC2 and LC90) were stained with pentamers, 8 days after restimulation using peptide pulsed frozen autologous PBMC. Each row represents a different volunteer. Each column represents a different cell line, primed with the peptide listed at the top of the column. The peptide contained within the vaccine immunogen is listed in full (left column), while dashed lines indicate amino acid homology with the prototype sequence. The pentamer corresponding to the peptide used to prime the cell line is shown (*y axis*). Cells are gated on live CD3$^+$ cells.

Figure 5-10 Development of HCV specific T cells, primed in vitro, in volunteer DC4



Naïve T cells were primed with autologous DCs matured with peptide 95H (KLSGLGLNAV,) then restimulated with the same peptide at Day 20 and stained sequentially with the matched pentamer (y axis). % $CD3^+Pentamer^+$ T cells is shown in the top right of each plot. Cells are gated on live $CD3^+$ cells.

Fresh samples are better than frozen samples

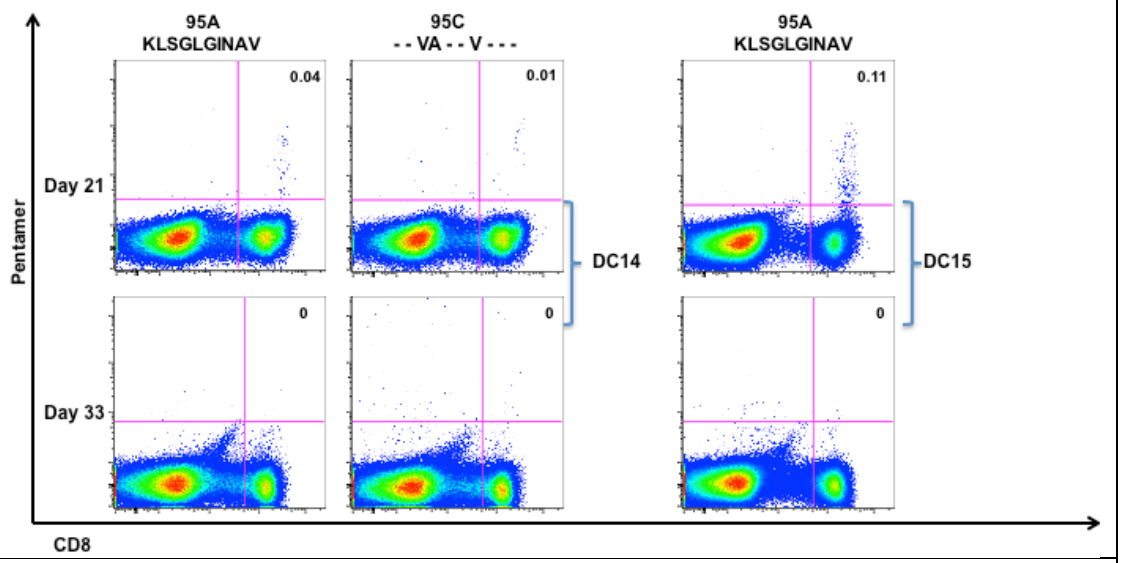
The frozen sample, LC90, failed to grow any successful cell lines. Furthermore, not only was DC development poor in this individual (Figure 5-6), staining at day 20 showed poor priming with a much lower proportion of $CD8^+$ cells (median $CD3^+$ $CD8^+$ T cells 41.75%; range 41.3- 42.2) in comparison to the fresh cell lines DC2 and DC4 (median $CD3^+$ $CD8^+$ T cells 65%; range 64.8-75.4) (data not shown) (Figure 5-8). Although using frozen cells would have streamlined the experiment considerably, this plan was abandoned after the poor priming seen with volunteer LC90 and further analysis of LC90 is not included in these results.

Cell lines must be re-plated in 6 well plates before adding feeder cells

I attempted one further refinement of the restimulation stages of this protocol. Previously, I had re-plated cell lines in 6 well plates when adding autologous feeder cells (see Materials and Methods). In 3 volunteers, I trialed adding feeder cells directly to the same 24 well plate in which cells had been primed. Although this would have conserved cell numbers and simplified the experiment, it was

not successful and resulted in the loss of pentamer positive populations. There were 3 positive cell lines identified at Day 21 which were no longer present at Day 33 following this modification (Volunteer DC14, line 95A and 95C, Volunteer DC15 line 95A). This modification was therefore not incorporated in the protocol.

Figure 5-11 Loss of HCV specific T cells after trying a new method of adding autologous feeder cells to primed naïve T cells.



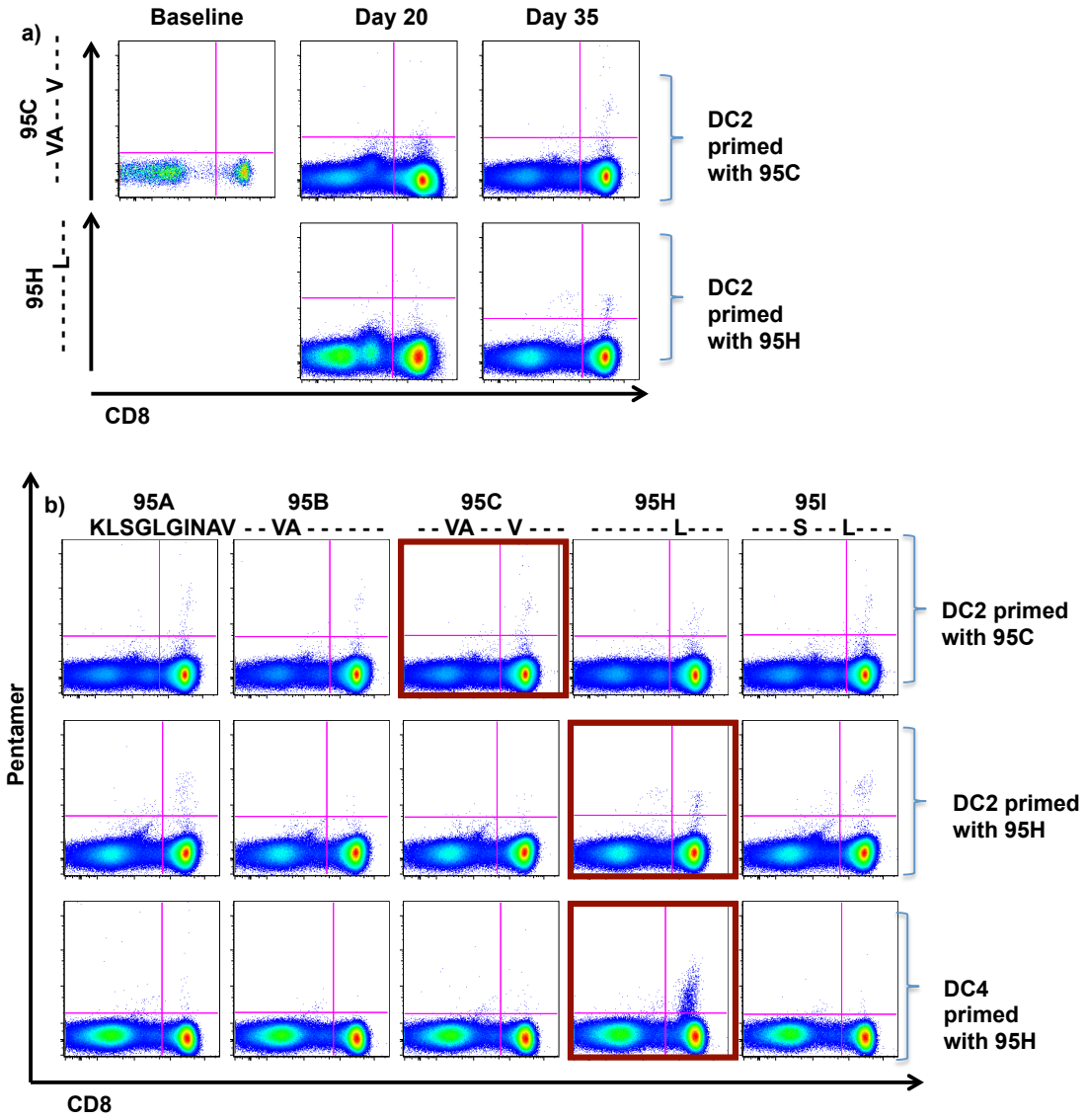
Pentamer staining of *in vitro* primed HCV specific T cells in two volunteers, DC14 and DC15. Cells were primed using peptides indicated at the top of each column and stained with matched pentamers (y axis). "-" indicates amino acid homology to the prototype sequence in the vaccine immunogen. Each row represents a different timepoint, showing the loss of HCV specific T cells following the addition of autologous feeder cells at Day 33, after an alteration to the protocol. % $CD3^+Pentamer^+$ T cells is shown in the top right of each plot. Cells are gated on live $CD3^+$ cells.

5.5.2 Results from the pilot experiment: there is a variant of epitope NS3$_{1406}$ which primes broadly cross-reactive T cells.

Initial results from the first two individuals tested (DC2 and DC4) demonstrated that it was possible to prime multiple HCV specific T cell lines (Figure 5-10 and Figure 5-12). In DC4 only 2 variants from Table 5-1 (95B and 95H) could be tested in this initial experiment (due to cell numbers), producing 1 successful 95H primed cell line which was cross-reactive with 95A (Figure 5-12b).

From DC2, T cell lines were primed to 2 out of the 5 variants tested from Table 5-1: 95C and 95H (Figure 5-12). The cross-reactivity of all three cell lines was tested by staining T cells from each line with pentamers matched to the other common sequence variants. Here, 95H primed T cells were cross-reactive with other genotype 1b variants, 95I and 95A. In contrast, cells primed by 95C recognised all 5 sequence variants tested by pentamer staining (Figure 5-12b). This suggested that it might indeed be possible to prime T cells for a universal genotype 1 vaccine. Could version 95C at epitope NS3$_{1406}$ be the key to such a vaccine?

Figure 5-12 The Universal genotype 1 HCV epitope? Pentamer staining of T cell lines primed with variants of peptide 95A (KLSGLGINAV).

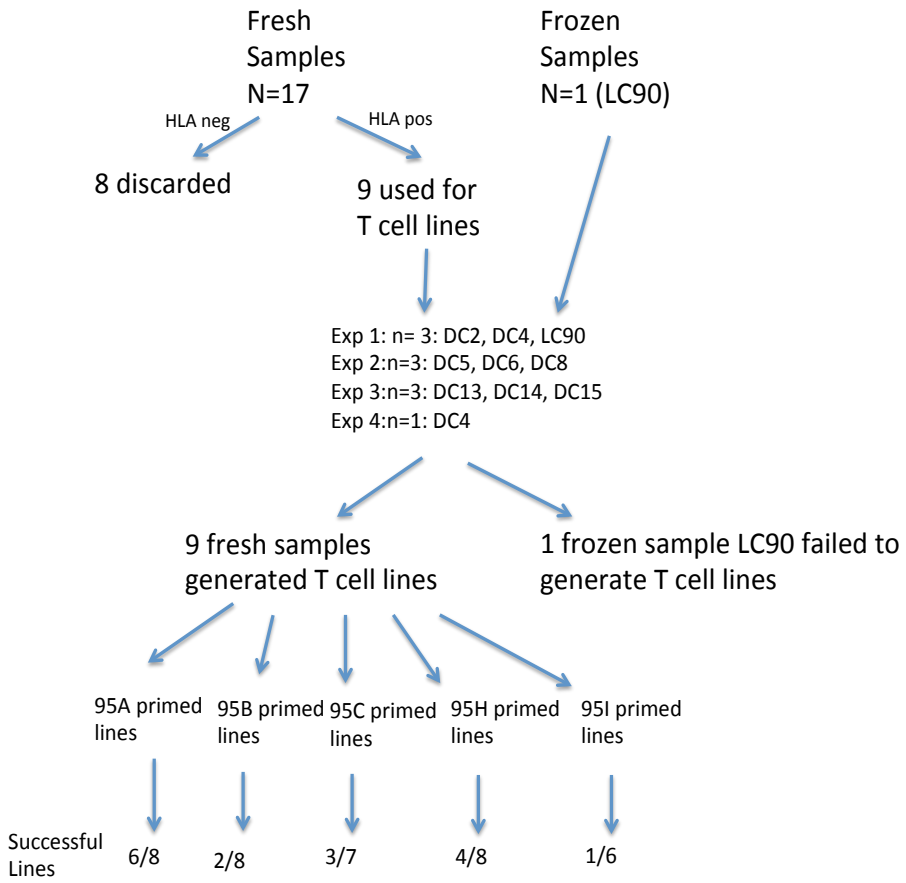


a) Development of HCV specific cells in volunteer DC2 over time. Two different cell lines, one primed by peptide 95B and one primed by peptide 95H are shown in each row. Each is stained with a pentamer matched to the priming peptide (*y* axis). **b**) Cross-reactivity of T cells primed by different peptides: 95C (top row) and 95H (middle and bottom rows), in volunteers DC2 and DC4. Each row represents a different cell line. Each cell line is stained with 5 pentamers, represented by a different column, and listed at the top of each column. "-" indicates amino acid homology with the prototype peptide sequence 95A (left column). The pentamer which matches the peptide used to prime a cell line is ringed in red. T cells primed by 95C (KLVALGVNAV) bind all 5 pentamers tested (top row). Cells are gated on live CD3$^+$ lymphocytes.

5.5.3 Is peptide 95C (KLVALGVNAV) the best candidate for an NS3$_{1406}$ containing vaccine immunogen?

I next proceeded to verify the possibility that peptide 95C could be part of a universal genotype 1 HCV vaccine, by using a larger sample size. In total, 17 fresh blood cones were obtained in 4 separate experiments, and 8 were discarded (not HLA-A2 positive). The 9 remaining cones were used to develop up to 5 different T cell lines per cone (cells permitting) for 35 days as described in Figure 5-1. The progress of this experiment is chartered in Figure 5-13 below.

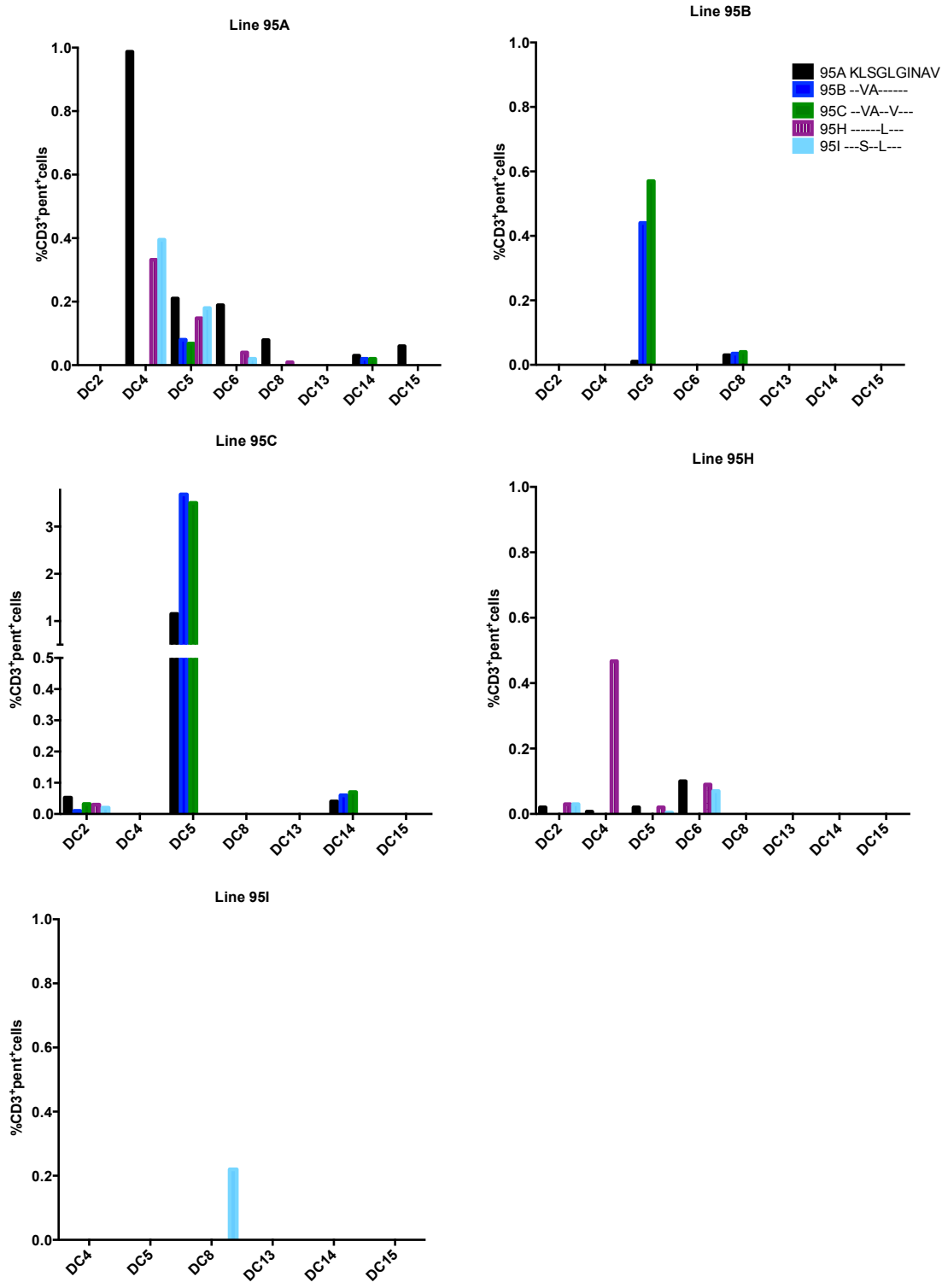
Figure 5-13 Progress of HCV specific T cell line culture



9 fresh whole blood cones and one frozen PBMC sample were selected for dendritic cell (DC) development and T cell priming. 4 separate experiments were performed named experiment (Exp) 1-4. Fresh cones were named DC2 to DC15 and the frozen PBMC sample was named LC90.

It was possible to prime T cell responses to all variants tested, although some were more immunogenic in terms of magnitude and frequency of T cell priming than others (Figure 5-14). The variant which most commonly primed T cells was the vaccine immunogen version, 95A, which successfully primed T cells in 6/8 individuals (75%). The common genotype 1b variant 95H was the next most frequently successful, priming responses in 4/8 (50%) volunteers. Responses in these 4 volunteers were low however: median $CD3^+$ $pentamer^+$ cells was 0.06%; range 0.02-0.47. In contrast, variant 95C primed responses in fewer individuals (3/7, 42.8%) however did produce the greatest magnitude response (3.7% $CD3^+$ $pentamer^+$ cells), nearly 4 times greater than the next largest response generated by peptide 95A (0.9% $CD3^+$ $pentamer^+$ cells). Two variants which did not appear immunogenic were variant 95I, which primed responses in only 1 person (DC8) and 95B which primed responses in 2/8 volunteers (DC5 and DC8).

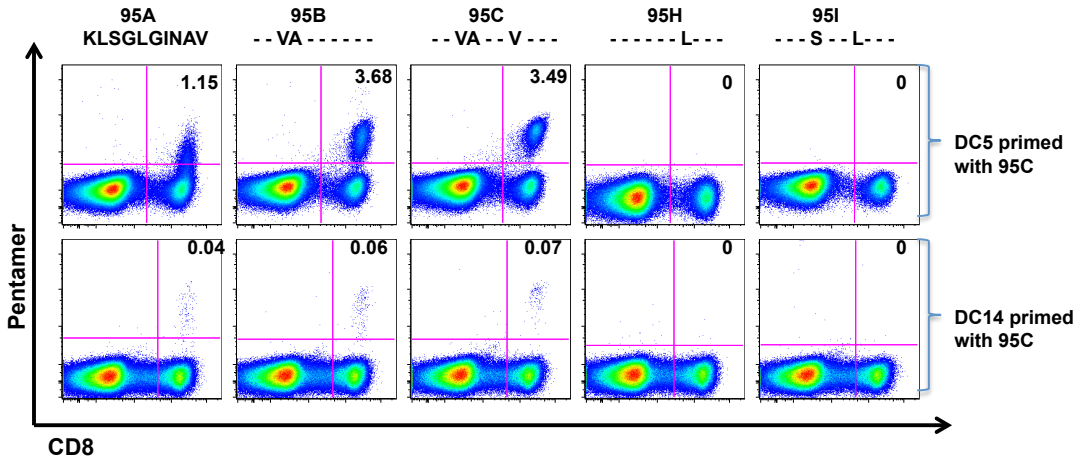
Figure 5-14 Magnitude and Cross-reactivity of T cell responses primed with HCV specific peptides in healthy HLA-A2 positive volunteers



Cell lines primed with different versions of peptide 95 are shown in each graph. Each cell line from each volunteer was stained with a different pentamer, represented by a coloured column. The vaccine immunogen sequence (95A) is listed in full, and " -" indicates amino acid homology with 95A. Individual volunteers (*x axis*) and %CD3$^+$ pentamer$^+$ cells (*y axis*) are shown.

The promising initial findings of universal cross-reactivity with peptide 95C priming, could not be replicated in either of the two other volunteers successfully primed with this variant (volunteers DC5 and DC14). Although there was good cross-reactivity to 95A and 95B, the common genotype 1b variants 95H and 95I were not recognised by these cell lines at all (Figure 5-15).

Figure 5-15 Cell lines primed with peptide KLVALGVNAV (95C) do not produce universally recognised HCV specific T cells



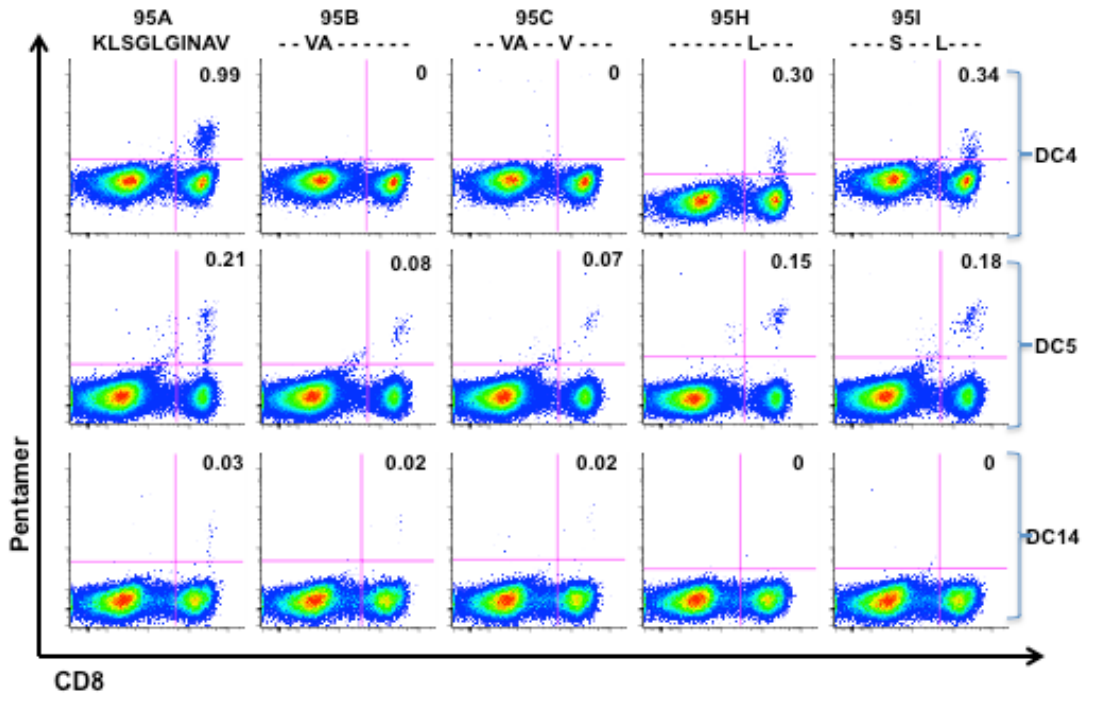
T cell lines from 2 healthy volunteers; DC5 (Day 33) and DC14 (Day 21) after priming with peptide KLVALGVNAV (95C). Each column represents staining with a different pentamer (*y axis*) listed at the top of the column. % $CD3^+$ $pentamer^+$ cells are shown in the top right corner of each plot. Cells are gated on live $CD3^+$ lymphocytes

Cross-reactivity was assessed in all other successful cell lines. The 5 cell lines primed by genotype 1a variants (95B and 95C) all recognised other genotype 1a variants, i.e. all 95B primed lines recognised 95C matched pentamers and vice versa, however apart from the initial result in volunteer DC2 (Figure 5-12), none demonstrated cross-reactivity to the 1b variants, 95H and 95I. Similarly, T cells primed by genotype 1b variants tended to recognise other 1b variants. 3/4 lines primed by 95H cross-recognised the other common 1b variant, 95I. The poorly immunogenic peptide 95I (which produced a response in only 1 individual-DC8) was not cross-reactive with any variants. This was also the only cell line which

did not show any cross-recognition of the vaccine immunogen peptide, 95A. Interestingly, all other cell lines primed by other peptide variants (95B, 95C and 95H) cross-recognised the uncommon 95A variant, present in the vaccine immunogen.

The cross-reactivity of 95A primed cell lines was inconsistent between volunteers. 2 lines (DC4 and DC6) were cross-reactive to both 95H and 95I, 1 line (DC8) recognised 95H alone, and 1 low magnitude T cell line from DC14 recognised the genotype 1a variants 95B and 95C. One volunteer, DC5 produced a broadly cross-reactive T cell line which recognised all variants (Figure 5-16).

Figure 5-16 Patterns of cross-reactivity in T cells primed by KLSGLGINAV (95A)

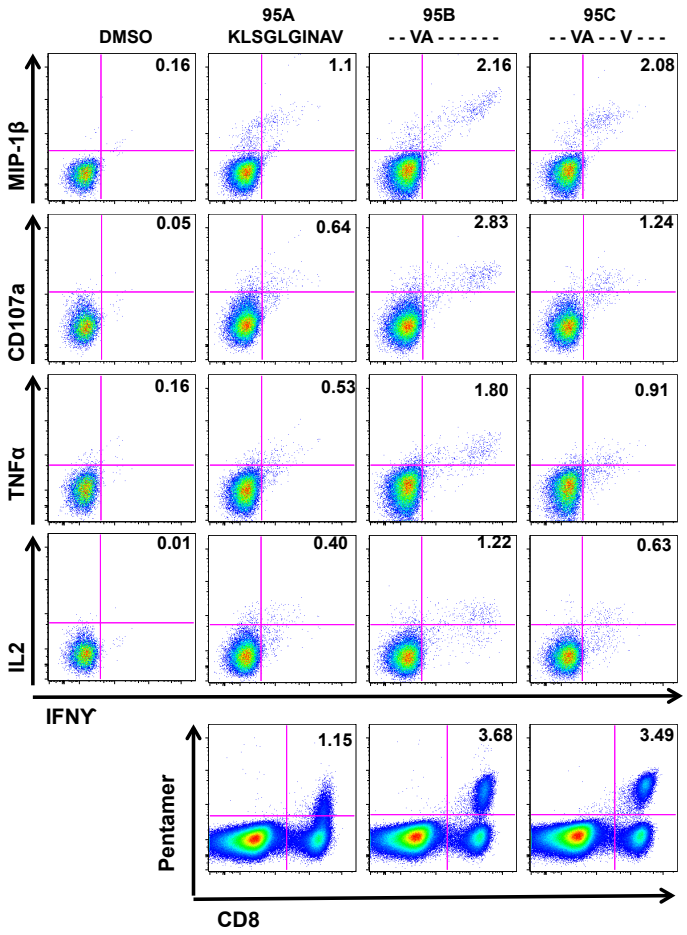


T cell lines from 3 healthy volunteers; DC4 (Day 21) DC5 (Day 33) and DC14 (Day 21) after priming with peptide KLSGLGINAV (95A). The top row (DC4) shows cross-reactivity with versions 95H and 95I. DC5 shows cross-reactivity to 95B, 95B, 95H and 95I. DC14 shows cross-reactivity to 95B and 95C. Each column represents staining with a different pentamer (*y axis*) listed at the top of the column. %CD3$^+$ pentamer$^+$ cells are shown in the top right corner of each plot. Cells are gated on live CD3$^+$ cells.

5.5.4 Functional recognition of different sequence variants

It is possible, although unlikely, that T cell responses, which appear to be cross-reactive via pentamer staining, may not actually be functional responses. That is, primed T cells may bind variant peptides presented by MHCI (and detectable by pentamer staining) but the TCRs are not triggered and there is no downstream signaling. To assess the functionality of cross-reactive responses, ICS was performed on 9 cell lines after pentamer staining.

Figure 5-17 Representative ICS staining in a cell line primed with peptide 95C (KLVALGVNAV) from healthy volunteer DC5

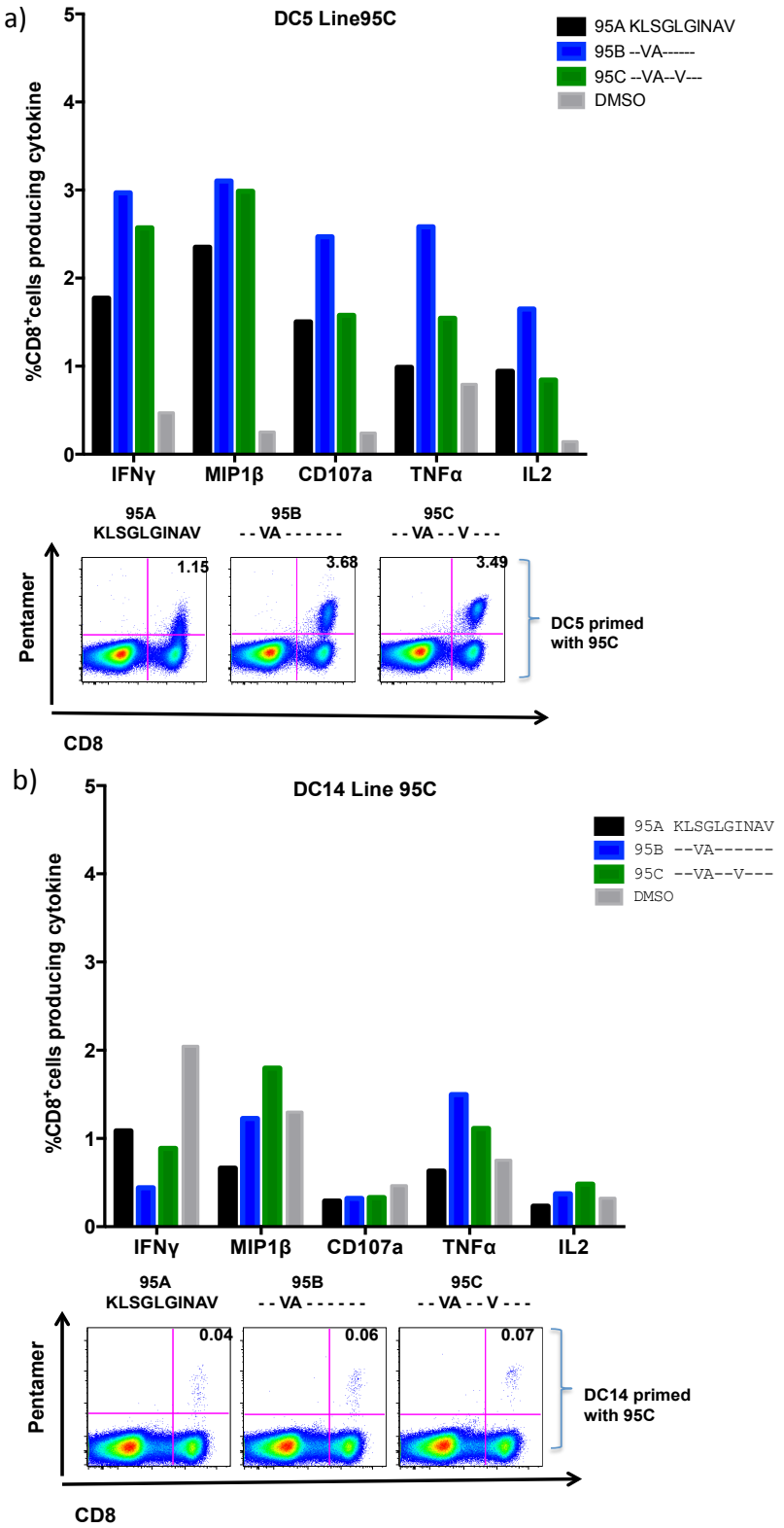


The top 4 rows represent ICS staining. The bottom row represents comparative pentamer staining. A cell line from volunteer DC5 was primed by peptide 95C and stimulated with different variants of epitope NS3 $_{1406}$ (95A, 95B, 95C,95H and 95I listed above each column) and stained for cytokines (*y axis*). Amino acid homology to the peptide contained within the vaccine immunogen is indicated by "-". Cells are gated on live CD8$^+$ cells. % CD8$^+$cells positive for both IFNγ and the *y axis* cytokine is indicated in the top right.

First, I assessed the functionality of the cell lines primed with variant 95C to confirm that the cross-reactive pentamer stains represented functional cross-reactive T cell responses. In the 95C primed line from volunteer DC5, polyfunctional cytokine production (IFNγ, MIP1β, CD107a, TNFα and IL2) was observed in response to stimulation by peptides 95A and 95B, as well as the autologous peptide 95C (Figure 5-18).

ICS data from DC14 was more difficult to interpret due to high levels of background and smaller populations of 95C specific T cells (Figure 5-14). The only response significantly above background was MIP1β production in response to stimulation by the autologous peptide 95C. There was some suggestion of TNFα production in response to stimulation by 95B and 95C although the 95A specific cells identified by pentamer staining could not be verified by ICS.

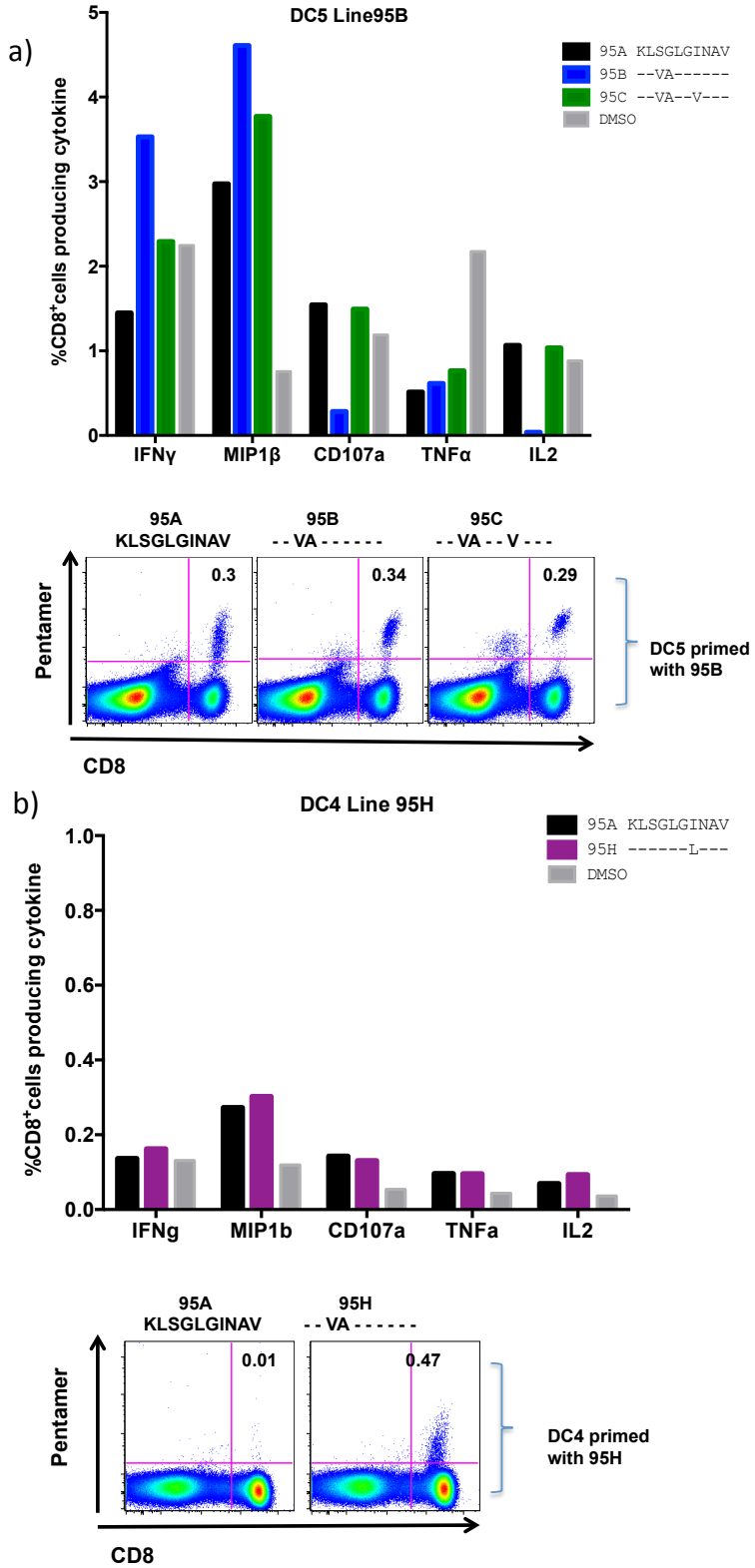
Figure 5-18 Functional cross-reactivity of cell lines primed with KLVALGVNAV (95C) in 2 healthy volunteers



Cell lines were stimulated by different peptide variants (95A, 95B, 95C), indicated by coloured columns. Subsequent cytokine production was assessed via ICS and compared with pentamer stains. Cell lines from 2 volunteers are shown: (**a**) DC5, Day 33 after monocyte separation and (**b**) DC14, Day 21 after monocyte separation % CD8⁺cells producing a given cytokine (*x* axis) is shown on the *y* axis. Cells are gated on live CD8⁺ cells.

Next I assessed cytokine production from lines 95B and 95H. Despite high levels of background in the 95B primed cell line (from volunteer DC5) there was a clear pattern of IFNγ and MIP1β production in response to stimulation by peptides 95B and 95C, with 2.43% and 1.46% IFNγ$^+$/MIP1β$^+$ CD8$^+$ cells respectively. (Figure 5-19a). Line 95H in DC4 produced only weak ICS responses, however stimulation by both 95A and 95H elicited detectable cytokine responses (Figure 5-19b).

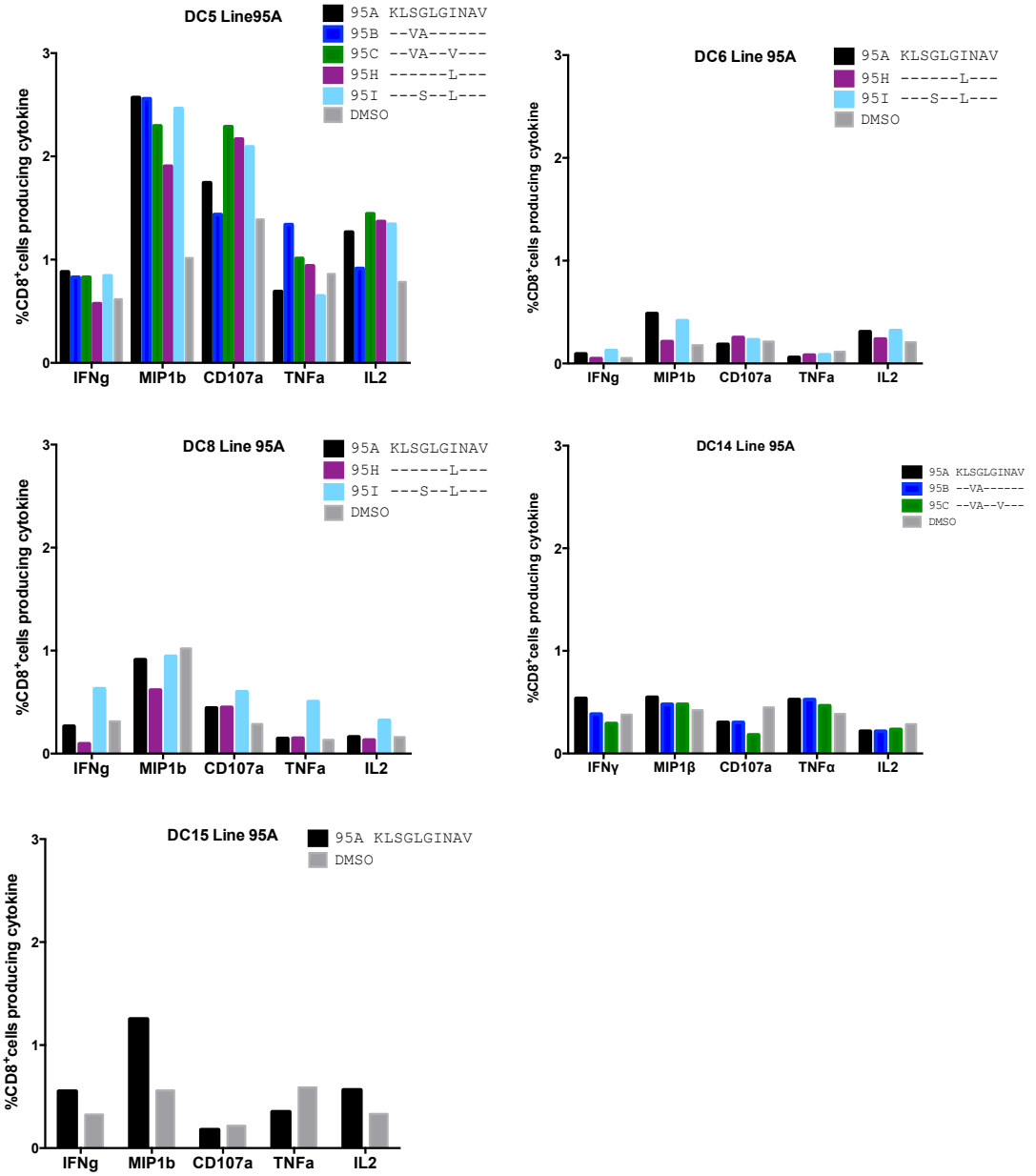
Figure 5-19 Functional cross-reactivity of cell lines primed with KLVALGINAV (95B) and KLSGLGLNAV (95H)



Cell lines were stimulated by different peptide variants, indicated by coloured columns. Subsequent cytokine production was assessed via ICS. Cell lines from 2 volunteers (DC4 and DC5) are shown with corresponding pentamer stains: **(a)** DC5 primed by 95B, Day 33 after monocyte separation **(b)** DC4, primed by 95H, Day 21 after monocyte separation % CD8$^+$cells producing a given cytokine (*x axis*) is shown on the *y axis*. Cells are gated on live CD8$^+$ cells.

Finally, I assessed ICS in 95A primed cell lines from 5 individuals (Figure 5-20). The unusual broadly cross-reactive response in DC5 revealed by pentamer staining, was reproduced by peptide stimulation, where all 5 peptide variants stimulated polyfunctional cytokine production. In volunteer DC14, where there was also a suggestion of unusual cross-reactivity with variants 95B and 95C, high levels of background made this impossible to confirm via ICS. In other individuals, it was difficult to show functional cross-reactivity with the variants detected by pentamer staining. In volunteer DC6 responses were too low to demonstrate cross-reactivity to 95H and I, except for MIP-1β production which was observed in response to 95A and 95H. Otherwise there was minimal cytokine production even in response to stimulation with the autologous peptide 95A. In DC8, responses to 95H and I were expected (as shown by pentamer staining) however background was high in this sample, and only CD107a production could be shown in response to both peptides. Peptide 95I was able to stimulate production of other cytokines (TNFα, IL2 and IFNγ) however this is hard to interpret in the context of low responses to stimulation by the autologous peptide 95A. Cell lines from DC15 were not cross-reactive for other variants by pentamer staining however ICS confirmed that 95A specific T cells produced multiple cytokines in response to 95A peptide stimulation.

Figure 5-20 Functional cross-reactivity of cell lines primed with KLSGLGINAV (95A)



Cell lines primed by peptide KLSGLGINAV (95A) from 4 volunteers (DC5, DC6, DC8, DC14, DC15) are shown, stimulated by different peptide variants to which they were cross-reactive via pentamer staining. Each variant is represented by coloured columns. Subsequent cytokine production was assessed via ICS. % CD8⁺ cells producing a given cytokine (*x axis*) is shown on the *y axis*. Cells are gated on live CD8⁺ cells.

5.6 Discussion

This is the first attempt to my knowledge, to use an *in vitro* model to systematically identify the optimal version of a T cell epitope to include in a vaccine immunogen. I chose to focus on epitope NS3$_{1406}$ for this work due to its immunodominance in both healthy volunteers and patients vaccinated with an immunogen containing this epitope (NSmut). Importantly NSmut contains only a rare variant (KLSGLGINAV) of this highly variable epitope.

Priming naïve HCV specific T cells is more difficult than priming Melan-A specific T cells

First I validated a previously described dendritic cell/ T cell priming protocol (Salio et al. 2001). I showed that this protocol successfully primed Melan-A specific T cells and produced mature DCs. I then modified this protocol to allow for the more difficult priming of HCV specific T cells, in particular to account for their lower precursor frequencies. It is not known what the T cell precursor frequency is for most of the variants tested in this thesis, except for one variant 95B (KLVALGINAV) which was shown in a study of 8 healthy volunteers to have a precursor frequency of 1:150,000 CD8$^+$ T cells (Schmidt et al. 2011). This is in contrast to the precursor frequency of Melan-A, specific T cells which has been shown to be 1:2,500 (Pittet et al. 1999). I established a successful HCV specific protocol by using fresh cells rather than frozen cells, growing T cell lines for long periods (up to 35 days) as well as using peptides for initial restimulation rather than autologous PBMC.

The NS3$_{1406}$ variant in NSmut (95A, KLSGLGINAV) primed T cells in the greatest number of individuals

As shown by Wolfl et al. not all NS3$_{1406}$ variants were equally immunogenic. That is, some variants primed very few T cell lines compared with other variants, despite being used to grow T cell lines from the same individuals. A particularly poor candidate for inclusion in a vaccine, if these *in vitro* results are replicated *in vivo*, was variant 95I. Although this represents nearly 20% of genotype 1b sequences it was poor at priming T cells with only 1 out of 6 cell lines growing pentamer detectable T cells.

The variant which was the most successful at priming T cell lines was variant 95A, which produced 6 successful lines out of 8 attempts. This is the variant contained within the original vaccine immunogen discussed in this thesis (NSmut). The next most successful was variant 95H which primed 50% (4/8) of cell lines attempted.

Why might different variants have different immunogenicity?

T cell Receptor /HLA binding avidity

There are many reasons why some variants might be more successful at priming naive T cells than others. This work has not explored the individual binding characteristics of the peptides representing each variant, although this would make interesting ongoing work. Peptides are likely to have different HLA and T cell receptor (TCR) binding affinity. For example, variant 95A may have a particularly strong avidity for HLA binding molecules whereas 95I may bind weakly, and not be available for presentation to T cells.

NS3$_{1406}$ is an HLA-A2 epitope. The anchor binding positions of the HLA-A2 molecule are well defined, with the strongest amino acid binders being a hydrophobic amino acid at position 2 such as leucine (L) or methionine (M) and leucine or valine (V) in position 9 (Parker et al. 1994). As shown in Table 5-2, these anchor positions are conserved between the different peptide variants with leucine at position 2 and alanine at position 9. Instead, mutations occur at positions 3, 4 and 7. These mutations could nonetheless still completely alter the binding shape of the peptide, despite the conservation of dominant anchor residues.

Table 5-2 Relative positions of amino acid differences between variants of NS3$_{1406}$

	1	2	3	4	5	6	7	8	9	10
95A	K	L	S	G	L	G	I	N	A	V
95B	–	–	**V**	**A**	–	–	–	–	–	–
95C	–	–	**V**	**A**	–	–	**V**	–	–	–
95H	–	–	–	–	–	–	**L**	–	–	–
95I	–	–	–	**S**	–	–	**L**	–	–	–

It is important to note that this culture system does expose cells to peptide for prolonged periods, therefore results may not be representative to those *in vivo* where antigens may be encountered more briefly or more infrequently. This system also bypasses antigen processing and presentation by adding peptide directly to the cell milieu, therefore it is not known whether some of these seemingly immunogenic variants such as 95C or 95H, are actually processed *in vivo*. Nonetheless the fact that they are found in circulating sequences worldwide offers the possibility that they may be. We know at least from the results of vaccinating patients in healthy volunteers with an immunogen containing 95A, that 95A can be successfully processed *in vivo*.

Naïve T cell precursor frequency may be different between variants

Another possibility to account for different immunogenicity of the different NS3$_{1406}$ variants is that there are different naive T cell precursor frequencies for each of these variants. Wolfl and others have postulated that this accounted for their difficulty prime T cells using a peptide not trialed here (KLVAMGINAV). If there were indeed a paucity of naïve T cells to a specific variant that could explain why for example 95I was so poorly immunogenic.

Prior T cell priming by similar antigens

It is also possible that these volunteers may have been exposed to a previous antigen (either endogenous or exogenous) which is similar to one or some of these variants. This has been described with the HCV epitope NS3$_{1073}$ where previous exposure to influenza virus was reported to result in cross-reactive T cell responses to this epitope (Wedemeyer et al. 2001). However, this finding was not replicated by another study using tetramers to assess cross-reactivity (Kasprowicz et al. 2008). This has not been reported at epitope NS3$_{1406}$, however if it were the case then the high immunogenicity of certain variants could be due to their similarity to previously encountered antigens and the existence of a memory population. This however seems less likely than the other possibilities.

Finally, it is important to keep in mind that individual variation in the capacity of different peptides to prime T cells in these experiments could be due to chance. It is possible that some experiments contained different numbers of naive precursors for the different variants as only total cell numbers were counted

before adding to DCs for priming. An attempt was made to account for this by repeating the experiment in a number of different individuals. This allowed a clear pattern to be established where if the *in vitro* results were to play out *in vivo,* 95I would certainly be a poor choice for a vaccine immunogen. Furthermore, if a genotype 1a variant were to be selected for an immunogen, 95C appears more likely to induce T cells than 95B.

Initially it appeared possible to prime a universally cross-reactive T cell response to NS3$_{1406}$

Of course, the ability to induce T cell responses is only one measure of immunogenicity. The purpose of this work was to not only find a version of epitope NS3$_{1406}$ which would induce T cell responses in a large number of people, but also to find a variant which would induce cross-reactive T cells to other common sequence variants.

Initially, results were promising and it seemed that finding a candidate immunogen with almost 'universal' genotype 1 cross-reactivity was possible. A single variant (95C) was able to prime T cell lines which were cross-reactive to all common sequence variants tested. Such T cells would have the potential to target all sequence variants that were found at a frequency of greater than 5% worldwide. However, this finding was not reproducible when priming with 95C was trialed in further volunteers. Although 2 volunteers produced 95C specific T cell lines which were able to cross react with the major genotype 1a variant (95B), they did not recognise the genotype 1b variants (95H and 95I).

Variant 95A primed T cells with a range of cross-reactive responses, but consistently recognised genotype 1b variants (95H and I)

It was expected, based on the *in vivo* IFN-γ ELISpot responses that 95A primed cell lines would range from being not cross-reactive with any variants, to being cross-reactive with variant 95I (as seen in 4 out of 5 healthy volunteers) and in some cases cross-reactive with 95H (see Figure 4-10).

These predicted patterns were observed in 4 out of the 6 cell lines primed by 95A. Two lines (DC4 and DC6) were cross-reactive to both 95H and 95I, one line (DC8) recognised 95H and one line (DC16) recognised only 95A. Two volunteers (DC5 and DC14), produced unexpected patterns of cross-reactivity. DC14 produced 95A primed lines which were cross-reactive with variants 95B and 95c but not with the genotype 1b variants 95H and 95I. It would have been useful to confirm these findings at a later time point, given the small magnitude of responses in this individual, however this was not possible due to cell numbers.

Surprisingly, a broadly cross-reactive response was seen in 1 volunteer (DC5) primed by 95A. DC5 produced T cells which recognised all common sequence variants. This finding was verified at 2 different time points (day 25 and day 33 following monocyte separation). It is noteworthy that DC5 had one of the highest magnitude responses to priming with 95A.

In fact, when the *in vivo* data from healthy volunteers vaccinated with NSmut containing 95A was examined closely, it showed that it was possible to detect low level T cell responses to both genotype 1b and 1a variants, if the primary response to vaccination with 95A was of sufficient magnitude. For example in

the case of volunteer 003-319, IFN-γ ELISpot responses to the genotype 1a variants 95B and 95C were detectable at low levels, however were less than 1% of the magnitude response to the priming peptide 95A (Figure 4-8, p165). This was observed at supra-physiological concentrations of peptide, therefore the *in vivo* translation of such low level cross-reactive responses remains to be seen. Nonetheless the magnitude of response cannot fully account for the broad cross-reactive responses identified in DC5 since DC4 produced a much larger pentamer positive 95A population after 95A priming, however that T cell population did not show any cross-reactivity to the genotype 1a variants 95B and 9C.

Genotype subtype specific cross-reactivity (1a or 1b) was observed using other peptide variants.

The other T cell lines primed by alternative NS3$_{1406}$ variants were also tested for cross-reactivity. 95I proved not only to be poorly immunogenic but also produced highly specific T cell lines, which were not cross-reactive to any other variants tested. However, priming with 95B and 95H consistently produced T cell lines which were cross-reactive for the other major variant of their genotype subtype i.e 95C and 95I respectively. This is likely due to the amino acid similarities within genotype 1a and genotype 1b variants (Table 5-2, p215 above). Nearly all the variants were cross-reactive with variant 95A, although the utility of this is limited given 95A is a rarely accounted viral sequence. Functional analysis by ICS showed that the cross-reactive responses identified by pentamer staining produced multiple cytokines including IFNγ, TNFα, IL2, CD107a and MIP-1β. This suggests that should such primed T cells encounter

these variant HCV sequences in the wild, they would be able to respond in a functional manner.

Limitations of this approach

Overall, although this *in vivo* priming system has limitations and there is some inter-individual variation, real life vaccination also carries its challenges and inter-individual variation. The concept behind this model is a valid one and important for the evaluation of variable and/or highly immunogenic epitopes for any future vaccine effort for this virus.

There was no single variant amongst those trialed, which was able to prime T cell responses to all common genotype 1 HCV sequences at this site in the genome. This does not however mean that such a variant does not exist. It may be possible to synthesise a variant artificially, even if it is not found in natural infection. Alternatively there may be a low prevalence variant not trialed in this work, which is able to prime multiple T cell responses. In fact variant 95A is such an example, where, despite representing only 4.3% of genotype 1b sequences and no genotype 1a sequences, it is highly immunogenic and almost always primed responses to other more common 1b variants.

Nonetheless it appears from this work, that it is not possible to prime T cell responses that will consistently recognise both genotype 1a and 1b sequences. In contrast, using a common genotype 1a sequence (like 95C) to prime T cells specific to genotype 1a variants and using a genotype 1b sequence like 95A or 95H to prime T cells which recognise most genotype 1b sequences is possible.

This may be due to the sequence similarities between the two common genotype 1a variants (95B and 95C) and the two gene 1b variants (95H and 95I) .

One further question which arises from this work is whether such highly variable epitopes are in fact detrimental to the overall success of an HCV vaccine. It may be that by diverting T cell responses to such an immunogenic and variable epitope, the vaccinated host would remain defenseless against the majority of circulating HCV strains. Such an epitope either requires the inclusion of more than one variant in the immunogen (in this case for example 95a and 95c to represent both genotype 1b and genotype 1a coverage) or perhaps should not be included in the immunogen at all. This would allow for the priming of T cell responses to other subdominant areas of the immunogen, which may be more effective (Im et al. 2011). The exploration of either of these strategies would make for interesting ongoing work as the search for an ideal HCV immunogen continues.

5.7 Key points/ Summary of results

- There is no ideal version of epitope $NS3_{1406}$ which consistently primes T cells able to recognise all common genotype 1 sequence variants.
- It is possible to generate HCV specific T cells from naive precursors in healthy volunteers. Such T cells can be assessed for cross-reactivity and functionality.
- It is harder to prime naïve T cell to HCV specific epitopes than to Melan-A epitopes.
- Not all variants of epitope $NS3_{1406}$ are equally immunogenic.
- It is likely that both a genotype 1a and 1b version of this epitope is needed to obtain full genotype 1 coverage from an $NS3_{1406}$ containing vaccine.

6 Concluding Remarks

The search for safe and effective treatments for HCV continues, in an attempt to combat this global disease. This work, on a therapeutic HCV vaccine, forms part of the effort to increase the rates of cure for patients, whilst minimising treatment side effects and costs. It also adds to the body of scientific knowledge around T cell immune responses to vaccination and well as vaccine design, paving the way for future endeavours in the field of prophylactic HCV vaccines or vaccines for other chronic viral infections.

Patients with HCV have an attenuated response to vaccination compared with healthy volunteers

The vaccination strategy described in this work focused on T cell responses as a therapeutic means of combating HCV, given the evidence for the importance of T cell in HCV clearance *in vivo*. Overall, vaccination was safe and well tolerated, with no evidence of immunopathology in patients, apart from one patient who had an elevation in transaminases. This was ultimately felt unlikely to be related to vaccination. It was possible to generate T cell responses to a level not previously described in other HCV therapeutic vaccine trials and in some cases, to levels greater than those seen in natural infection. Comparison to a control group of patients who received IFN/RBV without vaccination showed that T cell responses to non-structural proteins (included in the vaccine immunogen) were not induced during standard of care therapy, as shown by work in our group previously (Barnes et al. 2009).

However, T cell responses detected after vaccination in patients were of lower magnitude and poorer quality in comparison to those induced in healthy volunteers vaccinated with the same regimen. Furthermore the kinetics of T cell responses were different in patients and healthy volunteers. Healthy volunteers made robust T cell responses to priming, however responded more weakly to boosting, whereas the opposite kinetic profile was observed in patients.

Patients with chronic HCV may have generally poorer immune responses than healthy volunteers

Healthy volunteers and patients with chronic HCV represent very different hosts, and some of these differences may lead to a non-specific attenuation of vaccine immunogenicity in patients. As discussed in Chapter 3, lifestyle factors such as sleep patterns or concomitant drug use could have an impact on immunogenicity (Prather et al. 2012). Concurrent IFN therapy could also decrease T cell responses. Patients may also have other intercurrent infections, the significance of which is unknown. For example, Parvovirus 4 infection is frequently found in patients with blood borne viruses however is more rarely detected in healthy subjects (Simmons et al. 2012). Nonetheless, the generally strong IFN-γ ELISpot and proliferative responses to non-HCV antigens such as CMV and FEC, suggest that there is a more specific defect in anti-HCV immunity in response to vaccination.

Constant exposure to HCV antigen may explain the lower immunogenicity observed in patients with HCV

Sequence analysis revealed one explanation for the discrepancy in vaccine responses between patients and healthy volunteers. T cell responses to vaccination were made only at epitopes where patients' circulating virus was different to the vaccine immunogen sequence. Furthermore, in those patients that did make a T cell response, T cells either did not target circulating virus, or did so with a greatly decreased magnitude. Individuals whose viral sequence matched the immunogen sequence at a given epitope, did not make a T cell response to that epitope.

The findings from sequence analysis suggest that T cell exhaustion may play a critical role in the success or otherwise of therapeutic T cell strategies in chronic HCV and indeed in other chronic viral infections such as HBV. It may be that, despite a period of viral suppression with IFN/RBV, it is only possible to induce T cell responses to novel HCV antigens to which patients have not previously been exposed.

It is also possible that T cells detected after vaccination could be rested memory populations, from which virus has escaped. This seems likely at epitopes where a well described escape variant has been described such as epitope HSKKKCDEL. T cells specific to this variant were detected in one patient, which failed to target the escaped viral sequence HSK**R**KCDEL. In this case it is likely that HSKKKCDEL specific T cells were induced by the primary infecting virus and boosted by vaccination. Such examples suggest that there may be multiple explanations for the T cells detected after vaccination, which may well differ between epitopes,

depending on the likelihood of viral escape, T cell cross-reactivity and sequence variability at a given epitope.

T cell cross-reactivity is important for therapeutic and prophylactic vaccine design

The issue of cross-reactive T cell responses is critical for the success of an HCV T cell vaccine, whether it be therapeutic or prophylactic. A successful T cell vaccine for HCV must be able to contend with viral variation as well as take into account the multitude of HLA types present in a global population. Ideally a vaccine immunogen would induce T cells which were not only cross reactive to other circulating strains within a specific viral genotype, but would also cover other common genotypes such as genotype 3 in the UK or genotype 4 in Egypt. T cell cross-reactivity needs to be considered on an individual epitope level using peptide dilution assays. This thesis has demonstrated that T cell responses to epitopes such as $NS3_{1073}$ which have only two main sequence variants, will cover all genotype 1 HCV strains detected at >5% of the population. In contrast, T cell responses to epitope $NS3_{1406}$ do not target common genotype 1a strains, and only target common genotype 1b strains in some individuals.

Can we address the variable nature of HCV through immunogen design?

There are several possible adaptations which could be made to an HCV vaccine in order to address the intrinsic variability of the HCV genome.

Use a common sequence variant of each epitope

One option is to take only the most commonly found viral sequences at each epitope and insert those into the vaccine immunogen. However analysis of

immunogenic epitopes performed in this study show that many epitopes, in particular the most highly immunogenic epitope $NS3_{1406}$, are also highly variable. This means that there may be no single common sequence which could be selected at highly immunogenic sites. Furthermore, even epitopes which appear to be conserved may actually be quite variable when sequences from a specific HLA population (to which T cell responses targeting such an epitope are restricted) are examined. In this case, selecting the most common variant of a given epitope at a population level would lead to vaccination with a variant from which virus in patients with the relevant HLA type would have already escaped.

Exclude variable epitopes

A second option is to exclude such variable epitopes and choose only highly conserved epitopes or conserved regions. These regions may represent viral regions that are unable to mutate without a significant fitness cost, and therefore may represent ideal T cell targets. On the other hand they may be highly conserved because T cells are unable to target these regions. For example such areas of the genome may have poor HLA avidity or may not be processed and presented appropriately. They may also represent epitopes for which there is a low naive T cell precursor frequency (Wolfl et al. 2008). This approach could also lead to other subdominant epitopes becoming prominent, generating new T cells, as has been suggested in mouse models for an HIV vaccine (Im et al. 2011).

Choose a single cross-reactive version of each epitope

A third approach to combating viral variation is to evaluate different variants of each epitope, either occurring in natural infection or synthetically produced, and

identify the most cross-reactive version. I attempted to identify such a variant at the single most immunodominant epitope in healthy volunteers and patients- $NS3_{1406}$. This showed that there was no single version of this epitope, commonly encountered at a population level, which could induce T cells which would target all other circulating 1a and 1b strains. However, an *in vitro* model which evaluated the priming capabilities of each variant showed that it was possible to consistently prime cross-reactive T cells to either genotype 1a or genotype 1b variants occurring in >5% of the population. This outcome was more promising than that initially suggested by results from patients vaccinated with ChAd3-NSmut/Ad6-NSmut, where in many cases there was no cross-recognition of any of the HCV variants tested. The *in vitro* model suggests that inserting two versions of epitope $NS3_{1406}$ would cover 95% of genotype 1a and 1b sequences.

Such an approach, although labour intensive, could be performed for each epitope included in a vaccine immunogen. This system also lends itself to a program of immunogen design for other variable viruses, such as HIV, where variation is well characterised and epitope based vaccines have been trialed with preliminary success (Rosario et al. 2010). Although this *in vitro* model has limitations, inherent to any *in vitro* system, it nonetheless could also be used as part of an initial screening process to exclude epitopes that were clearly poorly immunogenic or poorly cross-reactive from future immunogen designs. This would then allow for likely immunogen candidates to be taken forward into animal and ultimately human studies. Of course, such an approach is based on looking at responses to single epitopes, whereas the immune response *in vivo*

situation, where T cell responses to multiple epitopes may be induced simultaneously, could be quite different.

T cells displayed features of exhaustion

Even if the problem of viral variability can be overcome through immunogen design, there remains the ongoing issue of T cell exhaustion, even when T cells are confronted with novel HCV antigens.

There was a relative deficit of CD4+ T cells

One striking observation was that there was a significant lack of CD4+ T cell help, which has been shown to be critical for the induction of effective CD8+ T cells in mouse LCMV models as well as for protection from reinfection with HCV in Chimpanzee models (Zajac et al. 1998; Grakoui et al. 2003). It is not clear why there should be a relative deficit in CD4+ compared with CD8+ T cells in these patients. It could be that other assays such as carboxyfluorescein diacetate succinimydl ester (CFSE) assays or cultured ELISpots may have been more effective at detecting CD4+ T cells than the thymidine proliferation assays and peptide pools used for *ex vivo* IFN-γ ELISpots in this study. Nonetheless, strongly CD4+ T cell proliferative responses, detected in healthy volunteers using the same assays as in HCV002TV, suggest that the absence of CD4+ T cell responses was a genuine finding (Barnes et al. 2012). Given the low magnitude CD8+ responses in most individuals, the lack of CD4+ T cells may simply represent a proportional decrease, since they represent a smaller subpopulation of T cells. It is possible that the presence of other immunomodulatory factors could affect CD4+ T cells more than CD8+ T cells.

Such immunomodulatory factors might include the presence of regulatory populations like T regulatory cells, or circulating factors such as IL-10 and TGFβ. Antigen specific T regulatory cells have been observed in HCV infected patients as have higher levels of IL-10 than healthy controls, both of which might serve to decrease HCV specific T cell responses below detectable levels (Godkin et al. 2008).

Strategies to overcome T cell exhaustion

It is possible that T cell exhaustion could be overcome by administering a vaccine with greater potency, which could elicit T cell responses of greater magnitude. Alternatively, vaccination could be administered after a longer period of viral suppression- such as at the end of treatment with IFN/RBV, when T cells have had a longer recovery phase. However it is not known how long T cells require in order to 'recover'; indeed recent data suggests that T cells from patients with chronic HCV may remain functionally impaired even after successful IFN/RBV treatment (Missale et al 2012). Other options described below include blocking regulatory or immunosuppressive pathways, or adding cytokines or other novel adjuvants to augment immune responses.

Blocking cell surface receptors or soluble factors

Programmed death receptor 1 (PD-1) is upregulated on exhausted T cells, and blockade of it, or its ligand PD1 ligand (PD-L1) could reverse some of the features of T cell exhaustion and enhance vaccine immunogenicity. It has been shown both *in vivo* in mice with LCMV and *in vitro* with T cells from HCV infected patients that blocking the PD-1 pathway with PD-L1 blockade restored

proliferative capacity and cytokine secretion to T cells (Urbani et al. 2006; Barber et al. 2006). The effectiveness of this approach is likely to depend not only on the safety of such immunomodulatory agents in humans, but also the characteristics of the T cell population in question. It has been shown that PD-L1 blockade of liver derived T cells is ineffective, probably due to the higher level of antigen exposure and therefore higher level of exhaustion in these T cells (Nobuhiro et al. 2008). This approach may therefore be ineffective in longstanding HCV infection with high viral loads.

Blockade of other inhibitory molecules such as CTLA-4 have been explored with limited success. Efforts to block CTLA-4 in mice showed some enhancement of anti-tumour T cell immunity, but no improvement of T cell function in chronic viral infection (LCMV) (Barber et al. 2006; Sotomayor et al. 1999).

Blockade of soluble factors is another potential pathway for enhancing immunogenicity. Blocking IL10 in mice has been shown to enhance the immunogenicity of an otherwise ineffective LCMV DNA vaccine and to enhance viral clearance (Brooks et al. 2008). Such a strategy either alone or in combination with other immunomodulatory agents could be a potent mechanism to reverse the inhibitory effects of longstanding HCV infection.

Novel Adjuvants

Cytokines and novel adjuvants have both been used to augment immunogenicity in vaccine trials for malaria, HIV, HBV and anti-cancer vaccines. Administration of exogenous IL2 has been shown to prevent CD8+ T cells becoming tolerised to antigens presented in the liver of mice, despite the absence of co-stimulatory

molecules (Schurich et al. 2010). This could prove particularly useful for inducing immunity to hepatotropic viruses such as HBV and HCV where the tolerogenic environment of the liver may contribute to T cell anergy. However the non-specific pro-inflammatory effects of IL2 may limit the translation of this work into humans. Other cytokines with more limited tissue distribution may have a better safety profile. For example, a study administering IFNλ3 in combination with an HIV vaccine in rhesus macaques, showed higher levels of long-lived activated CD8+ T cells (CD107a+ and CD38+/HLA-DR+) in the IFNλ3 group compared with the group who received vaccination alone (Morrow et al. 2010).

There also a large group of novel adjuvants which consist, in part, of various combinations of aluminum, oil and saponin based compound. Their mechanism of action is, in many cases, poorly understood, but in some cases they work by creating a depot effect for an administered vaccine (Pichichero et al. 2008). Such adjuvants have been shown to augment immunogenicity in HBV, HIV and cancer immunotherapy clinical trials, compared with the administration of vaccination alone (Pichichero et al. 2008).

Class II invariant chain

Another approach which could overcome exhaustion is to link the Class II invariant chain to antigen and thus improve either antigen processing or presentation. Adenoviral vector vaccines have been used to deliver LCMV derived antigens linked to the murine Class II invariant chain. Such vaccines produce higher magnitude CD4+ and CD8+ T cell responses and broader T cell responses in mice compared with the equivalent vaccines without the invariant

chain (Holst et al. 2008). Invariant chain adenoviral vaccines encoding the HCV NS3 proteins have also been trialled in murine models and been shown to be superior to NS3 containing vaccines alone, with the production of polyfunctional T cells which make IL-2, TNFα and IFNγ and result in increased levels of antigen specific memory populations (Mikkelsen et al. 2011). Such results would overcome many of the challenges of therapeutic vaccination, if they could be demonstrated in humans.

Limitations of the approach in HCV002TV

This study has focused on selected immune correlates of vaccine efficacy however these are not clearly defined in natural history studies. Nonetheless it is clear from our data that T cells induced by vaccination do not target circulating virus, so efficacy is unlikely. In this study, the effect of IFN on vaccine responses was not clear, likely due to the small sample size and large degree of inter-individual variation. This study has also focused on analysis of peripheral blood samples rather than examining what is happening in the liver itself, due to the ethical considerations and impracticality of obtaining liver biopsies. It may be that the most relevant cell populations have homed to the liver and are not detectable in peripheral blood at the timepoints samples were taken.

Although a range of different assays were performed in this study, it would have been useful to undertake more extensive epitope mapping from the beginning of the study and perhaps try to capture low frequency T cell responses with CD4+ pentamers or routine pentamer stains at key timepoints. More extensive phenotypic information would also been useful, either through FACS analysis of pentamer staining or through new detailed techniques such as cytometry

through time of flight, which allows more extensive T cell characterisation (Newell et al. 2012).

A similar process assessing cross-reactivity of different sequence variants at different epitopes in healthy volunteers would also have provided useful information for future vaccine design. These sequence analyses could also have included assessment of flanking regions, which have been shown in the case of a CD4+ influenza epitope, to alter T cell repertoire (Cole et al. 2011).

Future applications of this work

One of the most important findings from this work is that T cell responses to variable pathogens should not be reported without also reporting concurrent pathogen sequence data. The detection of T cells alone does not imply vaccine efficacy, since such T cells may fail to target circulating virus, as demonstrated in this thesis.

Many of the principles identified in this study can be applied to prophylactic and therapeutic vaccine design for HCV and also extrapolated to work on immunotherapy for other viruses or even malignancies. Although an effective prophylactic vaccine for HBV has existed for many years, there is still a strong interest in immunotherapy as a means of curing the many chronically infected individuals worldwide who remain on antiviral therapy for the foreseeable future. HBV is a less variable virus than HCV therefore some of the issues around T cell cross-reactivity may be less problematic for an HBV therapeutic vaccine, although there may still be problems related to T cell exhaustion and chronic viraemia. In the HIV field, the lessons learned around viral variation and T cell

responses to these adenoviruses may influence future vaccine design. Future viral vector vaccine trials may choose not to use heterologous adenoviruses, given the potential for cross-reactive antibodies. The next Oxford HCV trial (HCV003) was designed using a new boosting vector (MVA) to overcome this problem, and shows promising results (manuscript in preparation).

Although the search for the ideal therapeutic vaccine may not keep pace with other fast moving treatment options for HCV, a prophylactic vaccine remains both a realistic and highly desirable goal to prevent the 3-4 million new infections and their sequelae across the globe every year. Particular at risk populations such as men who have sex with men who are at risk of concurrent HIV/HCV infection, or IVDU populations, will be ideal groups in which to trial these vaccines in the coming years.

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Appendix

Peptides used in pools for IFNγ-ELISpot assays

Peptides for:	**NS3-5b_1b**			
Peptide length:	**15**			
Peptide overlap:	**11**			**Pool F**
Pep No	1	APITAYSQQTRGLLG	1 to 15	Minipool Fa
Pep No	2	AYSQQTRGLLGCIIT	5 to 19	Minipool Fa
Pep No	3	QTRGLLGCIITSLTG	9 to 23	Minipool Fa
Pep No	4	LLGCIITSLTGRDKN	13 to 27	Minipool Fa
Pep No	5	IITSLTGRDKNQVEG	17 to 31	Minipool Fa
Pep No	6	LTGRDKNQVEGEVQV	21 to 35	Minipool Fa
Pep No	7	DKNQVEGEVQVVSTA	25 to 39	Minipool Fa
Pep No	8	VEGEVQVVSTATQSF	29 to 43	Minipool Fa
Pep No	9	VQVVSTATQSFLATC	33 to 47	Minipool Fa
Pep No	10	STATQSFLATCVNGV	37 to 51	Minipool Fa
Pep No	11	QSFLATCVNGVCWTV	41 to 55	Minipool Fb
Pep No	12	ATCVNGVCWTVYHGA	45 to 59	Minipool Fb
Pep No	13	NGVCWTVYHGAGSKT	49 to 63	Minipool Fb
Pep No	14	WTVYHGAGSKTLAGP	53 to 67	Minipool Fb
Pep No	15	HGAGSKTLAGPKGPI	57 to 71	Minipool Fb
Pep No	16	SKTLAGPKGPITQMY	61 to 75	Minipool Fb
Pep No	17	AGPKGPITQMYTNVD	65 to 79	Minipool Fb
Pep No	18	GPITQMYTNVDQDLV	69 to 83	Minipool Fb
Pep No	19	QMYTNVDQDLVGWQA	73 to 87	Minipool Fb
Pep No	20	NVDQDLVGWQAPPGA	77 to 91	Minipool Fb
Pep No	21	DLVGWQAPPGARSLT	81 to 95	Minipool Fc
Pep No	22	WQAPPGARSLTPCTC	85 to 99	Minipool Fc
Pep No	23	PGARSLTPCTCGSSD	89 to 103	Minipool Fc
Pep No	24	SLTPCTCGSSDLYLV	93 to 107	Minipool Fc
Pep No	25	CTCGSSDLYLVTRHA	97 to 111	Minipool Fc
Pep No	26	SSDLYLVTRHADVIP	101 to 115	Minipool Fc
Pep No	27	YLVTRHADVIPVRRR	105 to 119	Minipool Fc
Pep No	28	RHADVIPVRRRGDSR	109 to 123	Minipool Fc
Pep No	29	VIPVRRRGDSRGSLL	113 to 127	Minipool Fc
Pep No	30	RRRGDSRGSLLSPRP	117 to 131	Minipool Fc
Pep No	31	DSRGSLLSPRPVSYL	121 to 135	Minipool Fd
Pep No	32	SLLSPRPVSYLKGSS	125 to 139	Minipool Fd
Pep No	33	PRPVSYLKGSSGGPL	129 to 143	Minipool Fd
Pep No	34	SYLKGSSGGPLLCPS	133 to 147	Minipool Fd
Pep No	35	GSSGGPLLCPSGHAV	137 to 151	Minipool Fd
Pep No	36	GPLLCPSGHAVGIFR	141 to 155	Minipool Fd
Pep No	37	CPSGHAVGIFRAAVC	145 to 159	Minipool Fd
Pep No	38	HAVGIFRAAVCTRGV	149 to 163	Minipool Fd
Pep No	39	IFRAAVCTRGVAKAV	153 to 167	Minipool Fd
Pep No	40	AVCTRGVAKAVDFVP	157 to 171	Minipool Fd
Pep No	41	RGVAKAVDFVPVESM	161 to 175	Minipool Fe
Pep No	42	KAVDFVPVESMETTM	165 to 179	Minipool Fe
Pep No	43	FVPVESMETTMRSPV	169 to 183	Minipool Fe

Pep No	44	ESMETTMRSPVFTDN	173	to	187	Minipool Fe	
Pep No	45	TTMRSPVFTDNSSPP	177	to	191	Minipool Fe	
Pep No	46	SPVFTDNSSPPAVPQ	181	to	195	Minipool Fe	
Pep No	47	TDNSSPPAVPQSFQV	185	to	199	Minipool Fe	
Pep No	48	SPPAVPQSFQVAHLH	189	to	203	Minipool Fe	
Pep No	49	VPQSFQVAHLHAPTG	193	to	207	Minipool Fe	
Pep No	50	FQVAHLHAPTGSGKS	197	to	211	Minipool Fe	
Pep No	51	HLHAPTGSGKSTKVP	201	to	215	Minipool Ff	
Pep No	52	PTGSGKSTKVPAAYA	205	to	219	Minipool Ff	
Pep No	53	GKSTKVPAAYAAQGY	209	to	223	Minipool Ff	
Pep No	54	KVPAAYAAQGYKVLV	213	to	227	Minipool Ff	
Pep No	55	AYAAQGYKVLVLNPS	217	to	231	Minipool Ff	
Pep No	56	QGYKVLVLNPSVAAT	221	to	235	Minipool Ff	
Pep No	57	VLVLNPSVAATLGFG	225	to	239	Minipool Ff	
Pep No	58	NPSVAATLGFGAYMS	229	to	243	Minipool Ff	
Pep No	59	AATLGFGAYMSKAHG	233	to	247	Minipool Ff	
Pep No	60	GFGAYMSKAHGIDPN	237	to	251	Minipool Ff	
Pep No	61	YMSKAHGIDPNIRTG	241	to	255	Minipool Fg	
Pep No	62	AHGIDPNIRTGVRTI	245	to	259	Minipool Fg	
Pep No	63	DPNIRTGVRTITTGA	249	to	263	Minipool Fg	
Pep No	64	RTGVRTITTGAPVTY	253	to	267	Minipool Fg	
Pep No	65	RTITTGAPVTYSTYG	257	to	271	Minipool Fg	
Pep No	66	TGAPVTYSTYGKFLA	261	to	275	Minipool Fg	
Pep No	67	VTYSTYGKFLADGGC	265	to	279	Minipool Fg	
Pep No	68	TYGKFLADGGCSGGA	269	to	283	Minipool Fg	
Pep No	69	FLADGGCSGGAYDII	273	to	287	Minipool Fg	
Pep No	70	GGCSGGAYDIIICDE	277	to	291	Minipool Fg	
Pep No	71	GGAYDIIICDECHST	281	to	295	Minipool Fh	
Pep No	72	DIIICDECHSTDSTT	285	to	299	Minipool Fh	
Pep No	73	CDECHSTDSTTILGI	289	to	303	Minipool Fh	
Pep No	74	HSTDSTTILGIGTVL	293	to	307	Minipool Fh	
Pep No	75	STTILGIGTVLDQAE	297	to	311	Minipool Fh	
Pep No	76	LGIGTVLDQAETAGA	301	to	315	Minipool Fh	
Pep No	77	TVLDQAETAGARLVV	305	to	319	Minipool Fh	
Pep No	**78**	**QAETAGARLVVLATA**	309	to	323	Minipool Fh	in blue: end of pool F
Pep No	**79**	AGARLVVLATATPPG	313	to	327	Minipool Ga	**Pool G**
Pep No	80	LVVLATATPPGSVTV	317	to	331	Minipool Ga	
Pep No	81	ATATPPGSVTVPHPN	321	to	335	Minipool Ga	
Pep No	82	PPGSVTVPHPNIEEV	325	to	339	Minipool Ga	
Pep No	83	VTVPHPNIEEVALSN	329	to	343	Minipool Ga	
Pep No	84	HPNIEEVALSNTGEI	333	to	347	Minipool Ga	
Pep No	85	EEVALSNTGEIPFYG	337	to	351	Minipool Ga	
Pep No	86	LSNTGEIPFYGKAIP	341	to	355	Minipool Ga	
Pep No	87	GEIPFYGKAIPIEAI	345	to	359	Minipool Ga	
Pep No	88	FYGKAIPIEAIRGGR	349	to	363	Minipool Ga	
Pep No	89	AIPIEAIRGGRHLIF	353	to	367	Minipool Gb	
Pep No	90	EAIRGGRHLIFCHSK	357	to	371	Minipool Gb	
Pep No	91	GGRHLIFCHSKKKCD	361	to	375	Minipool Gb	
Pep No	92	LIFCHSKKKCDELAA	365	to	379	Minipool Gb	
Pep No	93	HSKKKCDELAAKLSG	369	to	383	Minipool Gb	
Pep No	94	KCDELAAKLSGLGIN	373	to	387	Minipool Gb	
Pep No	95	LAAKLSGLGINAVAY	377	to	391	Minipool Gb	

Pep No	96	LSGLGINAVAYYRGL	381	to	395	Minipool Gb
Pep No	97	GINAVAYYRGLDVSV	385	to	399	Minipool Gb
Pep No	98	VAYYRGLDVSVIPTI	389	to	403	Minipool Gb
Pep No	99	RGLDVSVIPTIGDVV	393	to	407	Minipool Gc
Pep No	100	VSVIPTIGDVVVVAT	397	to	411	Minipool Gc
Pep No	101	PTIGDVVVVATDALM	401	to	415	Minipool Gc
Pep No	102	DVVVVATDALMTGYT	405	to	419	Minipool Gc
Pep No	103	VATDALMTGYTGDFD	409	to	423	Minipool Gc
Pep No	104	ALMTGYTGDFDSVID	413	to	427	Minipool Gc
Pep No	105	GYTGDFDSVIDCNTC	417	to	431	Minipool Gc
Pep No	106	DFDSVIDCNTCVTQT	421	to	435	Minipool Gc
Pep No	107	VIDCNTCVTQTVDFS	425	to	439	Minipool Gc
Pep No	108	NTCVTQTVDFSLDPT	429	to	443	Minipool Gc
Pep No	109	TQTVDFSLDPTFTIE	433	to	447	Minipool Gd
Pep No	110	DFSLDPTFTIETTTV	437	to	451	Minipool Gd
Pep No	111	DPTFTIETTTVPQDA	441	to	455	Minipool Gd
Pep No	112	TIETTTVPQDAVSRS	445	to	459	Minipool Gd
Pep No	113	TTVPQDAVSRSQRRG	449	to	463	Minipool Gd
Pep No	114	QDAVSRSQRRGRTGR	453	to	467	Minipool Gd
Pep No	115	SRSQRRGRTGRGRRG	457	to	471	Minipool Gd
Pep No	116	RRGRTGRGRRGIYRF	461	to	475	Minipool Gd
Pep No	117	TGRGRRGIYRFVTPG	465	to	479	Minipool Gd
Pep No	118	RRGIYRFVTPGERPS	469	to	483	Minipool Gd
Pep No	119	YRFVTPGERPSGMFD	473	to	487	Minipool Ge
Pep No	120	TPGERPSGMFDSSVL	477	to	491	Minipool Ge
Pep No	121	RPSGMFDSSVLCECY	481	to	495	Minipool Ge
Pep No	122	MFDSSVLCECYDAGC	485	to	499	Minipool Ge
Pep No	123	SVLCECYDAGCAWYE	489	to	503	Minipool Ge
Pep No	124	ECYDAGCAWYELTPA	493	to	507	Minipool Ge
Pep No	125	AGCAWYELTPAETSV	497	to	511	Minipool Ge
Pep No	126	WYELTPAETSVRLRA	501	to	515	Minipool Ge
Pep No	127	TPAETSVRLRAYLNT	505	to	519	Minipool Ge
Pep No	128	TSVRLRAYLNTPGLP	509	to	523	Minipool Ge
Pep No	129	LRAYLNTPGLPVCQD	513	to	527	Minipool Gf
Pep No	130	LNTPGLPVCQDHLEF	517	to	531	Minipool Gf
Pep No	131	GLPVCQDHLEFWESV	521	to	535	Minipool Gf
Pep No	132	CQDHLEFWESVFTGL	525	to	539	Minipool Gf
Pep No	133	LEFWESVFTGLTHID	529	to	543	Minipool Gf
Pep No	134	ESVFTGLTHIDAHFL	533	to	547	Minipool Gf
Pep No	135	TGLTHIDAHFLSQTK	537	to	551	Minipool Gf
Pep No	136	HIDAHFLSQTKQAGD	541	to	555	Minipool Gf
Pep No	137	HFLSQTKQAGDNFPY	545	to	559	Minipool Gf
Pep No	138	QTKQAGDNFPYLVAY	549	to	563	Minipool Gf
Pep No	139	AGDNFPYLVAYQATV	553	to	567	Minipool Gg
Pep No	140	FPYLVAYQATVCARA	557	to	571	Minipool Gg
Pep No	141	VAYQATVCARAQAPP	561	to	575	Minipool Gg
Pep No	142	ATVCARAQAPPPSWD	565	to	579	Minipool Gg
Pep No	143	ARAQAPPPSWDQMWK	569	to	583	Minipool Gg
Pep No	144	APPPSWDQMWKCLIR	573	to	587	Minipool Gg
Pep No	145	SWDQMWKCLIRLKPT	577	to	591	Minipool Gg
Pep No	146	MWKCLIRLKPTLHGP	581	to	595	Minipool Gg
Pep No	147	LIRLKPTLHGPTPLL	585	to	599	Minipool Gg
Pep No	148	KPTLHGPTPLLYRLG	589	to	603	Minipool Gg

Pep No	149	HGPTPLLYRLGAVQN	593	to	607	Minipool Gh	
Pep No	150	PLLYRLGAVQNEVTL	597	to	611	Minipool Gh	
Pep No	151	RLGAVQNEVTLTHPI	601	to	615	Minipool Gh	
Pep No	152	VQNEVTLTHPITKYI	605	to	619	Minipool Gh	
Pep No	153	VTLTHPITKYIMACM	609	to	623	Minipool Gh	
Pep No	154	HPITKYIMACMSADL	613	to	627	Minipool Gh	
Pep No	155	KYIMACMSADL**EVVT**	617	to	631	Minipool Gh	in red: end of NS3
Pep No	**156**	ACMSADL**EVVT**STWV	621	to	635	Minipool Gh	in blue: end of pool G
Pep No	**157**	ADL**EVVT**STWVLVGG	625	to	639	Minipool Ha	**Pool H**
Pep No	158	VVTSTWVLVGGVLAA	629	to	643	Minipool Ha	
Pep No	159	TWVLVGGVLAALAAY	633	to	647	Minipool Ha	
Pep No	160	VGGVLAALAAYCLTT	637	to	651	Minipool Ha	
Pep No	161	LAALAAYCLTTGSVV	641	to	655	Minipool Ha	
Pep No	162	AAYCLTTGSVVIVGR	645	to	659	Minipool Ha	
Pep No	163	LTTGSVVIVGRIILS	649	to	663	Minipool Ha	
Pep No	164	SVVIVGRIILSGRPA	653	to	667	Minipool Ha	
Pep No	165	VGRIILSGRPAIVPD	657	to	671	Minipool Ha	
Pep No	166	ILSGRPAIVPDREFL	661	to	675	Minipool Ha	
Pep No	167	RPAIVPDREFLYQEF	665	to	679	Minipool Hb	
Pep No	168	VPDREFLYQEFDEME	669	to	683	Minipool Hb	
Pep No	169	EFLYQEFDEMEECAS	673	to	687	Minipool Hb	
Pep No	170	QEFDEMEECASHLPY	677	to	691	Minipool Hb	
Pep No	171	EMEECASHLPYIEQG	681	to	695	Minipool Hb	
Pep No	172	CASHLPYIEQGMQLA	685	to	699	Minipool Hb	
Pep No	173	LPYIEQGMQLAEQFK	689	to	703	Minipool Hb	
Pep No	174	EQGMQLAEQFKQKAL	693	to	707	Minipool Hb	
Pep No	175	QLAEQFKQKALGLLQ	697	to	711	Minipool Hb	
Pep No	176	QFKQKALGLLQTATK	701	to	715	Minipool Hb	
Pep No	177	KALGLLQTATKQAEA	705	to	719	Minipool Hc	
Pep No	178	LLQTATKQAEAAAPV	709	to	723	Minipool Hc	
Pep No	179	ATKQAEAAAPVVESK	713	to	727	Minipool Hc	
Pep No	180	AEAAAPVVESKWRAL	717	to	731	Minipool Hc	
Pep No	181	APVVESKWRALETFW	721	to	735	Minipool Hc	
Pep No	182	ESKWRALETFWAKHM	725	to	739	Minipool Hc	
Pep No	183	RALETFWAKHMWNFI	729	to	743	Minipool Hc	
Pep No	184	TFWAKHMWNFISGIQ	733	to	747	Minipool Hc	
Pep No	185	KHMWNFISGIQYLAG	737	to	751	Minipool Hc	
Pep No	186	NFISGIQYLAGLSTL	741	to	755	Minipool Hc	
Pep No	187	GIQYLAGLSTLPGNP	745	to	759	Minipool Hd	
Pep No	188	LAGLSTLPGNPAIAS	749	to	763	Minipool Hd	
Pep No	189	STLPGNPAIASLMAF	753	to	767	Minipool Hd	
Pep No	190	GNPAIASLMAFTASI	757	to	771	Minipool Hd	
Pep No	191	IASLMAFTASITSPL	761	to	775	Minipool Hd	
Pep No	192	MAFTASITSPLTTQS	765	to	779	Minipool Hd	
Pep No	193	ASITSPLTTQSTLLF	769	to	783	Minipool Hd	
Pep No	194	SPLTTQSTLLFNILG	773	to	787	Minipool Hd	
Pep No	195	TQSTLLFNILGGWVA	777	to	791	Minipool Hd	
Pep No	196	LLFNILGGWVAAQLA	781	to	795	Minipool Hd	
Pep No	197	ILGGWVAAQLAPPSA	785	to	799	Minipool He	
Pep No	198	WVAAQLAPPSAASAF	789	to	803	Minipool He	
Pep No	199	QLAPPSAASAFVGAG	793	to	807	Minipool He	

Pep No	200	PSAASAFVGAGIAGA	797 to 811	Minipool He	
Pep No	201	SAFVGAGIAGAAVGS	801 to 815	Minipool He	
Pep No	202	GAGIAGAAVGSIGLG	805 to 819	Minipool He	
Pep No	203	AGAAVGSIGLGKVLV	809 to 823	Minipool He	
Pep No	204	VGSIGLGKVLVDILA	813 to 827	Minipool He	
Pep No	205	GLGKVLVDILAGYGA	817 to 831	Minipool He	
Pep No	206	VLVDILAGYGAGVAG	821 to 835	Minipool He	
Pep No	207	ILAGYGAGVAGALVA	825 to 839	Minipool Hf	
Pep No	208	YGAGVAGALVAFKVM	829 to 843	Minipool Hf	
Pep No	209	VAGALVAFKVMSGEM	833 to 847	Minipool Hf	
Pep No	210	LVAFKVMSGEMPSTE	837 to 851	Minipool Hf	
Pep No	211	KVMSGEMPSTEDLVN	841 to 855	Minipool Hf	
Pep No	212	GEMPSTEDLVNLLPA	845 to 859	Minipool Hf	
Pep No	213	STEDLVNLLPAILSP	849 to 863	Minipool Hf	
Pep No	214	LVNLLPAILSPGALV	853 to 867	Minipool Hf	
Pep No	215	LPAILSPGALVVGVV	857 to 871	Minipool Hf	
Pep No	216	LSPGALVVGVVCAAI	861 to 875	Minipool Hf	
Pep No	217	ALVVGVVCAAILRRH	865 to 879	Minipool Hg	
Pep No	218	GVVCAAILRRHVGPG	869 to 883	Minipool Hg	
Pep No	219	AAILRRHVGPGEGAV	873 to 887	Minipool Hg	
Pep No	220	RRHVGPGEGAVQWMN	877 to 891	Minipool Hg	
Pep No	221	GPGEGAVQWMNRLIA	881 to 895	Minipool Hg	
Pep No	222	GAVQWMNRLIAFASR	885 to 899	Minipool Hg	
Pep No	223	WMNRLIAFASRGNHV	889 to 903	Minipool Hg	
Pep No	224	LIAFASRGNHVSPTH	893 to 907	Minipool Hg	
Pep No	225	ASRGNHVSPTHYVPE	897 to 911	Minipool Hg	
Pep No	226	NHVSPTHYVPESDAA	901 to 915	Minipool Hg	
Pep No	227	PTHYVPESDAAARVT	905 to 919	Minipool Hh	
Pep No	228	VPESDAAARVTQILS	909 to 923	Minipool Hh	
Pep No	229	DAAARVTQILSSLTI	913 to 927	Minipool Hh	
Pep No	230	RVTQILSSLTITQLL	917 to 931	Minipool Hh	
Pep No	231	ILSSLTITQLLKRLH	921 to 935	Minipool Hh	
Pep No	232	LTITQLLKRLHQWIN	925 to 939	Minipool Hh	
Pep No	233	QLLKRLHQWINEDCS	929 to 943	Minipool Hh	
Pep No	234	RLHQWINEDC**STPC**S	933 to 947	Minipool Hh	
Pep No	**235**	WINEDC**STPC**SGSWL	937 to 951	Minipool Hh	in blue: end of pool H
Pep No	**236**	DC**STPC**SGSWLRDVW	941 to 955	Minipool Ia	in red: end of NS4
Pep No	237	PCSGSWLRDVWDWIC	945 to 959	Minipool Ia	
Pep No	238	SWLRDVWDWICTVLT	949 to 963	Minipool Ia	
Pep No	239	DVWDWICTVLTDFKT	953 to 967	Minipool Ia	
Pep No	240	WICTVLTDFKTWLQS	957 to 971	Minipool Ia	
Pep No	241	VLTDFKTWLQSKLLP	961 to 975	Minipool Ia	
Pep No	242	FKTWLQSKLLPQLPG	965 to 979	Minipool Ia	
Pep No	243	LQSKLLPQLPGVPFF	969 to 983	Minipool Ia	
Pep No	244	LLPQLPGVPFFSCQR	973 to 987	Minipool Ia	
Pep No	245	LPGVPFFSCQRGYKG	977 to 991	Minipool Ia	
Pep No	246	PFFSCQRGYKGVWRG	981 to 995	Minipool Ib	
Pep No	247	CQRGYKGVWRGDGIM	985 to 999	Minipool Ib	
Pep No	248	YKGVWRGDGIMQTTC	989 to 1003	Minipool Ib	
Pep No	249	WRGDGIMQTTCPCGA	993 to 1007	Minipool Ib	
Pep No	250	GIMQTTCPCGAQITG	997 to 1011	Minipool Ib	
Pep No	251	TTCPCGAQITGHVKN	1001 to 1015	Minipool Ib	

Pep No	252	CGAQITGHVKNGSMR	1005 to 1019		Minipool Ib
Pep No	253	ITGHVKNGSMRIVGP	1009 to 1023		Minipool Ib
Pep No	254	VKNGSMRIVGPKTCS	1013 to 1027		Minipool Ib
Pep No	255	SMRIVGPKTCSNTWH	1017 to 1031		Minipool Ib
Pep No	256	VGPKTCSNTWHGTFP	1021 to 1035		Minipool Ic
Pep No	257	TCSNTWHGTFPINAY	1025 to 1039		Minipool Ic
Pep No	258	TWHGTFPINAYTTGP	1029 to 1043		Minipool Ic
Pep No	259	TFPINAYTTGPCTPS	1033 to 1047		Minipool Ic
Pep No	260	NAYTTGPCTPSPAPN	1037 to 1051		Minipool Ic
Pep No	261	TGPCTPSPAPNYSRA	1041 to 1055		Minipool Ic
Pep No	262	TPSPAPNYSRALWRV	1045 to 1059		Minipool Ic
Pep No	263	APNYSRALWRVAAEE	1049 to 1063		Minipool Ic
Pep No	264	SRALWRVAAEEYVEV	1053 to 1067		Minipool Ic
Pep No	265	WRVAAEEYVEVTRVG	1057 to 1071		Minipool Ic
Pep No	266	AEEYVEVTRVGDFHY	1061 to 1075		Minipool Id
Pep No	267	VEVTRVGDFHYVTGM	1065 to 1079		Minipool Id
Pep No	268	RVGDFHYVTGMTTDN	1069 to 1083		Minipool Id
Pep No	269	FHYVTGMTTDNVKCP	1073 to 1087		Minipool Id
Pep No	270	TGMTTDNVKCPCQVP	1077 to 1091		Minipool Id
Pep No	271	TDNVKCPCQVPAPEF	1081 to 1095		Minipool Id
Pep No	272	KCPCQVPAPEFFTEV	1085 to 1099		Minipool Id
Pep No	273	QVPAPEFFTEVDGVR	1089 to 1103		Minipool Id
Pep No	274	PEFFTEVDGVRLHRY	1093 to 1107		Minipool Id
Pep No	275	TEVDGVRLHRYAPAC	1097 to 1111		Minipool Id
Pep No	276	GVRLHRYAPACRPLL	1101 to 1115		Minipool Ie
Pep No	277	HRYAPACRPLLREEV	1105 to 1119		Minipool Ie
Pep No	278	PACRPLLREEVTFQV	1109 to 1123		Minipool Ie
Pep No	279	PLLREEVTFQVGLNQ	1113 to 1127		Minipool Ie
Pep No	280	EEVTFQVGLNQYLVG	1117 to 1131		Minipool Ie
Pep No	281	FQVGLNQYLVGSQLP	1121 to 1135		Minipool Ie
Pep No	282	LNQYLVGSQLPCEPE	1125 to 1139		Minipool Ie
Pep No	283	LVGSQLPCEPEPDVA	1129 to 1143		Minipool Ie
Pep No	284	QLPCEPEPDVAVLTS	1133 to 1147		Minipool Ie
Pep No	285	EPEPDVAVLTSMLTD	1137 to 1151		Minipool Ie
Pep No	286	DVAVLTSMLTDPSHI	1141 to 1155		Minipool If
Pep No	287	LTSMLTDPSHITAET	1145 to 1159		Minipool If
Pep No	288	LTDPSHITAETAKRR	1149 to 1163		Minipool If
Pep No	289	SHITAETAKRRLARG	1153 to 1167		Minipool If
Pep No	290	AETAKRRLARGSPPS	1157 to 1171		Minipool If
Pep No	291	KRRLARGSPPSLASS	1161 to 1175		Minipool If
Pep No	292	ARGSPPSLASSSASQ	1165 to 1179		Minipool If
Pep No	293	PPSLASSSASQLSAP	1169 to 1183		Minipool If
Pep No	294	ASSSASQLSAPSLKA	1173 to 1187		Minipool If
Pep No	295	ASQLSAPSLKATCTT	1177 to 1191		Minipool If
Pep No	296	SAPSLKATCTTHHVS	1181 to 1195		Minipool Ig
Pep No	297	LKATCTTHHVSPDAD	1185 to 1199		Minipool Ig

Pep No	298	CTTHHVSPDADLIEA	1189	to	1203	Minipool Ig
Pep No	299	HVSPDADLIEANLLW	1193	to	1207	Minipool Ig
Pep No	300	DADLIEANLLWRQEM	1197	to	1211	Minipool Ig
Pep No	301	IEANLLWRQEMGGNI	1201	to	1215	Minipool Ig
Pep No	302	LLWRQEMGGNITRVE	1205	to	1219	Minipool Ig
Pep No	303	QEMGGNITRVESENK	1209	to	1223	Minipool Ig
Pep No	304	GNITRVESENKVVVL	1213	to	1227	Minipool Ig
Pep No	305	RVESENKVVVLDSFD	1217	to	1231	Minipool Ig
Pep No	306	ENKVVVLDSFDPLRA	1221	to	1235	Minipool Ih
Pep No	307	VVLDSFDPLRAEEDE	1225	to	1239	Minipool Ih
Pep No	308	SFDPLRAEEDEREVS	1229	to	1243	Minipool Ih
Pep No	309	LRAEEDEREVSVPAE	1233	to	1247	Minipool Ih
Pep No	310	EDEREVSVPAEILRK	1237	to	1251	Minipool Ih
Pep No	311	EVSVPAEILRKSKKF	1241	to	1255	Minipool Ih
Pep No	312	PAEILRKSKKFPAAM	1245	to	1259	Minipool Ih
Pep No	313	LRKSKKFPAAMPIWA	1249	to	1263	Minipool Ih
Pep No	314	KKFPAAMPIWARPDY	1253	to	1267	Minipool Ih
Pep No	315	AAMPIWARPDYNPPL	1257	to	1271	Minipool Ih
Pep No	316	IWARPDYNPPLLESW	1261	to	1275	Minipool Ii
Pep No	317	PDYNPPLLESWKDPD	1265	to	1279	Minipool Ii
Pep No	318	PPLLESWKDPDYVPP	1269	to	1283	Minipool Ii
Pep No	319	ESWKDPDYVPPVVHG	1273	to	1287	Minipool Ii
Pep No	320	DPDYVPPVVHGCPLP	1277	to	1291	Minipool Ii
Pep No	321	VPPVVHGCPLPPIKA	1281	to	1295	Minipool Ii
Pep No	322	VHGCPLPPIKAPPIP	1285	to	1299	Minipool Ii
Pep No	323	PLPPIKAPPIPPPRR	1289	to	1303	Minipool Ii
Pep No	324	IKAPPIPPPRRKRTV	1293	to	1307	Minipool Ii
Pep No	325	PIPPPRRKRTVVLTE	1297	to	1311	Minipool Ii
Pep No	326	PRRKRTVVLTESSVS	1301	to	1315	Minipool Ij
Pep No	327	RTVVLTESSVSSALA	1305	to	1319	Minipool Ij
Pep No	328	LTESSVSSALAELAT	1309	to	1323	Minipool Ij
Pep No	329	SVSSALAELATKTFG	1313	to	1327	Minipool Ij
Pep No	330	ALAELATKTFGSSES	1317	to	1331	Minipool Ij
Pep No	331	LATKTFGSSESSAVD	1321	to	1335	Minipool Ij
Pep No	332	TFGSSESSAVDSGTA	1325	to	1339	Minipool Ij
Pep No	333	SESSAVDSGTATALP	1329	to	1343	Minipool Ij
Pep No	334	AVDSGTATALPDQAS	1333	to	1347	Minipool Ij
Pep No	335	GTATALPDQASDDGD	1337	to	1351	Minipool Ij
Pep No	336	ALPDQASDDGDKGSD	1341	to	1355	Minipool Ij
Pep No	337	QASDDGDKGSDVESY	1345	to	1359	Minipool Ik
Pep No	338	DGDKGSDVESYSSMP	1349	to	1363	Minipool Ik
Pep No	339	GSDVESYSSMPPLEG	1353	to	1367	Minipool Ik
Pep No	340	ESYSSMPPLEGEPGD	1357	to	1371	Minipool Ik
Pep No	341	SMPPLEGEPGDPDLS	1361	to	1375	Minipool Ik
Pep No	342	LEGEPGDPDLSDGSW	1365	to	1379	Minipool Ik
Pep No	343	PGDPDLSDGSWSTVS	1369	to	1383	Minipool Ik
Pep No	344	DLSDGSWSTVSEEAS	1373	to	1387	Minipool Ik
Pep No	345	GSWSTVSEEASEDVV	1377	to	1391	Minipool Ik
Pep No	346	TVSEEASED**VVCC**SM	1381	to	1395	Minipool Ik
Pep No	**347**	EASED**VVCC**SMSYTW	1385	to	1399	Minipool Ik
Pep No	**348**	D**VVCC**SMSYTWTGAL	1389	to	1403	Minipool La
Pep No	349	CSMSYTWTGALITPC	1393	to	1407	Minipool La

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Pool L

Pep No	350	YTWTGALITPCAAEE	1397 to 1411		Minipool La
Pep No	351	GALITPCAAEESKLP	1401 to 1415		Minipool La
Pep No	352	TPCAAEESKLPINAL	1405 to 1419		Minipool La
Pep No	353	AEESKLPINALSNSL	1409 to 1423		Minipool La
Pep No	354	KLPINALSNSLLRHH	1413 to 1427		Minipool La
Pep No	355	NALSNSLLRHHNMVY	1417 to 1431		Minipool La
Pep No	356	NSLLRHHNMVYATTS	1421 to 1435		Minipool La
Pep No	357	RHHNMVYATTSRSAG	1425 to 1439		Minipool La
Pep No	358	MVYATTSRSAGLRQK	1429 to 1443		Minipool Lb
Pep No	359	TTSRSAGLRQKKVTF	1433 to 1447		Minipool Lb
Pep No	360	SAGLRQKKVTFDRLQ	1437 to 1451		Minipool Lb
Pep No	361	RQKKVTFDRLQVLDD	1441 to 1455		Minipool Lb
Pep No	362	VTFDRLQVLDDHYRD	1445 to 1459		Minipool Lb
Pep No	363	RLQVLDDHYRDVLKE	1449 to 1463		Minipool Lb
Pep No	364	LDDHYRDVLKEMKAK	1453 to 1467		Minipool Lb
Pep No	365	YRDVLKEMKAKASTV	1457 to 1471		Minipool Lb
Pep No	366	LKEMKAKASTVKAKL	1461 to 1475		Minipool Lb
Pep No	367	KAKASTVKAKLLSVE	1465 to 1479		Minipool Lb
Pep No	368	STVKAKLLSVEEACK	1469 to 1483		Minipool Lc
Pep No	369	AKLLSVEEACKLTPP	1473 to 1487		Minipool Lc
Pep No	370	SVEEACKLTPPHSAK	1477 to 1491		Minipool Lc
Pep No	371	ACKLTPPHSAKSKFG	1481 to 1495		Minipool Lc
Pep No	372	TPPHSAKSKFGYGAK	1485 to 1499		Minipool Lc
Pep No	373	SAKSKFGYGAKDVRN	1489 to 1503		Minipool Lc
Pep No	374	KFGYGAKDVRNLSSK	1493 to 1507		Minipool Lc
Pep No	375	GAKDVRNLSSKAVNH	1497 to 1511		Minipool Lc
Pep No	376	VRNLSSKAVNHIHSV	1501 to 1515		Minipool Lc
Pep No	377	SSKAVNHIHSVWKDL	1505 to 1519		Minipool Lc
Pep No	378	VNHIHSVWKDLLEDT	1509 to 1523		Minipool Ld
Pep No	379	HSVWKDLLEDTVTPI	1513 to 1527		Minipool Ld
Pep No	380	KDLLEDTVTPIDTTI	1517 to 1531		Minipool Ld
Pep No	381	EDTVTPIDTTIMAKN	1521 to 1535		Minipool Ld
Pep No	382	TPIDTTIMAKNEVFC	1525 to 1539		Minipool Ld
Pep No	383	TTIMAKNEVFCVQPE	1529 to 1543		Minipool Ld
Pep No	384	AKNEVFCVQPEKGGR	1533 to 1547		Minipool Ld
Pep No	385	VFCVQPEKGGRKPAR	1537 to 1551		Minipool Ld
Pep No	386	QPEKGGRKPARLIVF	1541 to 1555		Minipool Ld
Pep No	387	GGRKPARLIVFPDLG	1545 to 1559		Minipool Ld
Pep No	388	PARLIVFPDLGVRVC	1549 to 1563		Minipool Le
Pep No	389	IVFPDLGVRVCEKMA	1553 to 1567		Minipool Le
Pep No	390	DLGVRVCEKMALYDV	1557 to 1571		Minipool Le
Pep No	391	RVCEKMALYDVVSTL	1561 to 1575		Minipool Le
Pep No	392	KMALYDVVSTLPQVV	1565 to 1579		Minipool Le
Pep No	393	YDVVSTLPQVVMGSS	1569 to 1583		Minipool Le
Pep No	394	STLPQVVMGSSYGFQ	1573 to 1587		Minipool Le
Pep No	395	QVVMGSSYGFQYSPG	1577 to 1591		Minipool Le
Pep No	396	GSSYGFQYSPGQRVE	1581 to 1595		Minipool Le
Pep No	397	GFQYSPGQRVEFLVN	1585 to 1599		Minipool Le
Pep No	398	SPGQRVEFLVNTWKS	1589 to 1603		Minipool Le
Pep No	399	RVEFLVNTWKSKKNP	1593 to 1607		Minipool Lf
Pep No	400	LVNTWKSKKNPMGFS	1597 to 1611		Minipool Lf
Pep No	401	WKSKKNPMGFSYDTR	1601 to 1615		Minipool Lf
Pep No	402	KNPMGFSYDTRCFDS	1605 to 1619		Minipool Lf

Pep No	403	GFSYDTRCFDSTVTE	1609	to	1623	Minipool Lf
Pep No	404	DTRCFDSTVTENDIR	1613	to	1627	Minipool Lf
Pep No	405	FDSTVTENDIRVEES	1617	to	1631	Minipool Lf
Pep No	406	VTENDIRVEESIYQC	1621	to	1635	Minipool Lf
Pep No	407	DIRVEESIYQCCDLA	1625	to	1639	Minipool Lf
Pep No	408	EESIYQCCDLAPEAR	1629	to	1643	Minipool Lf
Pep No	409	YQCCDLAPEARQAIK	1633	to	1647	Minipool Lf
Pep No	410	DLAPEARQAIKSLTE	1637	to	1651	Minipool Lg
Pep No	411	EARQAIKSLTERLYI	1641	to	1655	Minipool Lg
Pep No	412	AIKSLTERLYIGGPL	1645	to	1659	Minipool Lg
Pep No	413	LTERLYIGGPLTNSK	1649	to	1663	Minipool Lg
Pep No	414	LYIGGPLTNSKGQNC	1653	to	1667	Minipool Lg
Pep No	415	GPLTNSKGQNCGYRR	1657	to	1671	Minipool Lg
Pep No	416	NSKGQNCGYRRCRAS	1661	to	1675	Minipool Lg
Pep No	417	QNCGYRRCRASGVLT	1665	to	1679	Minipool Lg
Pep No	418	YRRCRASGVLTTSCG	1669	to	1683	Minipool Lg
Pep No	419	RASGVLTTSCGNTLT	1673	to	1687	Minipool Lg
Pep No	**420**	VLTTSCGNTLTCYLK	1677	to	1691	Minipool Lg
Pep No	**421**	SCGNTLTCYLKASAA	1681	to	1695	Minipool Ma
Pep No	422	TLTCYLKASAACRAA	1685	to	1699	Minipool Ma
Pep No	423	YLKASAACRAAKLQD	1689	to	1703	Minipool Ma
Pep No	424	SAACRAAKLQDCTML	1693	to	1707	Minipool Ma
Pep No	425	RAAKLQDCTMLVNAA	1697	to	1711	Minipool Ma
Pep No	426	LQDCTMLVNAAGLVV	1701	to	1715	Minipool Ma
Pep No	427	TMLVNAAGLVVICES	1705	to	1719	Minipool Ma
Pep No	428	NAAGLVVICESAGTQ	1709	to	1723	Minipool Ma
Pep No	429	LVVICESAGTQEDAA	1713	to	1727	Minipool Ma
Pep No	430	CESAGTQEDAASLRV	1717	to	1731	Minipool Ma
Pep No	431	GTQEDAASLRVFTEA	1721	to	1735	Minipool Mb
Pep No	432	DAASLRVFTEAMTRY	1725	to	1739	Minipool Mb
Pep No	433	LRVFTEAMTRYSAPP	1729	to	1743	Minipool Mb
Pep No	434	TEAMTRYSAPPGDPP	1733	to	1747	Minipool Mb
Pep No	435	TRYSAPPGDPPQPEY	1737	to	1751	Minipool Mb
Pep No	436	APPGDPPQPEYDLEL	1741	to	1755	Minipool Mb
Pep No	437	DPPQPEYDLELITSC	1745	to	1759	Minipool Mb
Pep No	438	PEYDLELITSCSSNV	1749	to	1763	Minipool Mb
Pep No	439	LELITSCSSNVSVAH	1753	to	1767	Minipool Mb
Pep No	440	TSCSSNVSVAHDASG	1757	to	1771	Minipool Mb
Pep No	441	SNVSVAHDASGKRVY	1761	to	1775	Minipool Mc
Pep No	442	VAHDASGKRVYYLTR	1765	to	1779	Minipool Mc
Pep No	443	ASGKRVYYLTRDPTT	1769	to	1783	Minipool Mc
Pep No	444	RVYYLTRDPTTPLAR	1773	to	1787	Minipool Mc
Pep No	445	LTRDPTTPLARAAWE	1777	to	1791	Minipool Mc
Pep No	446	PTTPLARAAWETARH	1781	to	1795	Minipool Mc
Pep No	447	LARAAWETARHTPVN	1785	to	1799	Minipool Mc
Pep No	448	AWETARHTPVNSWLG	1789	to	1803	Minipool Mc
Pep No	449	ARHTPVNSWLGNIIM	1793	to	1807	Minipool Mc
Pep No	450	PVNSWLGNIIMYAPT	1797	to	1811	Minipool Mc
Pep No	451	WLGNIIMYAPTLWAR	1801	to	1815	Minipool Md
Pep No	452	IIMYAPTLWARMILM	1805	to	1819	Minipool Md
Pep No	453	APTLWARMILMTHFF	1809	to	1823	Minipool Md

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Pool M

Pep No	454	WARMILMTHFFSILL	1813	to	1827	Minipool Md		
Pep No	455	ILMTHFFSILLAQEQ	1817	to	1831	Minipool Md		
Pep No	456	HFFSILLAQEQLEKA	1821	to	1835	Minipool Md		
Pep No	457	ILLAQEQLEKALDCQ	1825	to	1839	Minipool Md		
Pep No	458	QEQLEKALDCQIYGA	1829	to	1843	Minipool Md		
Pep No	459	EKALDCQIYGACYSI	1833	to	1847	Minipool Md		
Pep No	460	DCQIYGACYSIEPLD	1837	to	1851	Minipool Md		
Pep No	461	YGACYSIEPLDLPQI	1841	to	1855	Minipool Md		
Pep No	462	YSIEPLDLPQIIERL	1845	to	1859	Minipool Me		
Pep No	463	PLDLPQIIERLHGLS	1849	to	1863	Minipool Me		
Pep No	464	PQIIERLHGLSAFSL	1853	to	1867	Minipool Me		
Pep No	465	ERLHGLSAFSLHSYS	1857	to	1871	Minipool Me		
Pep No	466	GLSAFSLHSYSPGEI	1861	to	1875	Minipool Me		
Pep No	467	FSLHSYSPGEINRVA	1865	to	1879	Minipool Me		
Pep No	468	SYSPGEINRVASCLR	1869	to	1883	Minipool Me		
Pep No	469	GEINRVASCLRKLGV	1873	to	1887	Minipool Me		
Pep No	470	RVASCLRKLGVPPLR	1877	to	1891	Minipool Me		
Pep No	471	CLRKLGVPPLRVWRH	1881	to	1895	Minipool Me		
Pep No	472	LGVPPLRVWRHRARS	1885	to	1899	Minipool Me		
Pep No	473	PLRVWRHRARSVRAR	1889	to	1903	Minipool Mf		
Pep No	474	WRHRARSVRARLLSQ	1893	to	1907	Minipool Mf		
Pep No	475	ARSVRARLLSQGGRA	1897	to	1911	Minipool Mf		
Pep No	476	RARLLSQGGRAATCG	1901	to	1915	Minipool Mf		
Pep No	477	LSQGGRAATCGKYLF	1905	to	1919	Minipool Mf		
Pep No	478	GRAATCGKYLFNWAV	1909	to	1923	Minipool Mf		
Pep No	479	TCGKYLFNWAVKTKL	1913	to	1927	Minipool Mf		
Pep No	480	YLFNWAVKTKLKLTP	1917	to	1931	Minipool Mf		
Pep No	481	WAVKTKLKLTPIPAA	1921	to	1935	Minipool Mf		
Pep No	482	TKLKLTPIPAASQLD	1925	to	1939	Minipool Mf		
Pep No	483	LTPIPAASQLDLSGW	1929	to	1943	Minipool Mf		
Pep No	484	PAASQLDLSGWFVAG	1933	to	1947	Minipool Mg		
Pep No	485	QLDLSGWFVAGYSGG	1937	to	1951	Minipool Mg		
Pep No	486	SGWFVAGYSGGDIYH	1941	to	1955	Minipool Mg		
Pep No	487	VAGYSGGDIYHSLSR	1945	to	1959	Minipool Mg		
Pep No	488	SGGDIYHSLSRARPR	1949	to	1963	Minipool Mg		
Pep No	489	IYHSLSRARPRWFML	1953	to	1967	Minipool Mg		
Pep No	490	LSRARPRWFMLCLLL	1957	to	1971	Minipool Mg		
Pep No	491	RPRWFMLCLLLLSVG	1961	to	1975	Minipool Mg		
Pep No	492	FMLCLLLLSVGVGIY	1965	to	1979	Minipool Mg		
Pep No	493	LLLLSVGVGIYLLPN	1969	to	1983	Minipool Mg		
Pep No	**494**	LLLSVGVGIYLLPNR	1970	to	1984	Minipool Mg	in blue: end of pool M	