

Characterisation Of The Immune Response To PARV4

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To Nan and Bumpa

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Abstract

PARV4 is a novel human parvovirus initially identified in an intravenous drug user at risk of HIV infection. PARV4 is a small single stranded DNA virus principally absent from the general population, but common in HCV- and HIV-infected individuals. Until 2009, most published PARV4 studies related to the prevalence of PARV4 in various risk groups. PARV4 has been detected in the liver of HCV-patients and the bone marrow of HIV-patients. Parvovirus B19, the closest related virus, elicits a strong immune response and can cause serious disease. Thus, this project was initiated to characterise the immune response to PARV4, and investigate the clinical significance of this virus.

Cohorts of HCV-infected, HIV-infected, HCV-HIV co-infected, healthy and acute parvovirus B19-infected individuals were screened for humoral and cellular responses in both acute and chronic PARV4 infection. HCV- and HIV-related disease progression was also assessed relative to PARV4 infection. This study demonstrates that the highest prevalence of PARV4 infection is found in HCV-HIV co-infected intravenous drug users, and provides additional evidence for parenteral transmission. I present here the first data on the cellular immune response to PARV4 in acute and chronic infection and define PARV4 as a persistent virus. Although no clear correlation could be found between PARV4 and HCV or HIV disease progression, the high prevalence rates emphasize the importance of investigations into emerging infections such as PARV4.

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Publications

High frequencies of exposure to the novel human parvovirus PARV4 in hemophiliacs and injection drug users, as detected by a serological assay for PARV4 antibodies. (2009), Sharp, C.P., Lail, A., Donfield, S., **Simmons, R.**, Leen, C., Klenerman, P., Delwart, E., Gomperts, E.D. & Simmonds, P., *J Infect Dis*, 200, 1119-25.

High Frequency, Sustained T Cell Responses to PARV4 Suggest Viral Persistence In Vivo. (2011), **Simmons, R.**, Sharp, C., Sims, S., Kloverpris, H., Goulder, P., Simmonds, P., Bowness, P. & Klenerman, P. *J Infect Dis*, 203, 1378-87.

PARV4 Infection and Clinical Outcome in High-Risk Populations. (2012) **Simmons, R.**, Sharp, C., McClure, C.P., Rohrbach, J., Kovari, H., Frangou, E., Simmonds, P., Irving, W., Rauch, A., Bowness, P., Klenerman, P. and the Swiss HIV Cohort Study, *J Infect Dis*, 205, 1816-1820.

Abbreviations used in this thesis

AAV	Adeno-associated virus
AIDS	Acquired Immuno-deficient syndrome
ALT	Alanine amino-transferase
ARF	Alternative Reading Frame
BCL	B cell line
BCR	B cell receptor
CMV	Cytomegalovirus
CNS	Central nervous system
ConA	Concavidin A
CSF	Cerebrospinal fluid
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked Immunosorbent assay
ELISpot	Enzyme-linked Immunosorbent spot assay
Epitope RMT	Epitope RMTENIVEV
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FEC	Flu, EBV and CMV epitopes
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBoV	Human Bocavirus
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HLA-A2 RMT	HLA-A*0201 tetramer for peptide RMTENIVEV
HSV	Herpes Simplex virus
IDU	Intravenous Drug User
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
KIR	Killer-cell immunoglobulin-like receptor
MHC	Major histocompatibility complex
MSM	Men who have sex with men
NAT	Nucleic Acid Testing
NS	Non-structural protein
ORF	Open Reading Frame
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PMA	Phorbol Myristate Acetate
PRR	Pathogen recognition receptor
REACH	"Risk Evaluation Assessment of Community Health prospective study of IDUs" cohort
R10	RPMI 1640 supplemented with 10% FCS, 50,000 U penicillin, 50mg streptomycin and 1mmol L-glutamine
SAT	Small alternatively translated protein
SD	Standard deviation
SFU/10⁶ PBMC	Spot forming units per million PBMC
SNP	Single nucleotide polymorphism
TAE	Tris-Acetate EDTA (40mM Tris, 20mM Acetic Acid, 1mM EDTA, pH 8.0)
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VP	Viral capsid protein
WIMM	Weatherall Institute for Molecular Medicine

Chapter 1

Introduction

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Introduction

The aetiology of many clinical conditions is still unknown, thus there is a constant search for uncharacterised viruses. In 2005, Jones *et al.* used DNase sequence-independent single primer amplification (DNase-SISPA) to identify a new virus: PARV4 (Jones *et al.*, 2005). PARV4 was found in the plasma of a patient with acute viral infection symptoms, at risk of carrying human immunodeficiency virus 1 (HIV-1). The following years saw a series of publications on the prevalence of PARV4 viral DNA, but no studies had characterised the immune response to what was becoming a common virus in high-risk populations.

Parvovirus B19, the closest related virus to PARV4, elicits a strong humoral and cellular response. With the purpose of defining the clinical significance of PARV4, I have looked into the immune response to PARV4, the effect on Hepatitis C virus (HCV) and HIV-1 disease progression, and the particular case of acute PARV4 infection.

In this chapter, I first present a brief outline of the important processes of the immune system involved in anti-viral responses, then introduce the family of parvoviruses that infect humans, before concentrating on PARV4, the focus of this thesis.

1. The anti-viral immune response

The human body is an intricate assembly of cells that work together to allow a living being to function - but it is a delicate system that is constantly under attack from the outside world, be it through physical injury, the climate or invading microorganisms. Elie Metchnikov (1845–1916) described how the simplest of multicellular organisms, such as sponges and corals, defend themselves against microbial infection by the process of phagocytosis (Kaufmann, 2008). This marked the beginning of immunology.

The immune system has evolved to fight against a wide range of infections, through the innate and adaptive branches. The innate immune system is immediate and non-specific, and provides the initial defence against a pathogen. The adaptive branch, which is comprised primarily of B and T cells, specifically targets pathogen epitopes and develops 'memory' populations that elicit long lasting protection. Among the many infectious agents that challenge the human body, viruses represent a major threat. They have evolved many mechanisms to evade the immune response.

Whereas the innate and adaptive branches of the immune system have to be activated, some antiviral proteins are constitutively expressed and form part of intrinsic immunity. The Fv1, TRIM5 and APOBEC families of proteins are well characterised and inhibit retroviral infection by inhibiting viral capsid binding to its receptor, or hypermutating and destabilising retroviral genomes (reviewed by Bieniasz, 2004).

The following sections of this chapter will describe the different arms of the immune system and their involvement in the clearance of viral infections.

1.1. The innate immune system

1.1.1. Pattern recognition receptors

The innate immune system has evolved to recognise pathogen-associated molecular patterns (PAMPs), which are common across a range of pathogens. These PAMPs are recognised by a number of different receptors, known as pattern recognition receptors (PRRs) found both on the surface of a cell, as well as within the cytosol and endosomal compartments. One or more of these receptors, therefore, can detect different pathogens. The Toll-like receptors (TLRs) are the best characterised PRRs; there are 10 TLRs identified in humans, which are found on the surface of dendritic cells or macrophages, or in the membranes of endosomes (Takeda et al., 2003). Of these, TLR-3, 7, 8 and 9 are particularly important in the detection of viruses. TLR3 detects viral double stranded (ds) RNA, while TLR7 and 8 are specific for single stranded (ss) RNA, and TLR9 is responsible for recognising ssDNA. There is some evidence that ssDNA viruses of the *Parvovirinae* family, to which belongs PARV4, are recognised by TLR9 (Zhu et al., 2009). TLR triggering leads to the expression of a large range of genes, including the interferon regulatory factors (IRFs). These transcription factors activate the transcription of type 1 interferons: interferons α and β (IFN α and IFN β , reviewed by Tailor et al., 2006).

Other PRRs include the cytoplasmic viral sensors: receptors RIG-I (retinoic acid inducible gene I) and MDA5 (melanoma differentiation-associated gene 5) which can detect viral dsRNA. They in turn activate IRFs that lead to the production of IFN α and IFN β (Tailor et al., 2006).

1.1.2. Type I interferons

IFN α and IFN β act in several ways. Through binding their receptor IFNAR, they induce the expression of a large range of genes known as interferon-stimulated genes (ISGs). Protein kinase R (PKR) is a product of one of these ISGs and can inhibit the initiation of translation and editing of RNA, thereby reducing host and viral protein synthesis (reviewed in Sadler et al., 2008). The expression of 2',5'-oligoadenylate synthetase (OAS) leads to the activation of RNase-L which is able to degrade ssRNA, and has been shown to degrade RSV RNA (Player et al., 1998). Mx proteins are also activated by type I interferons, and MxA has been shown to inhibit the replication of HBV and Crimean-Congo hemorrhagic fever virus (Andersson et al, 2004, Gordien et al., 2001).

Type 1 interferons can also boost the adaptive the immune response by increasing the expression of major histocompatibility complex (MHC) molecules on the cell surface, thereby presenting more viral antigen to T cells. They are also able to activate NK cells through inducing the production of IL-15 (Nguyen et al., 2002). Both these processes ultimately promote the destruction of infected cells through the release of cytotoxic granules. Type 1 interferons also induce the maturation of dendritic cells and macrophages, which leads to the expression of co-stimulatory molecules CD80, CD86 and CD40 on the surface, augmenting their role as professional antigen-presenting cells. Thus, type I interferons provide a link between the innate and adaptive immune responses to virus (reviewed by Sadler et al., 2008; Perry et al., 2005; Murphy, 2008).

1.1.3. Natural killer and natural killer T cells

Natural Killer (NK) cells are lymphocytes that are able to recognise and kill transformed cells, non-self and infected cells without prior sensitisation. They detect these cells through a range of different receptors such as killer-cell immunoglobulin-like receptors (KIRs), that can be inhibitory or stimulatory. They also express Fc receptors able to bind antibody-antigen complexes. NK cells are further activated by type 1 interferons, IFN- γ and interleukin 12 (IL-12).

KIRs recognise MHC class I molecules presented at the surface of almost all cells within the body. The absence of these self-MHC I molecules on a cell's surface leads to the activation of NK cells, and consequently the killing of the target cell. NKG2D is an activating receptor and recognises MICA, MICB and the UL 16-binding protein (ULBP) family, which are expressed in response to cellular stress during viral infection for example (Obeidy and Sharland, 2009). Like CD8⁺ T cells, NK cells can trigger the death of a foreign or infected cell through the release of granzymes and perforin. Thus, NK cells play an important role in controlling the infection while the adaptive response develops (Biron, 1997, Warrell, 2010, Chapel, 2006).

Natural killer T (NKT) cells are so named because they share both NK and T cell markers. They express $\alpha\beta$ TCR molecules on their surface, but these are restricted by non-classical MHC class I-like molecule CD1d that bind to lipids or glycolipids. They provide an important link between the innate and adaptive branches, and improve disease outcome in viral infections such as respiratory syncytial virus and induce a Th1 response in hepatitis C virus (HCV) infection (van Dommelen and Degli-Esposti, 2004).

1.1.4. Dendritic cells

Dendritic cells (DCs) provide an essential link with the adaptive immune system, and are known as professional antigen presenting cells (APCs). DCs are myeloid cells that have a specialised morphology, their dendrites, that allow them to patrol tissues for any invading microbe. Once they detect a pathogen through PRRs, they mature and migrate to adjacent lymphoid tissues where they prime the adaptive immune system. They process antigen as outlined below, and present viral peptides bound to MHC molecules on their surface. Mature DCs have increased expression of MHC class II molecules. However, the MHC-antigen complex alone is not sufficient to elicit a T cell response. Through the expression of co-stimulatory molecules CD80 and CD86 (B7.1 and B7.2), DCs are able to prime naïve T cells. Along with the production of cytokines, T cells have the necessary signals to mount a specific immune response (Figure 1.1) (Warrell, 2010, Lenschow et al., 1996).

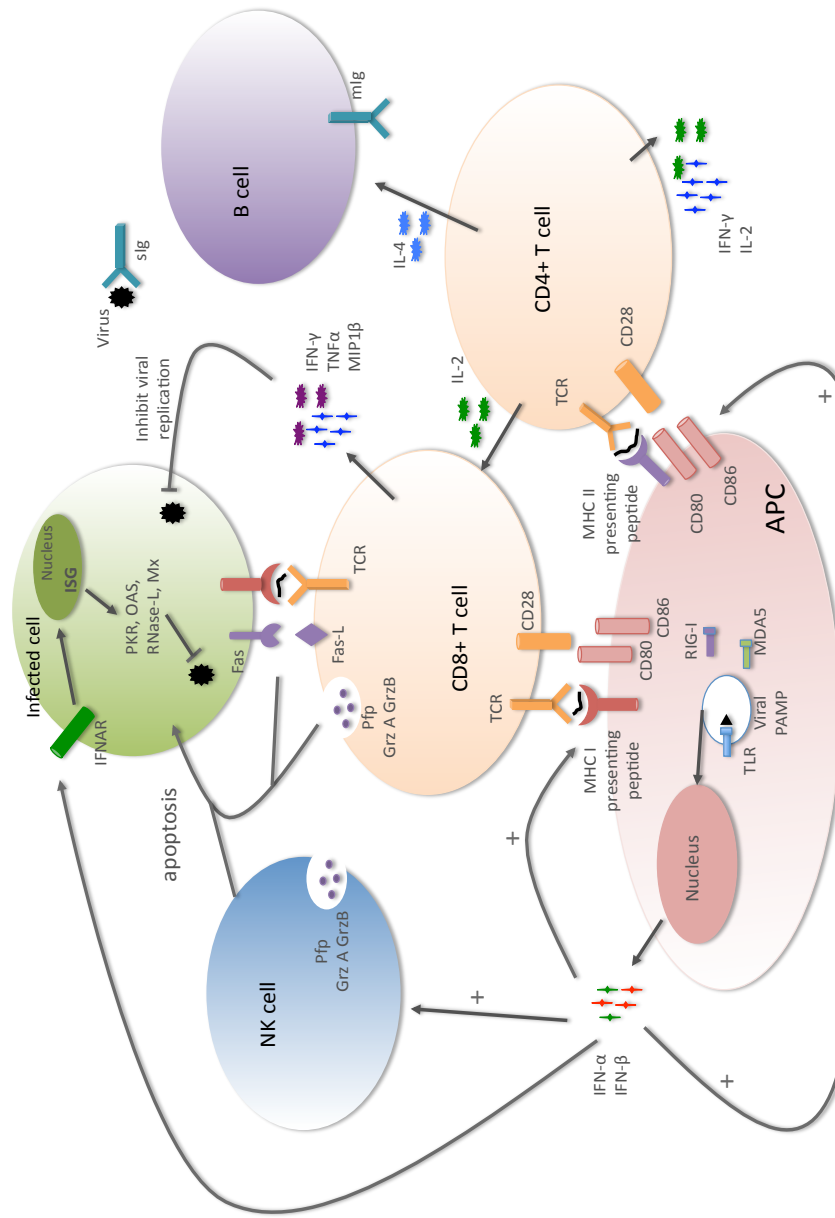


Figure 1.1 The anti-viral immune response. The anti-viral response is made up of the balanced interplay of several types of cells, including NK cells, APCs, CD8⁺ and CD4⁺ T cells. They are able to recognise the invading pathogen through a variety of receptors, eventually leading to the capture of virus or the destruction of infected cells. APC: antigen-presenting cell; Grz A, B: granzymes A and B; Pfp: perforin protein; Fas-L: Fas ligand; mlg: membrane-bound immunoglobulin; sig: secreted immunoglobulin; TLR: toll-like receptor; PAMP: pathogen-associated molecular pattern; IFNAR: type 1 interferon receptor; PKR: protein kinase R; OAS: 2',5'-oligoadenylate synthetase, Mx: myxovirus protein.

1.2. The adaptive immune system

The adaptive immune system tackles two targets during a viral infection: the virus itself and the infected host-cells. The humoral system is responsible for neutralising the virion through the release of antibodies. Cell-mediated immunity recognises viral peptides presented on the surface of an infected cell which targets it for destruction.

1.2.1. B cells

B cells mature in the bone marrow and have a receptor (BCR) able to recognise antigen. The BCR is made up of a Fab region that binds antigen, and an Fc region that initiates signalling via binding to the Fc receptor. Together these make up a membrane-bound immunoglobulin molecule, which is composed of a heavy chain and a light chain. Each chain is made up of variable (V) and joining (J) genetic segments that exist in multiple copies. The heavy chains have an extra diversity (D) segment. Additionally, light chains come from one of two loci creating a κ or λ light chain. These then combine with the constant (C) segments that make up the heavy chains. Moreover, the BCR undergoes somatic hypermutation which increases its affinity to antigen over the course of the antibody response. The BCR is a membrane-bound immunoglobulin (mIg). When a B cell binds to antigen, with the aid of helper T cells it matures into a plasma cell which secretes immunoglobulins (sIg) for this antigen. In early infection, IgM are produced, so called because of the μ constant chain that is used. Through

class switching, IgG (γ constant chain) are secreted which are long lasting and provide protection against reinfection.

By binding to the virion, antibodies can directly interfere with the virion binding the host-cell surface receptors thereby the virus is prevented from entering and replicating within a new cell. Such antibodies are known as neutralising and are a highly efficient way for the immune response to prevent infections. Viruses such as HIV and HCV rapidly mutate their envelope proteins in order to escape this pressure. HCV, for example, contains a hyper-variable region within its envelope protein that allows it to rapidly escape the action of neutralising antibodies.

When an antigen is coated with antibodies, a process known as opsonisation, it can then be readily recognised through Fc receptors and phagocytosed by macrophages. Antibodies can also target pathogens or infected cells through involving the complement system. When IgM or IgG bind to antigen, their Fc region changes conformation to allow binding of components of the classical complement pathway. The subsequent activation of the components of the complement leads to the lysis of the infected cell, or pathogen, through the formation of a membrane pore complex. Alternatively, the Fc component of IgG bound to antigen on the surface of a cell can also be recognised by low-affinity Fc γ RIII receptors expressed on the surface of NK cells, monocytes and neutrophils. This also leads to lysis of the infected cell and this process is known as antibody-dependent cellular cytotoxicity (Chapel, 2006, Warrell, 2010).

1.2.2. T cells

a. The T cell receptor (TCR)

All T cells express a T cell receptor (TCR) on their surface. There are two types of TCRs: the receptor found on most T cells is made up of alpha and beta chains ($\alpha\beta$ TCR). The second is made up of gamma and delta chains ($\gamma\delta$ TCR). TCRs need to be able to recognise all pathogens in the context of MHC presentation, and so are extremely diverse. This is possible through gene rearrangement as for BCRs. Like the BCRs, the TCR chains α and β have V, J and C domains. The β chains also have D segments. These are spliced and recombined, and further diversity is created at the junctions through a deoxynucleotide transferase enzyme. The heterodimerisation of the α and β creates a unique TCR, and the CDR3 hypervariable regions confer the specificity to the TCR.

The gene segments which make up γ chains of $\gamma\delta$ TCRs are found within the $\text{TCR}\alpha$ locus. There are fewer V segments for $\gamma\delta$ chains than for $\alpha\beta$ chains, but there is more variability in the junctional regions in $\gamma\delta$ TCRs. These amino acids lie at the centre of the TCR binding site. The $\gamma\delta$ TCR V regions also resemble immunoglobulin V segments more than those of $\alpha\beta$ TCRs (Murphy, 2008).

$\alpha\beta$ and $\gamma\delta$ TCRs are found on T cells in conjunction with the CD3 complex which allows transduction of signal into the cell. $\alpha\beta$ TCRs link up with CD8 or CD4 co-receptors, thereby creating two classes of T cells: the CD8⁺ cytotoxic T cells and the CD4⁺ helper T cells. These co-receptors are absent from $\gamma\delta$ TCRs.

b. CD8⁺ T cell, CD4⁺ T cell and $\gamma\delta$ ⁺ T cell functions

As their name implies, cytotoxic CD8⁺ T cells are responsible for killing infected cells by the secretion of perforin and granzymes through an immune synapse, or through Fas ligand (Fas-L) – Fas interactions (Anthony et al., 2010). CD8⁺ T cells secrete many cytokines including IFN- γ , TNF α , MIP-1 α and MIP-1 β (Figure 1.1). IFN- γ , whether expressed by T cells, NK or NKT cells, has a central role in antiviral immunity as this cytokine can increase lysosomal activity in macrophages and increase the expression of class I MHC on all cells, and class II MHC on APCs, thereby promoting the presentation and recognition of viral antigen. IFN- γ can also increase chemokine expression to promote leukocyte migration and NK cell activity, leading to the destruction of infected cells. To support this activity, IFN- γ also skews the immune response towards a Th1 phenotype (reviewed by Schroder et al., 2004). The expression of intrinsic defence factors also inhibits viral infection. For example, IFN- γ can induce the expression of tetherin that prevents the release of HIV-1 retrovirus particles lacking Vpu accessory protein (Neil et al., 2008; Le Tortorec et al., 2011).

TNF α can directly inhibit viral replication, induce apoptosis in infected cells and promote an inflammatory response through the expression of adhesion molecules (reviewed by Kafrouni et al., 2003). Similarly, MIP-1 α and β (macrophage-inflammatory protein-1 α and β) are chemokines that attract B cells, CD8⁺ and CD4⁺ T cells thereby recruiting further antiviral effector cells (Schall et al., 1993).

CD4⁺ T cells can help other cells such as B cells, CD8⁺ T cells, DCs and macrophages. Th1 cells, which produce IL-2 and IFN- γ amongst other cytokines, enhance the function of CD8⁺ T cells and macrophages, while Th2 cells, which

produce IL-4, IL-5, IL-10 and IL-13, support the humoral response through Ig class switching. Th17 cells, so named because of their production of IL-17, are important anti-microbial cells at epithelial and mucosal barriers, and are involved in autoimmunity and immunopathology (Mosmann et al., 1986, Weaver et al., 2006).

$\gamma\delta$ TCR T cells recognise a different spectrum of antigens to $\alpha\beta$ TCR T cells, and do not require antigen processing and MHC presentation to recognise antigen (Chien et al., 1996). Hence they are thought to bridge the innate and adaptive immune responses (Sciammas and Bluestone, 1999). In most studies, the antigens that activate $\gamma\delta^+$ T cells appear to be 'generic', such as a phospho-antigen, a heat shock-derived protein (Bukowski et al., 1995) or another host molecule stimulated by viral infection, rather than viral antigen (Ponniah et al., 1996).

$\gamma\delta^+$ T cells are important in the antiviral immune response. For example, $\gamma\delta^+$ T cells increase and are expanded in the peripheral blood of HIV-infected patients (Boullier et al., 1995, De Maria A, 1992) where they could play an essential role as they can respond to infection without themselves being infected with HIV-1 (Sciammas and Bluestone, 1999). Moreover, $\gamma\delta^+$ T cells in patients with Herpes Simplex Virus-1 (HSV-1) can lyse HSV-1 infected target cells (Maccario et al., 1993).

c. MHC molecules and antigen presentation

CD8⁺ and CD4⁺ T cells, and more specifically their $\alpha\beta$ TCRs, can only recognise peptide when presented by an MHC molecule (Zinkernagel and Doherty, 1974).

This is called MHC restriction. The MHC molecules in humans are called human leukocyte antigens (HLA) I and II. HLA class I is made up of HLA-A, B and C, HLA class II is made up of HLA-DP, DQ and DR. Unlike the TCRs which are specific for individual epitopes, HLA molecules can bind to many peptides, but at the same time are very selective. Thymus selection during T cell development selects for TCRs that recognise self-MHC molecules, but leads to the apoptosis of cells carrying TCRs that bind too strongly to self-peptides. There are over 5000 known HLA class I alleles, and over 1500 HLA class II alleles although some are more common in different ethnic groups (Robinson et al., 2011, Gonzalez-Galarza et al., 2011). Most nucleated cells express MHC class I on their surface, but MHC class II is restricted to cell types such as dendritic cells, B cells, activated T cells, macrophages and some epithelia cells. IFN- γ can lead to the expression of MHC class II by non-professional antigen-presenting cells during inflammation (Chapel, 2006, Murphy, 2008).

Although MHC class I and II have a different molecular structure, they have a similar peptide-binding groove. Nonetheless, MHC class I molecules have a very restricted groove, allowing them to bind peptides that are optimally 8 or 9 amino acids in length (Bjorkman et al., 1987). MHC class II molecules on the other hand, have a more open structure and can bind peptides over 13 amino acids long (Brown et al., 1993).

Cell-mediated immunity is based on the ability of infected cells to present viral peptide on their surface through antigen processing. There are two main pathways of antigen processing: the endogenous pathway that processes antigens found within the cytoplasm, and the endocytic pathway that processes proteins found in endosomes that have been taken up from the interstitial space

(Figure 1.2). The proteasome degrades proteins into short peptides, which are transported into the endoplasmic reticulum (ER) by Transporter associated with antigen presenting (Tap) protein where it interacts with tapasin protein before binding to folded MHC class I molecules. The endocytic pathway breaks up proteins through low pH in endosomes, and the peptides are delivered to MHC class II compartments where they can bind to MHC class II molecules. MHC class I and II molecules bound to peptide are then transported to the surface of the cell, where they can present the viral antigen to CD8⁺ T cells for MHC class I, and CD4⁺ T cells for MHC class II (Heath and Carbone, 2001).

There is a third mechanism called cross-presentation, whereby cells that are not infected by viruses are still able to present viral peptides to CD8⁺ T cells, i.e. capturing exogenous antigen and presenting it on an MHC I molecule. Although the exact mechanism is still unclear, this process occurs through antigen in endosomes being diverted to the cytosol, and degraded by the proteasome before continuing on the MHC I presentation pathway.

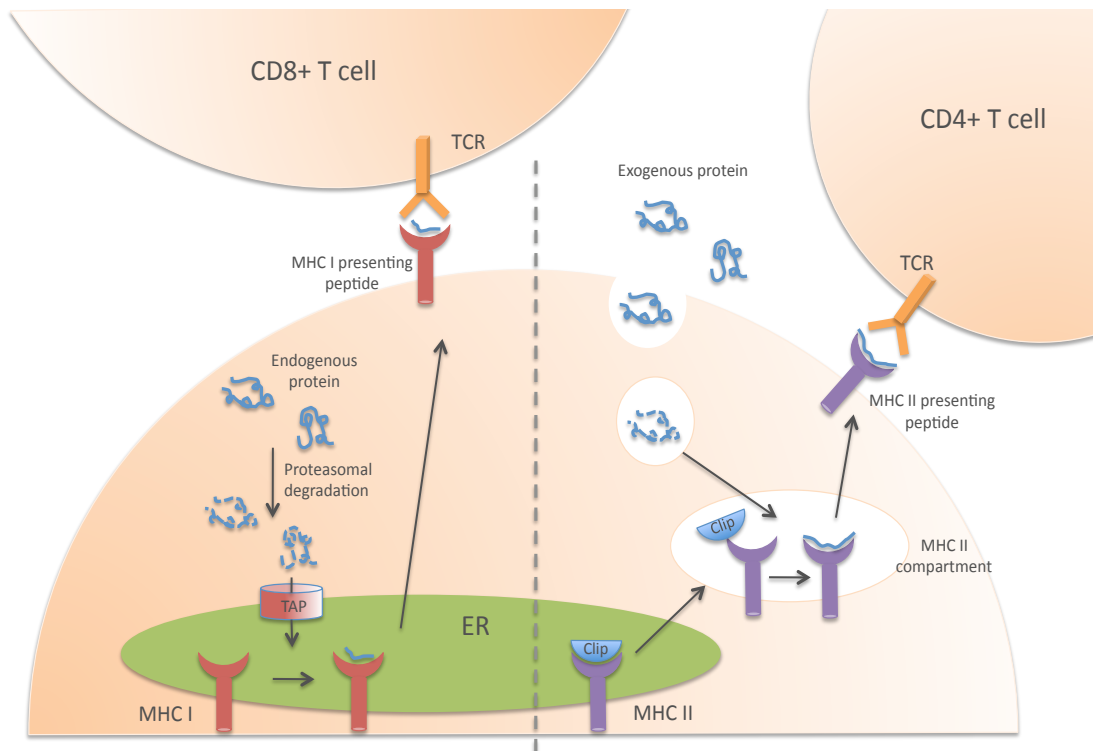


Figure 1.2 Antigen-processing pathways. There are two main pathways through which antigen is processed. The endogenous pathway (left panel) processes endogenous proteins that are degraded by the proteasome. These are transported through the TAP protein to the endoplasmic reticulum (ER), where they bind to MHC class I molecules before returning to the surface of the cell. There, this complex is recognised by the T cell receptor (TCR) of CD8⁺ T cells. The endocytic pathway takes up proteins from the interstitial space into endosomes. MHC II molecules are synthesised in the ER where Clp protein prevents any peptide binding. Exogenous proteins are degraded in a pH dependent process in late endosomes, and are taken up by MHC class II proteins in the MHC class II compartment once Clp is released, before returning to the surface to present antigen to CD4⁺ T cells. (Figure based on Heath and Carbone, 2001)

1.3. Immune memory

The defining characteristics of the adaptive immune response are that it is specific and provides immunological “memory”, in the form of an enhanced and faster response upon re-exposure to a given pathogen. As mentioned above, plasma cells secrete IgG that confer long-lasting protection. Since the advent of MHC class I tetramers, it has been possible to classify virus-specific CD8⁺ T cells into several subsets depending on surface markers such as CD45RA, CCR7 and CD62L.

CD45RA is a high molecular weight isoform of CD45R, a tyrosine phosphatase that regulates signalling through the TCR-CD3 complex. Naïve T cells that have not encountered antigen are characteristically CD45RA⁺. These cells also express CD62L, an adhesion molecule that binds to the lymph node receptor CD62, and CCR7, a chemokine receptor that also directs the migration of T cells to lymph nodes (reviewed by van Lier et al., 2003). When T cells are activated, they lose their expression of CD45RA, CD62L and CCR7. Effector T cells are thus CD62L⁻ and CCR7⁻, allowing them to leave the lymph nodes and migrate to peripheral tissues to fight infection (Sallusto et al., 1999).

After the acute T cell response, a contraction phase occurs, where only 10% of antigen-specific cells will persist and compose the memory T cell pool (Murali-Krishna et al., 1998). Memory T cells are divided into central and effector memory T cells. Central memory T cells (T_{CM}, CCR7⁺ CD45RA⁻) eventually lose their effector functions and home to organs in the absence of antigen; although they are still able to respond within hours if infection occurs. They are able to produce IL-2 and proliferate when the TCR is activated under suboptimal

conditions. Effector memory T cells (T_{EM} , $CCR7^- CD45RA^-$) have the ability to produce IFN- γ and TNF- α (reviewed in Wherry and Ahmed, 2004; de Jong et al., 1991). They can provide a more immediate response as they are scattered throughout the tissues. Terminally differentiated $CCR7-CD45RA^+$ T cells are known as effector memory $CD45RA^+$ T cells (T_{EMRA}) due to their strong production of perforin and their cytotoxic ability (Hamann et al., 1997).

CD27 and CD28 have also been used to describe the maturity of memory T cells (Appay et al., 2002). CD27 is a protein that binds to CD70 on activated lymphocytes creating a negative feedback loop and down-regulating CD27. The expression of CD27 leads to T cell expansion and the activation of B cells. CD28 binds to co-stimulatory molecules CD80 and CD86 expressed on APCs, which leads to T cell activation. Continuous stimulation of T cells may also lead to the down-regulation of CD28 (reviewed by van Lier et al., 2003; Denoeud and Moser, 2011).

Although the phenotype of T cells is similar in acute infections, it differs significantly depending on the nature of the virus. CMV-specific T cells show the most mature phenotype and can represent up to 10% of $CD8^+$ T cells to a single CMV epitope (Lanzavecchia and Sallusto, 2000, Gillespie et al., 2000). CMV-specific T cells are principally $CD27-CD28-CCR7-CD45RA^+$, whereas most HIV-specific T cells are $CD27^+CD28-CCR7-CD45RA^-$. HIV-infected individuals with higher frequencies of $CD27^-$ T cells have been shown to progress slower to AIDS; thus, suggesting the CD27-CD70 pathway may be important for controlling persistent viruses (van Baarle et al., 2002). This has led to the hypothesis that antigen exposure can skew T cell memory differentiation, and affect a T cell's ability to clear infection (van Lier et al., 2003).

For several decades immunologists have debated whether immunological memory is antigen-dependent or independent, i.e. whether a constant source of antigen is required to maintain protective memory cells, or whether other mechanisms are in place to allow these cells to persist after infection is cleared.

One of the first hypotheses was that memory T cells were long-lived and thus could be maintained beyond the course of infection (Gray, 2002, Antia et al., 2005). However, experiments showed that memory cells have, in fact, a faster turnover rate than naïve cells (Sprent, 2003, Tough and Sprent, 1994). To be maintained, these cells required continued antigenic stimulation (Gray and Matzinger, 1991, Oehen et al., 1992).

Additionally, many viruses are persistent, and several infections, thought to be cleared and leave no antigen to stimulate further cells, have also been shown to persist in various forms. This is exemplified by lymphocytic choriomeningitis (LCMV) where low levels of neutralizing antibodies were detected several months after an infection appeared to have been cleared, indicating the presence of antigen (Ciurea et al., 1999, Planz et al., 1997). This has also been suggested in HCV (reviewed in Lloyd et al., 2007).

Gray (2002) recalls the importance of follicular dendritic cells (FDCs) in lymphoid tissues and their ability to retain antigen on their surface for years (Tew and Mandel, 1979). The adoptive transfer of memory B cells into mice that lack FDCs failed to elicit a long-lasting IgG response, demonstrating the importance of FDCs in maintaining B cell memory (Fu et al., 2000).

Zinkernagel (2002) makes the distinction between antigen-independent memory that is not biologically relevant, and protective memory that is antigen-dependent. Gray (2002) underlines that it is the effector functions that are

protective, rather than simply the presence of memory cells, and that for this functional state, antigen is required.

Lessons can also be learnt from the advent of vaccination. Live attenuated viral vaccines often yield a longer-lasting protective immune response than killed vaccines. This may be due to the quantity of antigen produced by the live vaccine, or to the inflammatory cytokines secreted as part of the anti-vaccine immune response that promote effector functions in B and T cells (Gray, 2002). Heat-killed *Listeria monocytogenes* was able to elicit a strong CD8⁺ T cell response, although it was not protective (Lauvau et al., 2001). As Gray suggests, this has important consequences for the treatment of viral infection, where the persistence of antigen that may cause immunopathology, may also be required for protection (Gray, 2002).

The opposed school of thought argues that antigen is not required for maintaining immunological memory. Lau and colleagues showed that LCMV-specific T cells persisted in non-immunised mice after the adoptive transfer of CD8⁺ T cells from LCMV-immunised mice (Lau et al., 1994). Many other experiments have suggested that antigen is not required, including the maintenance of memory cells after the adoptive transfer of T cells into MHC-deficient hosts (reviewed by Antia et al., 2005).

Antia et al. (2005) classify antigen-independent mechanisms of memory maintenance into three models:

1. Bystander stimulation, based on the principle that infection also triggers the activation of non-specific “bystander cells” through the action of inflammatory cytokines (Tough et al., 1996)

2. Crossreactive stimulation, which suggests that specific T cells may be activated by cross-reactive epitopes. For example, LCMV epitope-specific CD8⁺ memory T cells were stimulated by infection with unrelated viruses such as Pichinde virus and vaccinia virus (Selin et al., 1994)
3. Homeostatic regulation of turnover: Freitas et al. (1993) first suggested that a homeostatic system must be in place to regulate the total population size of memory CD8⁺ T cells. As the body is infected with further viruses, there is competition for resources by CD8⁺ T cells which can lead to homeostatic deletion, or attrition (Gray, 2002, Selin et al., 1999). IL-7 and IL-15 have a crucial role in maintaining the memory CD8⁺ T cell pool at a constant size, and enabling cell proliferation (reviewed by Wherry and Ahmed, 2004; Antia et al., 2005). CD127, the IL-7 receptor, is expressed by memory cells that are not activated by antigen but that are maintained in the memory pool, providing evidence for the absence of antigen (Wherry and Ahmed, 2004, Huster et al., 2004)

These models are not mutually exclusive, as the activation of innate immune cells and the subsequent production of type 1 interferons can induce the secretion of IL-15 by DCs and macrophages, thereby triggering memory CD8⁺ T cell division (reviewed by Gray, 2002).

As mentioned above, homeostatic deletion of memory cells that are not exposed to antigen implies that even if memory cells are maintained in the absence of antigen, they are disadvantaged in the advent of a new infection. Hence Gray suggests that:

“Although memory might not, in experimental terms, be absolutely dependent on antigen, it is strongly potentiated by it, and in the normal,

intact immune system, antigen protects the memory-cell population from terminal attrition”

This emphasizes the importance, but not necessarily the requirement, of antigen for maintaining immunological memory (Gray, 2002).

1.4. Immune regulation

The immune system is carefully regulated so as to effectively fight infection while limiting the destruction of self. This is accomplished through the elimination of self-reactive cells as part of central tolerance and through the action of regulatory T cells in the periphery. Regulatory CD4⁺ T cells are characteristically Foxp3⁺ and can regulate the proliferation and effector functions of T cells, NK cells, NKT cells, B cells and APCs (reviewed in Sakaguchi et al., 2008, and Jiang and Chess, 2006). There are several mechanisms of suppression by regulatory T cells, which are either contact-dependent, for example, CTLA-4 expressed on these cells can inhibit the activation of effector T cells by competing with CD28 and binding to CD80 and CD86, or they can be cytokine-mediated, such as through the expression of TGF- β , IL-10 and galectin 1. The release of TGF- β induces the expression of the transcription factor Foxp3 in naïve T cells, and IL-10 which can attenuate DC function and promote the conversion of conventional T cells into regulatory T cells. The expression of galectin 1 on regulatory T cells leads to cell cycle arrest and apoptosis of conventional T cells (reviewed by Sakaguchi et al. 2010, Shevach, 2009).

CD8⁺ T cells can also have a regulatory function. These CD8⁺ suppressor T cells have an important role in the discrimination of self and non-self. T cells with a

high avidity for self-peptides are eliminated in the thymus through negative selection, allowing intermediate and low-avidity TCR-expressing T cells into the periphery. T cells with intermediate-avidity for self-antigens express higher levels of heat shock protein 60 (Hsp60). Peptides derived from this protein can bind to class 1b MHC molecule Qa-1 (HLA-E in humans). CD8⁺ suppressor cells are Qa-1-restricted and downregulate these cells. This has the indirect effect of increasing the proportion of peripheral T cells with high avidity for foreign antigens, which is beneficial for protective immunity (Chen et al., 2007; Jiang et al., 2010; Jiang and Chess, 2006).

When an immune response is excessive and uncontrolled, it can cause immunopathology, which is exemplified when Foxp3⁺ T cells are missing in immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome. Immunopathology is also at the centre of diseases such as chronic autoimmune liver disease in some patients following hepatitis B virus (HBV) infection. Dengue virus can trigger the production of antibodies that cross-react with endothelial cells and cause tissue damage (Lin et al., 2003). Over-reactive T cells can also be destructive for infected tissues, such as in liver pathology seen in chronic HCV infection (Guidotti and Chisari, 2006, Chapel, 2006).

1.5. Viral immune escape

Against this army of defenders, pathogens should have little chance of surviving. However, viruses are particularly apt at evading the immune system and establishing persistent infection. There are several ways of escape that Wherry and Klennerman (2011) classify into three groups:

- 1) immune ignorance: when the virus is able to “hide” from the immune system either by remaining in an immunoprivileged site, such as the brain, through latency (e.g. herpes viruses, Banks and Rouse, 1992), or by downregulating MHC processing pathways (eg. HBV, adenoviruses, Paabo et al., 1989). EBV is able to dampen the immune response by releasing an inhibitory cytokine that is a homologue of IL-10 (Salek-Ardakani et al., 2002)
- 2) immune escape through viral sequence mutation and functional flexibility of viral proteins such as in HCV and HIV (Altman and Feinberg, 2004)
- 3) immune exhaustion where virus-specific T cells progressively lose effector functions and their ability to proliferate (eg HCV, Urbani et al., 2006)

These mechanisms often lead to persistent viral infection and chronic disease.

2. Parvoviruses

The *Parvoviridae* family of viruses can infect vertebrates (sub-family *Parvovirinae*) and insects (sub-family *Densovirinae*) (Figure 1.3). They are non-enveloped icosahedral particles of 18-25nm in diameter, packaging around 5kb of ssDNA. Their size is reflected in their name “parvovirus”, derived from the Latin, *parvus*, for small. These viruses were first identified as the aetiology of feline diseases characterised by enteritis, panleukopenia and cerebellar ataxia (Knipe David M, 2007).

The parvovirus family has expanded considerably in the last few years. There are now four parvoviruses known to infect humans, each with several genotypes: adeno-associated virus (AAV), human bocavirus (HBoV), parvovirus B19 and PARV4 (Figure 1.3). Each family member differs in their biology, their pathology and their cellular tropism, but they share a similar genomic structure with two open reading frames (HBoV has a third, Figure 1.4 and Table 1.1) (Heegaard and Brown, 2002). Moreover, none of the parvoviruses encode a DNA polymerase and they rely on actively dividing host-cells, or the co-infection with a helper virus to replicate (Brown, 2010).

As parvoviruses use the host's DNA polymerase they are thought to be fairly stable and undergo little genetic change. Sharp *et al.* discovered PARV4 and HBoV homologues in chimpanzees and gorillas. As parvovirus infections are restricted in host species, they hypothesised that parvoviruses have co-evolved with their hosts over the 90 million years of mammalian evolution (Sharp et al., 2010a). The conservation in sequences seen in human isolates suggests a recent spread to humans (Sharp et al., 2010a, Norja et al., 2006, Manning et al., 2006).

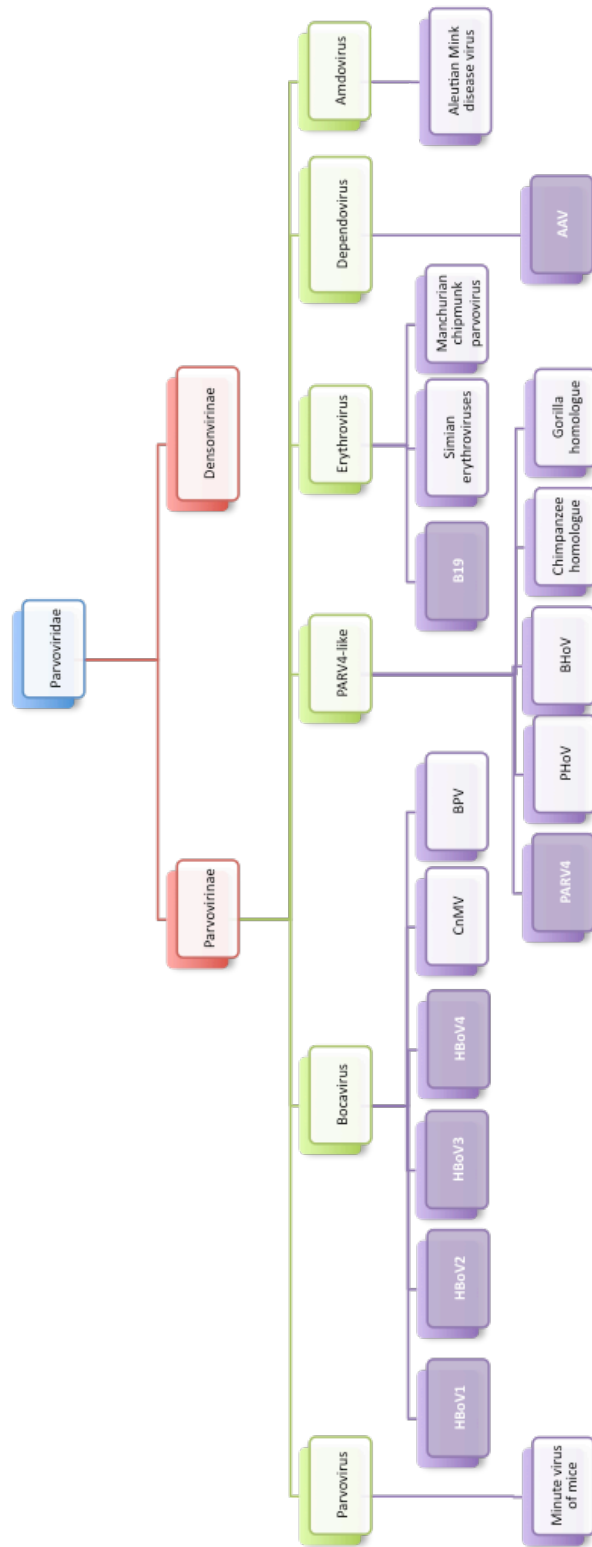


Figure 1.3 Parvovirus family tree. In blue is the family name, in red the sub-families, in green the genera and in purple the species names. Purple filled boxes are the species known to infect humans. Only viruses mentioned in this thesis are included. All Densovirinae infect insects so have been excluded from this diagram. HBoV: Human Bocavirus, CnMV: canine minute virus, BPV: bovine parvovirus, PHoV: porcine hokovirus, BHoV: bovine hokovirus, AAV: adeno-associated virus. (Designed using data from studies: Lau et al., 2008, Knipe et al., 2007, Allander et al., 2005, Kapoor et al., 2010c, Sharp et al., 2010a)

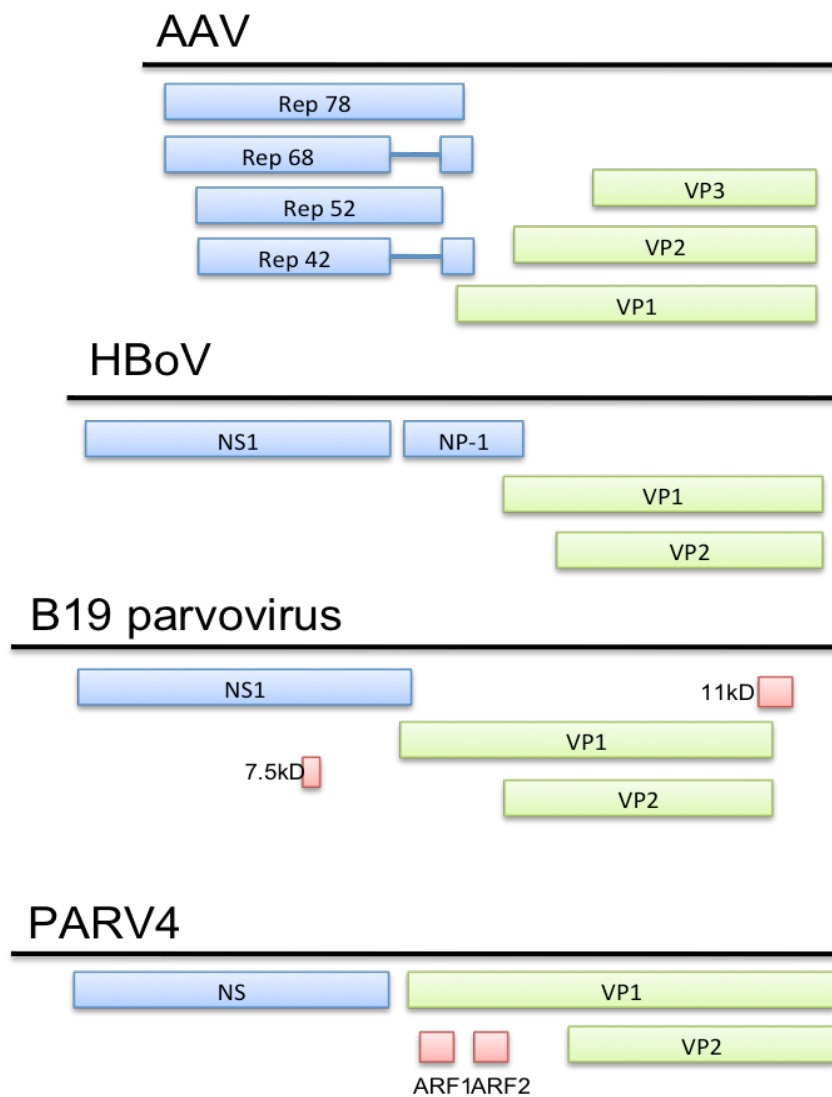


Figure 1.4 Genomic structures of human parvoviruses. Black lines represent the size of the genome, and the coloured boxes represent the proteins encoded by each genome (to scale). (Design based on references Lindner and Modrow, 2008, Corcoran and Doyle, 2004, Simmonds et al., 2008, Russell and Kay, 1999).

	Discovered	Prevalence	Pathology	Transmission
AAV	1965	70% seropositive in healthy population	None	Respiratory Mucosal Blood products
B19	1974	70% seropositive in healthy population	Fifth disease Arthropathy Hydrops fetalis Pure red cell aplasia Chronic anaemia Transient aplastic crisis	Respiratory Mother to foetus Blood products
Bocavirus	2005	Near 100% of healthy population 1.5 - 20% DNA found in children	Upper and lower respiratory tract infections and diarrhoea	Respiratory Fecal - oral
PARV4	2005	0 - 5% DNA in healthy population 85% in HIV+ IDUs >30% healthy Central African population	Unknown	Parenteral/ blood products, more?

Table 1.1 Comparison of human parvoviruses. AAV: adeno-associated virus; IDU: intravenous drug user

In the following sections each virus will be discussed in greater detail: AAV, HBoV and parvovirus B19, followed by a more extensive review of PARV4.

2.1. Adeno-associated virus

Adeno-associated viruses (AAVs) were the first parvoviruses to be detected in humans. They are members of the *Dependovirus* genus, so named because of their requirement for a helper virus to replicate, such as adenovirus or herpes virus.

2.1.1. Genome structure

AAVs package 4.7kb of ssDNA and have two open reading frames (ORFs): *rep* and *cap*, encoding several Rep proteins and VP proteins respectively (Coura Rdos and Nardi, 2007) (Figure 1.4). AAV is able to integrate the host genome as the Rep protein binds to the host chromosome and recombination occurs through shared sequences in the terminal hairpin structure (Weitzman et al., 1994). In the absence of a helper virus, only the Rep protein is expressed and there is limited DNA replication (Brown, 2010).

2.1.2. Prevalence, disease and immune response

There are 5 common human serotypes: AAVs-1, -2, -3, -8 and -9, which have different cellular tropisms as their capsids bind to different receptors (Akache et al., 2006, Wu et al., 2006, Zaiss and Muruve, 2008, Gao et al., 2004). Many studies have suggested that 80% of adults are seropositive for AAV2 (Erles et al., 1999,

Madsen et al., 2009). However recent experiments have challenged this estimate and suggested the prevalence of antibodies may be as low as 30% (Calcedo et al., 2009, Halbert et al., 2006).

AAVs are not known to cause disease, and with their selective tropism, they are very good candidates for gene therapy. They are being developed to treat a wide range of diseases (Coura Rdos and Nardi, 2007, Daya and Berns, 2008). AAVs elicit a strong humoral response, while the cellular response in humans was thought to be as small as it is in animal models (Hernandez et al., 1999, Zaiss and Muruve, 2005, Snyder et al., 1999, Chirmule et al., 1999). However, a phase I study in 2006 was limited by the host immune response to this virus (Manno et al., 2006). Moreover, recent studies have shown that AAV2 can elicit robust T cell responses, e.g. 46% of healthy donors made detectable responses to the virus, and T cell responses were cross-reactive to different serotypes (Madsen et al., 2009, Mingozzi and High, 2011, Mingozzi and High, 2007, Mingozzi et al., 2007). Several MHC class I and class II epitopes have been identified both in mice and humans (Madsen et al., 2009, Sabatino et al., 2005, High et al., 2004).

2.2. Human bocavirus (HBoV)

Named because of its sequence similarity to bovine and canine parvoviruses, homologues of human bocavirus (HBoV) have also been detected in chimpanzees and gorillas (Sharp et al., 2010a, Kapoor et al., 2010a). HBoV was discovered in 2005 and though near 100% of the population worldwide is seropositive, viral DNA is prevalent only in young children under 2 years old (Lindner et al., 2008c, Lindner et al., 2008b, Lindner et al., 2008a, Wang et al., 2010, Bastien et al.,

2007). HBoV is not often detected in adults, but has been found in several immunosuppressed individuals (Manning et al., 2006, Kupfer et al., 2006).

2.2.1. Genome structure

HBoV is a 5.2 kb ssDNA virus, made up of three reading frames that encode non-structural proteins NS and NP-1, and structural proteins VP1/VP2 (Lindner and Modrow, 2008) (Figure 1.4). HBoV has 4 genetic variants, named 'types' or 'species' 1 to 4, making it more variable than PARV4 or B19 (Arthur et al., 2009, Kapoor et al., 2010c, Kapoor et al., 2009).

2.2.2. Prevalence and disease

HBoV is found primarily within nasopharyngeal aspirates (NPA), but also in blood, urine and faeces (Pozo et al., 2007, Wang et al., 2010, reviewed by Allander, 2008). HBoV is frequently detected in conjunction with other respiratory viruses, such as respiratory syncytial virus, adenovirus and human rhinovirus. Several studies have shown HBoV is likely to be the aetiology of some upper and lower tract respiratory infections (Allander et al., 2005, Manning et al., 2006, Fry et al., 2007, reviewed by Lindner et al., 2008a). The symptoms associated with HBoV infection include pneumonia, bronchitis, cough, fever and wheezing (Lindner et al., 2008c). High viral loads have been associated with acute disease that is symptomatic, especially when HBoV is detected in peripheral blood (Allander et al., 2007, Fry et al., 2007). A peak of infection has been detected during the winter months, however this is not consistent in all studies (Arnold et al., 2006, Allander et al., 2005).

As previously mentioned, HBoV is also found in faeces, and HBoV2 and HBoV3 have particularly been associated with gastroenteritis in several publications (Arthur et al., 2009, Chow et al., 2010, Arnold et al., 2006, Vicente et al., 2007, Pham et al., 2011, Kapoor et al., 2010c), much like their animal homologues bovine parvovirus (BPV) and canine minute virus (CaMV) (Carmichael et al., 1994, Durham et al., 1985). From these data, it is assumed HBoV is transmitted through the respiratory and faecal-oral routes.

2.2.3. Immune response

HBoV antibodies have been shown to increase with age (Wang et al., 2010), and can target both NS and VP1/VP2 proteins (Shirkoochi et al., 2010). There is little data on the cellular immune response to HBoV. CD4 helper T cells responding to VP2 capsid protein are thought to be important as they are able to proliferate, produce IFN- γ , IL-10 and IL-13 when stimulated with antigen (Lindner et al., 2008c, Kumar et al., 2011). IL-13 is of particular interest because of its role in respiratory symptoms and pathology such as asthma. Correspondingly, HBoV infection has been linked to asthma exacerbation (Vallet et al., 2009).

Though only recently discovered, the prevalence of HBoV has been extensively studied, as respiratory infections are the leading cause of hospitalisation in children. However, despite all the evidence there is still some discussion as to the exact role of HBoV in these respiratory and enteric illnesses, and whether it truly is the causative agent (Martin et al., 2010, Williams, 2010). Further research is required to define the biology of this clinically significant virus.

2.3. Parvovirus B19

Parvovirus B19, also known as human erythrovirus, was discovered in 1975 and until 2005 was the only pathogenic parvovirus known to infect humans.

2.3.1. Genome structure

Parvovirus B19 is a 5.6 kb ssDNA virus composed of two ORFs. ORF1 encodes non-structural protein NS1, essential for viral replication, and ORF2 encodes capsid proteins VP1 and VP2, where VP2 makes up 95% of the capsid (Modrow and Dorsch, 2002) (Figure 1.4). VP1 and VP2 are identical except that VP1 has an additional VP1 unique region (VP1u) 227 amino acids long, which contains a phospholipase A2 domain that is essential for virus entry (Servant-Delmas et al., 2010).

Parvovirus B19 has three genotypes that differ by 13-14%. There is a geographical segregation of these genotypes with 1 and 2 (A6 and LaLi-like) mainly found in Western countries, whereas genotype 3 (V9-like) has predominantly been detected in sub-Saharan Africa and South America (Parsyan et al., 2007, Candotti et al., 2004, Sharp et al., 2010a, Sanabani et al., 2006). It has been suggested that there is a temporal evolution of the genotypes as genotype 2 is predominantly found in older people compared to genotype 1 (Norja et al., 2006). Additionally, genotype 3 is suggested to have diverged over 500 years ago, making it the oldest variant (Parsyan et al., 2007), also suggesting parvovirus B19 may have originated in Africa (Hubschen et al., 2009). Furthermore, simian homologues have been found in cynomolgus monkeys and rhesus and pig-tailed macaques, chipmunks and cows (Sharp et al., 2010a, Green

et al., 2000, O'Sullivan et al., 1994, Yoo et al., 1999). Despite these differences, all genotypes show similar functional characteristics and represent a single serotype (Ekman et al., 2007).

2.3.2. Prevalence and transmission

It is estimated that over 70% of the general adult population is seropositive for B19 (Rohrer et al., 2008, Kerr et al., 1999, Gilbert et al., 2005). Seroprevalence increases with age with 2-10% in under 5 year olds, 40-60% in adults over 20 years, reaching 85% in the elderly (Servant-Delmas et al., 2010). B19 is transmitted mainly through respiratory droplets, but can also be passed on through blood products or from mother to foetus. Infection is most frequent during the winter and spring, and peaks every 3 to 4 years (Brown and Young, 1997).

B19 replicates in the bone marrow in progenitor erythroid cells, as it uses the host cell machinery to replicate (Young and Brown, 2004). The virus enters the cell through structural protein VP binding to globoside (erythroid P antigen) on the surface of the cell: individuals who lack P antigen are resistant to B19 infection (Brown et al., 1994). Integrin complex $\alpha 5\beta 1$ and Ku80 are also required for cell entry (Munakata et al., 2005, Weigel-Kelley et al., 2003). B19 is also able to enter non-permissive cells, and can become cytotoxic through an excessive production of NS1 (Liu et al., 1992). The NS1 protein induces IL-6 expression, and has an endonuclease activity thought to be critical for viral replication and the induction of apoptosis in infected cells (Young and Brown, 2004, Modrow and Dorsch, 2002). Apoptosis is initiated through the activation of the TNF

receptor signalling pathway and several caspases (Sol et al., 1999, Moffatt et al., 1998). Death of the host cell is the major contributor to B19 pathology: as more erythroid progenitor cells are infected and destroyed, ultimately this reduces the number of erythrocytes produced.

2.3.3. Disease

30% of individuals infected with B19 remain asymptomatic, whilst most suffer a mild short-lived illness (reviewed by Brown and Young, 1997). Disease is manifested through blocking erythropoiesis and induction of inflammation, the consequences of which depend on the immune status of the host. In the healthy individual, if infection is not self-limiting and asymptomatic, B19 can cause arthropathy in adults and erythema infectiosum, or Fifth disease, in children (Corcoran and Doyle, 2004, Young and Brown, 2004). In an individual with a hematologic disorder it can cause transient aplastic crisis, and in an immunocompromised patient it is responsible for chronic anaemia, pure red cell aplasia or even hydrops fetalis and foetal death (Corcoran and Doyle, 2004, Young and Brown, 2004). Adding to the risks associated with parvovirus B19 is its transmission via blood products, and the clinical groups mentioned above are specifically prone to require transfusions. In response to this, the FDA and the Council of Europe have established mandatory nucleic acid testing and a maximum viral titre allowed, to limit B19 transmission (Council of Europe, 2005). This improvement is reflected in the recent decrease of B19 DNA levels in blood products (Fryer et al., 2007c, Modrow et al., 2011).

A few studies have also linked B19 to meningitis, encephalitis, myocarditis and hepatitis (reviewed by Young and Brown, 2004). B19 has been implicated in several autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE). It is thought this is mainly through molecular mimicry. Peptides derived from VP1 and VP2 protein may trigger the production of cross-reactive auto-antibodies able to recognise keratin, collagen and cardiolipin (Lunardi et al., 1998, Lunardi et al., 2008).

Initially, B19 was thought to be a typical acute virus that could be rapidly cleared by the humoral immune system. However, many studies have now shown that the virus persists in tissues such as bone marrow, skin, liver, brain, myocardium and synovium, years after symptoms have cleared (Corcioli et al., 2008, Lefrere et al., 2005, Manning et al., 2007). Notably, mRNA was not detected in most of these tissues, except a couple in bone marrow. This implies the virus is not actively replicating, but in a latent phase (Corcioli et al., 2008).

2.3.4. The immune response to B19

The humoral response to parvovirus B19 is well characterised, and is critical in the acute phase of infection. The production of neutralising antibody against VP1 and VP2 correlates with the disappearance of viral DNA from blood. VP1u is an immunodominant site for antibodies (Zuffi et al., 2001, Musiani et al., 2000). Anti-B19 IgG provides protection against reinfection (reviewed by Young and Brown, 2004) and immunoglobulin therapy may be beneficial to infected individuals (Schwarz et al., 1990).

Th1 cytokines (IL-2, IL-12, IL-15) are the most dominant in acute infection. Several pro-inflammatory cytokines (IL-1 β , GM-CSF, IL-6, TNF α , IL-8) are also elevated within the first few weeks of infection (Isa et al., 2007).

The role of T cells is evident in immunosuppressed individuals who present with exacerbated disease (Heegaard and Schmiegelow, 2002). In healthy asymptomatic individuals, the highest and most frequent T cell responses are to NS1, whereas in persistently infected individuals (with B19 DNA in their bone marrow) most responses are against VP proteins. The authors suggest the lack of NS1 responses in the persistently infected may be due to cellular exhaustion due to continuous antigen exposure (Isa et al., 2006).

In general, CD4⁺ T cell responses tend to target VP1/2 epitopes, while CD8⁺ T cells focus on NS1 peptides. Both acutely infected (serum IgM⁺) and remotely infected (serum IgM⁻ IgG⁺) individuals elicit broad CD4⁺ T cell responses to VP proteins, although the responses in remotely infected individuals were only detected after short-term T cell culture (Kasprowicz et al., 2006, Franssila et al., 2001). An immunodominant HLA-DRB1*1501-restricted epitope has been identified and is targeted by many acute and remotely infected individuals (Kasprowicz et al., 2006). These antigen-specific CD4⁺ T cells are CD62L⁺ CCR7⁺, thus have a central memory phenotype. Two studies found that the production of IFN- γ by CD4⁺ T cells was reduced in acutely infected individuals and in children (Isa et al., 2007, Corcoran et al., 2000).

CD8⁺ T cell responses to B19 are usually strong, with up to 4.5% of CD8⁺ cells found to be epitope-specific in acutely infected individuals (Isa et al., 2005). However these cells have a different phenotype to B19-specific CD4⁺ T cells as they display an activated effector phenotype (CD38⁺ CD57⁺ perforin⁺ and

CD62L^{lo} CCR7⁻). There are 9 known B19 CD8⁺ T cell epitopes (Tolfvenstam et al., 2001, Norbeck et al., 2005, Isa et al., 2005, Kasprowicz, 2005), notably HLA-A*2402 restricted FYTPLADQF which uses a highly conserved TCR (BV5.1), which is seen in other persistent viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), or during continuous antigenic exposure, such as with *Influenza* (Wang et al., 1995, Annels et al., 2000, Lawson et al., 2001). Both CD4⁺ and CD8⁺ T cell responses are long-lived and detectable several decades after the resolution of symptoms. The level of response and the phenotype of these cells are similar to other persistent viruses such as CMV (Kasprowicz et al., 2006, Isa et al., 2005, Franssila et al., 2001).

AAV, HBoV and B19 are all similar in the way they are transmitted and their requirement for actively dividing cells to replicate. However, their clinical significance varies (Table 1.1). These members of the *Parvoviridae* family may shed light on the biology of less-well characterised member PARV4.

3. PARV4

PARV4 was identified in plasma in 2005 while looking for new uncharacterised DNA viruses in patients with acute viral infection symptoms, at risk of carrying HIV-1 (Jones et al., 2005). The patient carrying PARV4 was an intravenous drug user (IDU), hepatitis B virus (HBV) positive, HIV-1 negative and his symptoms included: fatigue, night sweats, pharyngitis, neck stiffness, vomiting, diarrhoea, arthralgias and confusion (Jones et al., 2005). tBLASTx searches linked the viral sequence to non-structural and capsid proteins of other parvoviruses, and showed PARV4 to have 45% nucleotide identity with parvovirus B19 (Fryer et al., 2007c).

PARV4 particles have been visualised using immune electron microscopy and are approximately 20-22nm in diameter (Tuke et al., 2009) (Figure 1.5). Below, the genome, prevalence, viral transmission, disease pathology and the immune response to PARV4 are discussed in more detail.

3.1. Genome

3.1.1. Genome structure and protein function

The PARV4 genome of 5.6kb ssDNA is composed of two ORFs and two alternative reading frames (ARFs, Figure 1.4). ORF1 is 1992 nucleotides long, encoding 664 amino acids, and is homologous to non-structural (NS) proteins in the other human parvoviruses.

*For copyright reasons this figure has been removed.
It can be found in:
Figure 1 from Tuke et al., 2009*

Figure 1.5 Immune electron micrograph of PARV4 particles seen in plasma. This sample was from a HCV antibody-negative, HCV RNA-positive individual with a PARV4 viral load of 5×10^8 DNA copies/ml. Horizontal bar represents 100nm. (From Tuke et al., 2009)

ORF2 is 2745 nucleotides long, encoding 915 amino acids and is homologous to capsid proteins (VP1 and VP2) in the other parvoviruses. VP1 and VP2 are identical in sequence, except that VP1 has a unique N-terminal region of an additional 362 amino acids (Sharp et al., 2009). These two ORFs do not overlap like in parvovirus B19 but are separated by approximately 103-107 nucleotides depending on the genotype (Fryer et al., 2007a, Simmonds et al., 2008). VP1 is also thought to be larger, around 100kDa compared to 90kDa for VP1 proteins in the other human parvoviruses (Lau et al., 2008).

The NS protein contains parvovirus-conserved motifs associated with rolling circle replication and ATPase (Fryer et al., 2007a, Ding et al., 2002, Astell et al., 1987). Zadori *et al.* showed that parvoviruses require a phospholipase A2 enzymatic activity to be infectious (Zadori et al., 2001). The sequence motifs are YxGxG and HDxxY and can be found in PARV4 VP protein. However, the functions of these proteins are still unclear.

Very little is known about the entry, replication and exit of PARV4. The first stumbling block is that its tissue tropism is unknown, let alone the cell type or receptor it may bind to. However, the process of replication can be speculated from extensive studies on animal parvoviruses and B19. Parvoviruses enter the cell by endocytosis and are transported close to the nucleus in endosomes (Bartlett et al., 2000). The pH change in late endosomes causes the capsid to change conformation and expose the phospholipase A2 domain on the Vp1u region. This enzyme can cleave the endosomal membrane, thereby delivering the virus to the nuclear periphery (Harbison et al., 2008, Servant-Delmas et al., 2010). Some parvoviruses also contain a nuclear localization signal (NLS) which allows them to move into the nucleus, although this has not been studied in B19,

neither does there appear to be a clear NLS sequence in the PARV4 NS protein (based on sequence motifs from Yang et al., 1992 and Vihinen-Ranta et al., 1997). Parvovirus B19 protein NS1 recruits cellular transcription factors and has an endonuclease and helicase function important in viral replication (Servant-Delmas et al., 2010). NS1 also has a role in apoptosis as it interacts with the TNF receptor signaling pathway, triggering the activation of caspases 3 and 6 (Servant-Delmas et al., 2010). None of these processes have been studied in PARV4.

The two ARFs are found in the ORF2 sequence, in a region of suppression of variability (Figure 1.6) (Simmonds et al., 2008). ARF1 and ARF2 are 67 and 86 amino acids respectively, and are conserved across all three PARV4 genotypes. ARF1 encodes a protein with sequence similarity to the small alternatively translated (SAT) protein found in other parvoviruses such as minute virus of mice (Simmonds et al., 2008). This SAT protein was also identified in PARV4-like viruses in pigs, with little variation within each virus group, but significant differences between different host species. The role of SAT proteins is unknown, however it is speculated they are toxic to cells, and may cause cell lysis thereby allowing viral spreading (Zadori et al., 2005).

The closest sequence homologue to PARV4 is found in the chimpanzee, where ORF1 and ORF2 differ by only 18% and 15% in nucleotides, respectively. This corresponds to 9% and 7% in amino acid sequence (Sharp et al., 2010a). The next closest in sequence homology are porcine and bovine hokoviruses PHoV and BHoV, with 40% divergence with ORF1 and 35% with ORF2 (Adlhoch et al., 2010, Lau et al., 2008, Szelei et al., 2010). The close relationship in sequence between these viruses justifies their classification into a new genus.

*For copyright reasons this figure has been removed.
It can be found in:
Figure 2 from Simmonds et al., 2008*

Figure 1.6 Pairwise comparison of genotypes 1, 2 and 3 (genotype 3 is represented by isolate NG_OR). There is a significantly more conserved area between the three genotypes around the two alternative reading frames ARF1 and ARF2. (From Simmonds et al., 2008)

3.1.2. Genotypes

PARV4 sequences are divided into three genotypes that differ in nucleotide sequence by 8.5% and 6.6% in NS and VP respectively (Simmonds et al., 2008), similar to differences seen between B19 genotypes (Servant et al., 2002, Hokynar et al., 2002). Substitutions are confined mainly to synonymous sites, making the amino acid differences much smaller: 2.8% and 1.7% divergence for NS and VP respectively (Simmonds et al., 2008, Szelei et al., 2010) (Figure 1.6). Pairwise comparison suggested the genotypes were not the product of recombination, unlike that seen in the HBoV genotypes (Simmonds et al., 2008).

A temporal succession between genotype 1 and 2 (previously known as PARV5 Fryer et al., 2007a) has been suggested, also seen for B19 parvovirus (Fryer et al., 2007c, Schneider et al., 2008b, Manning et al., 2007). There may also be a difference in geographical distribution, as is seen in HCV genotypes: genotypes 1 and 2 are found mainly in Western countries, whereas genotype 3 has only been detected in sub-Saharan Africa (Panning et al., 2010, Simmonds et al., 2008, Fryer et al., 2007a, Botto et al., 2009) (Figure 1.7). Based on the high substitution rate for parvoviruses reported by Shackelton et al. (2006) and the highly conserved PARV4 isolates identified, Manning *et al.* proposed a very recent origin for PARV4 in IDUs with a divergence in the last 20 years (Manning et al., 2007). It is also possible that, due to the small size of PARV4, it cannot afford to mutate much before losing vital functions.

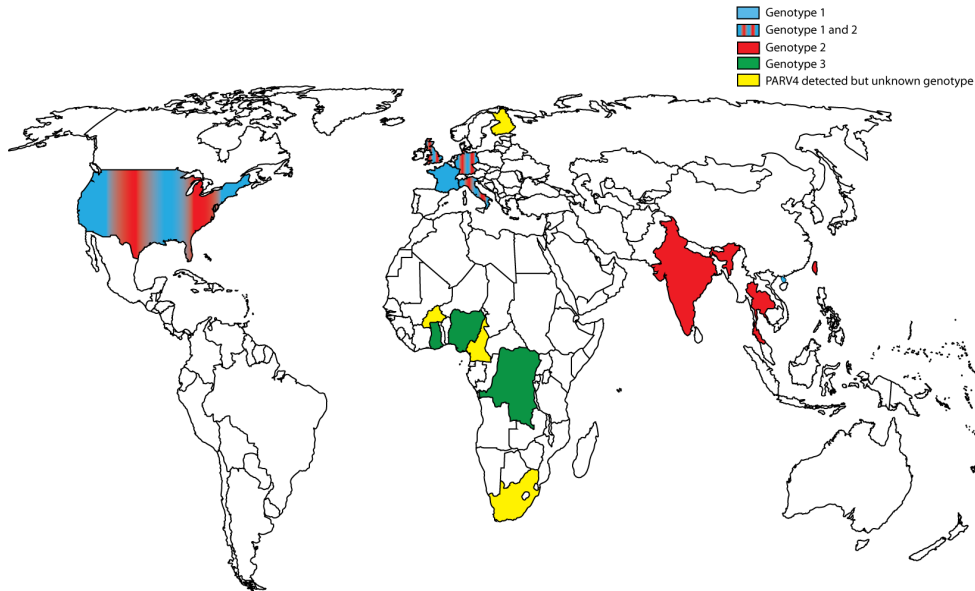


Figure 1.7 Geographical distribution of PARV4 genotypes. This map was created using a blank template (Adobe, 2009) and references: Yang et al., 1992, Lau et al., 2008, Lurcharchaiwong et al., 2008, Touinssi et al., 2011, Corcioli et al., 2010, Panning et al., 2010, Tuke et al., 2009, Botto et al., 2009, Biagini et al., 2008, Simmonds et al., 2008, Schneider et al., 2008a, Schneider et al., 2008b, Vallerini et al., 2008, Fryer et al., 2007c, Fryer et al., 2007a, Manning et al., 2007, Longhi et al., 2007, Fryer et al., 2006, Fryer et al., 2007d, Benjamin, 2011.

3.2. Tissue tropism

PARV4 has been found in the plasma, which suggests there is a viremic phase during which viral DNA can spread to different organs (Szelei et al., 2010). The exact tissue tropism of PARV4 is unknown, but PARV4 DNA has been found in many tissues.

PARV4 DNA was detected in 8%, 15%, 46% and 71% of bone marrow and lymphoid samples of HIV-infected patients in different studies, but was absent from bone marrow samples of HIV-negative individuals (Corcioli et al., 2010, Manning et al., 2007, Longhi et al., 2007, Simmonds et al., 2008).

Another study found 8 positive liver samples from individuals that were not viremic. This same cohort showed one PARV4-positive bone marrow sample but the liver sample from the same patient was negative (Schneider et al., 2008b). A further study, looking at the presence of PARV4 DNA in different organs determined that the liver and myocardium were the most frequently infected (7/17 [41%] autopsy samples from HIV-negative individuals). Additionally, viral DNA has been found in the skin, synovium, cerebrospinal fluid (CSF), lung and kidney but is absent from respiratory samples, urine, peripheral blood mononuclear cells (PBMCs) and brain (frontal or occipital lobe) (Manning et al., 2007, Corcioli et al., 2010, Manning et al., 2006, Botto et al., 2009, Vallerini et al., 2008, Benjamin, 2011).

Corcioli *et al.* suggest that the bone marrow is unlikely to be the site of replication as they found 5 individuals who had PARV4 DNA in their bone marrow who were not viremic, implying that virus may simply persist in the bone marrow. The absence of PARV4 in urine and bronchial lavages suggests the

virus does not shed through the kidney or lung (Corcioli et al., 2010, Botto et al., 2009). Together, these studies suggest that PARV4 is able to infect several organs, but the site of replication remains unknown.

3.3. Prevalence and transmission of PARV4

This section presents the prevalence of PARV4 in several risk groups, from HCV- and HIV-infected IDUs to healthy individuals, and how this provides clues on the transmission routes of PARV4.

3.3.1. PARV4 and parenteral transmission

a. HCV-infected individuals

PARV4 is most frequently found in IDUs, often co-infected with HCV. PARV4 DNA or antibodies have been found in 8-33% HCV⁺ IDUs (median 22%) (Simmonds et al., 2007, Lurcharchaiwong et al., 2008, Fryer et al., 2007d, Sharp et al., 2009). HCV⁺ haemophiliacs and haemodialysis patients show similar rates with 27% PARV4 prevalence (Sharp et al., 2009, Touinssi et al., 2011). Additionally, in large screens of individuals, PARV4 positive samples were predominantly from HCV⁺ individuals, ranging from 54% to 71% (Schneider et al., 2008b, Lau et al., 2008, Tuke et al., 2009). This trend was broken by one study in France that found that out of 93 blood donors infected with HCV, none were PARV4 viremic (Servant-Delmas et al., 2009). The authors suggest that geographical differences may exist, but also that a serology assay may be more sensitive. Indeed, the highest values have been detected using antibody screening assays (Sharp et al., 2009). This

low rate may also be due to the nation-wide selective exclusion of IDUs from blood donations, which may have skewed the results (Etablissement Francais du Sang, 2011).

b. HCV- HIV-coinfected individuals

When individuals are co-infected with HCV and HIV-1, the rate of PARV4 infection is consistently high: HIV-1-HCV co-infected haemophiliacs were 55% seropositive, while 65% to 85% of IDUs were infected with PARV4 (median 74%) (Yang et al., 2011, Sharp et al., 2009, Longhi et al., 2007, Simmonds et al., 2007, Lahtinen et al., 2011). The only exception was seen in Thailand, where only 3% of the HIV⁺ HCV⁺ IDUs were PARV4 viremic; this might have been because the level of viral DNA was below the threshold of detection, as PARV4 DNA is harder to detect than PARV4 antibodies (Lurcharchaiwong et al., 2008).

c. Parenteral transmission

The frequent co-infection of PARV4 in HIV-1 and HCV patients strongly suggests a shared route of transmission. PARV4 is consistently found in IDUs and the highest prevalence is found in HIV-HCV co-infected IDUs (85%) likely reflecting higher exposure. This increased use of shared needles may also explain the high IgM levels observed in some studies (Yang et al., 2011, Lahtinen et al., 2011). There is another possible explanation to these rates: PARV4 may reactivate or only persist in immunosuppressed patients, thereby explaining the higher prevalence in HIV-1 patients. However, Manning *et al.* showed that PARV4 viral load is not affected by the level of immunosuppression, as measured by CD4⁺ T

cell counts (Manning et al., 2007). The absence of PARV4 in several immunosuppressed HIV⁺ MSM groups also excludes the possibility of permissive infection due to a weakened immune system (Simmonds et al., 2007). Parenteral transmission is also supported by the high prevalence of PARV4 in haemophiliacs (Simmonds et al., 2007, Sharp et al., 2009).

3.3.2. HIV⁺ individuals and sexual transmission

PARV4 is also associated with HIV-1 infection, with three studies reporting variable rates in HIV⁺ MSM cohorts (0%, 24% and 71%) (Simmonds et al., 2007, Sharp et al., 2009, Yang et al., 2011). Again, the higher levels were detected through the use of serological assays. Other cohorts reported between 15% and 20% PARV4 DNA, and 0% to 59% PARV4 IgG in HIV⁺ heterosexuals or MSMs (Longhi et al., 2007, Sharp et al., 2009, Yang et al., 2011, Simmonds et al., 2008). Sharp *et al.* also found that 36% of blood donors in South Africa who were HIV⁺ were also PARV4 seropositive (Sharp et al., 2010b).

As PARV4 is found in individuals with no parenteral risk, there is evidence that the virus could be transmitted sexually. One group found two HIV⁺ heterosexual patients who were PARV4⁺, whose only risk came from unprotected sexual exposure (Simmonds et al., 2008). Additionally, a recent study in Taiwan found 71% PARV4 IgG in HIV⁺ MSM (non-IDUs), a level on a par with HIV-HCV co-infected IDUs. However, it is possible this was due to unreported IV drug use (Yang et al., 2011). With lower levels in HIV⁺ MSM in Western countries, sexual exposure was not thought to be a primary route of transmission, but cannot be excluded.

3.3.3. Transmission through blood products

B19 is principally transmitted through the respiratory route. PARV4 was absent from both samples from siblings of PARV4-infected haemophiliacs living in the same household, and from respiratory aspirates (Manning et al., 2006, Botto et al., 2009, Sharp et al., 2009). Hence PARV4 is not thought to be transmitted respiratorily. B19 can also be transmitted through blood products, and there is evidence this may also be a problem with PARV4.

a. PARV4 is common in blood products

Due to their small size and lack of envelope, parvoviruses are relatively resistant to inactivation methods such as solvent/detergent or heating (Fryer et al., 2007b). Several studies have reported the presence of PARV4 in blood products. Initially the level of contamination appeared to be relatively low (7/137 plasma pools, Fryer et al., 2006), and multiply transfused patients or haemodialysis patients did not appear to be infected with PARV4 (Biagini et al., 2008, Servant-Delmas et al., 2009). However further research indicated these levels could rise to 16% in factor VIII concentrated products (Fryer et al., 2007c), to 21% in some commercially available plasma pools (Fryer et al., 2007b), and 33% in multiple coagulation factor concentrates (Schneider et al., 2008a).

b. A problem in transplantation and transfusion

Touinssi *et al.* also found that in another cohort, an average of 31% haemodialysis patients were infected with PARV4 (range 0-61%) suggesting transfusion patients are at high risk (Touinssi et al., 2011). One study looked at

commercially available factor VIII concentrates from pigs and found 100% prevalence of a PARV4-like homologue (Szelei et al., 2010). With an increasing need and practice of xenotransplantation, it is essential to study these viruses that may become a problem to the receiving patient. Mueller *et al.* discuss PARV4, along with herpesviruses and hepatic viruses, as potential threats in transplantation from pigs to humans (Mueller et al., 2011).

There is evidence that PARV4 may be transmitted during transplantation. Kidney and lung transplant recipients were found to have 3% and 14% PARV4 viremia respectively, with evidence this infection may have occurred during transplantation (Biagini et al., 2008, Touinssi et al., 2011). The risk is increased during transplantation due to the possibility of virus reactivation.

Additionally, it has been recently shown that many viruses previously thought unable to cross the species barrier may do so, as human herpesviruses are capable of productive infection of porcine cells (Kim et al., 2010).

The presence of virus in blood products is also a problem for parvovirus B19. However, naturally occurring high levels of anti-B19 neutralising antibodies in plasma abrogated part of the problem by inactivating the virus (Brown and Simmonds, 2007). With little exposure of the population to PARV4, neutralising antibodies are unlikely to be common.

c. Detection assays for blood safety

Nucleic acid amplification techniques (NAT) to detect parvovirus B19, carried out by manufacturers since 2004, are sequence-specific and may miss different genotypes of the same virus (Baylis et al., 2004). There is no such practice for

PARV4, and PARV4 DNA has been difficult to detect in peripheral blood in comparison to other tissues, warranting the use of different assays, such as the serology detection developed by Sharp *et al.* (Schneider *et al.*, 2008b, Manning *et al.*, 2007, Lau *et al.*, 2008, Sharp *et al.*, 2009).

In the 1980s and 1990s new safety regulations, such as excluding high-risk populations, were implemented in response to the HIV-1 and HCV epidemics, leading to safer blood products. This is reflected in a study where PARV4 DNA was found in 23% of factor VIII products with an expiry date pre-1990 compared to 2% of products from post-1990 (Fryer *et al.*, 2007c).

Recent data suggests that B19 is more sensitive to heating in certain liquid preparations and to lower pH compared to other animal parvoviruses (Blumel *et al.*, 2002). Inactivation occurs not through capsid disintegration but through loss of viral DNA (Mani *et al.*, 2007). The effect of these treatments on PARV4 is unknown. Nonetheless Fryer *et al.* show that PARV4 DNA was only detectable in 8% of inactivated manufactured factor VIII products compared to 20% untreated, thereby suggesting that PARV4 may be sensitive to inactivation treatments (Fryer *et al.*, 2007c). Schneider *et al.* presented a comparative study of several coagulation factors inactivated in different ways, showing the efficiency at eliminating PARV4. Pasteurisation at 60°C was particularly effective (Schneider *et al.*, 2008a).

No PARV4 was detected in 78 multiply transfused patients, nor in a recent large-scale study of plasma pools representing 69,632 blood donations (Servant-Delmas *et al.*, 2009, Eis-Hubinger *et al.*, 2010). PARV4 was also absent from coagulation factors manufactured between 2007 and 2008 (Modrow *et al.*, 2011). These results reflect recent improvements in the safety of purification

methods. In conclusion, although the risk is limited, with little knowledge on the biology and clinical significance of PARV4, blood products should be monitored for the safety of those receiving them.

d. PARV4 and HBV-infected individuals

Few studies have looked at the co-infection of PARV4 with HBV, even though PARV4 was initially identified in an individual infected with this hepatic virus. Two groups have found that PARV4 was absent from plasma and nucleic acid extracts from HBV-infected individuals (Lurcharchaiwong et al., 2008, Eis-Hubinger et al., 2010). Nonetheless, another study did find that 3 out of 8 of the PARV4 positive samples were HBV positive, and 39% of HBV+ haemodialysis patients were also PARV4 viremic (Tuke et al., 2009, Touinssi et al., 2011). However, this was part of a study that found similar rates in HCV+ haemodialysis patients, and may simply reflect the transmission route of PARV4 through treatment.

3.3.4. PARV4 in healthy individuals

a. PARV4 is absent from the general population in the West

PARV4 DNA is essentially absent from the general healthy population in Western countries: the average prevalence is 1.8% ranging from 0 to 5.1% (median 1%) (Fryer et al., 2006, Fryer et al., 2007b, Simmonds et al., 2007, Lurcharchaiwong et al., 2008, Vallerini et al., 2008, Servant-Delmas et al., 2009, Biagini et al., 2008, Tuke et al., 2009, Botto et al., 2009). This was also corroborated in serology studies developed in 2009, with 0% PARV4 IgG found in 3 different cohorts

(Sharp et al., 2010b, Lahtinen et al., 2011, Sharp et al., 2009). A study in France was the exception to this trend as PARV4 DNA was found in 24% of blood donors in South East France (Touinssi et al., 2010). These results are questionable though, as the real-time PCR assay was repeated with a different probe, resulting in only 5% prevalence.

b. The unusual cases in Taiwan, central Africa and India

Until recently, studies across the world had shown that PARV4 was virtually absent from the general population. Nonetheless, a study in Taiwan revealed 24/55 healthcare workers (44%) had detectable anti-PARV4 IgG in their blood. This high rate implies possible occupational exposure.

In 2010, Panning *et al.* published that 8.6% of a cohort of 279 infants from Ghana had detectable PARV4 viremia (Panning et al., 2010). The study showed that there was a lower risk of PARV4 infection for children who had access to a kitchen (as an indicator of higher household hygiene) and who didn't live close to a river. The rate was also lower if the child's water source was a pipe rather than a well, pump or borehole, however this did not reach statistical significance. PARV4 was also more prevalent in older children (median 24 months), thereby excluding the possibility of prenatal or perinatal transmission. The authors suggest PARV4 may be food-borne or transmitted through contact with contaminated objects (Panning et al., 2010). The high prevalence of PARV4 homologues found in non-human primates could also be explained if PARV4 was transmitted through an environmental source (Sharp et al., 2010a). Sharp *et al.* suggest that arthropod transmission is unlikely as DNA viruses tend to be

species specific, and furthermore, no other parvoviruses are known to be carried by arthropods (Sharp et al., 2010b).

A second study within the same year also found that 25-37% of the general adult population in Cameroon, the Democratic Republic of the Congo and Burkina Faso were PARV4 IgG seropositive (Sharp et al., 2010b). Parenteral exposure was excluded as a risk factor. It is possible that PARV4 is transmitted in a similar way to HBV in Africa, through saliva or small traces of blood (Kiire, 1996). The data on PARV4 to date suggest that it may have a different epidemiological pattern in Africa. This is supported by the PARV4 sequences found in Ghana clustering with the first isolated genotype 3 sequence, identified in a Nigerian woman (Simmonds et al., 2008, Panning et al., 2010). Moreover, the sequences from two neighbouring villages in Ghana formed a monophyletic subclade which suggests the local epidemic spread of PARV4 (Panning et al., 2010). A recent study that found PARV4 in the cerebrospinal fluid of two children in India (discussed in section 3.4.2) also supports other transmission routes than through needle use, that are not restricted to Africa.

In conclusion, whereas PARV4 is clearly transmitted mainly through parenteral exposure in the West, sexual, occupational and/or environmental exposure are proposed to play a more predominant role in Taiwan, India and Africa. With the high rates of circulation of PARV4 in non-human primates (higher than in the human population), it is possible PARV4 originated in Africa, much like HIV-1, although their epidemiological patterns are evidently different.

3.4. Persistence and disease

3.4.1. Persistence

Many parvoviruses are rapidly cleared from the body. However B19 establishes life-long persistence, and the presence of viral DNA detected in autopsy tissue suggests PARV4 may do the same. In lung transplant recipients PARV4 DNA was detected in plasma from day 0 when the transplant occurred, to 1097 days later (median 206 days over 14 patients) suggesting PARV4 does persist over time (Touinssi et al., 2011). Viral antigen may persist in tissue rather than in plasma as several studies have found viral DNA in solid tissue, but none in peripheral blood (Longhi et al., 2007, Heegaard et al., 2002, Norja et al., 2006, Manning et al., 2007, Isa et al., 2006, Schneider et al., 2008b).

3.4.2. Disease

There is no clear link yet between PARV4 infection and disease. This has largely been made difficult to investigate due to the complications of co-infection with HCV and HIV-1.

a. PARV4 in transplantation and transfusion

A recent study looking at the prevalence of PARV4 in diverse tissues suggested focussing the investigation of the clinical significance of PARV4 on heart and liver diseases, as PARV4 DNA was most frequently detected in these organs (Corcioli et al., 2010).

PARV4 has been detected in plasma and solid tissue in the context of transplantation in several individuals. Schneider *et al.* (2008b) showed that 12

out of 13 PARV4 positive samples were from livers removed during transplant, and the last was from autopsy tissue. Reasons for transplantation were cirrhosis and/or primary HCC (hepatocellular carcinoma), especially due to chronic HCV infection. Additionally, 33% of immunosuppressed patients were PARV4 viremic as opposed to 5.5% in immunocompetent individuals suggesting reactivation of the virus may occur. This leads to speculate whether PARV4 contributed to disease progression.

However, no difference was seen in the clinical outcome between PARV4-positive or negative individuals having had a lung transplant (Touinssi et al., 2011). PARV4 DNA was detected in 31% of haemodialysis patients, but the authors found no correlation between viremia and the duration of dialysis or other clinical markers (Touinssi et al., 2011). Additionally, the presence of hepatic viruses HBV and HCV did not alter the PARV4 viral titre, which may reflect the control of viruses by the immune system of the host (Touinssi et al., 2011). The absence of PARV4 in immunosuppressed HIV⁺ MSMs in another study suggests that a deficient immune system does not allow preferential infection (Simmonds et al., 2007).

b. PARV4 may be a subclinical infection

The study on Ghanaian infants looked for signs of acute infection, including fever, rash or myalgia (Panning et al., 2010). None of these symptoms were detected around the time of sample collection. Moreover, in IDUs from another cohort, no differences in symptoms were observed 6 months prior to interview between PARV4 seropositive and seronegative individuals, whereas there was

an observed increase in persistent drug injection, lending of injecting equipment, history of imprisonment and unemployment in PARV4 seropositives (Lahtinen et al., 2011). A comparison between HIV-infected IDUs pre-AIDS and with AIDS (55% versus 85% PARV4 DNA respectively) suggested that even though individuals with AIDS were more prone to PARV4 infection, this was most likely due to an increased exposure to the virus.

216 'febrile' individuals either MSM or IDU, who were suffering from symptoms associated with acute HIV-1 (but tested HIV-1 RNA negative), were studied to look at PARV4 disease. 6% of this cohort was PARV4 viremic, compared to 2% of a healthy cohort. Again, this difference was put down to higher exposure to the virus rather than a correlation with disease. Similarly, the two patients in whom PARV4 genotype 3 was isolated presented with a number of symptoms, including pneumonia and pancytopenia. However, these were most likely due to the AIDS they had acquired (Simmonds et al., 2008).

Out of 84 individuals of an Italian cohort with acute viral symptoms, 1 was PARV4 viremic. This patient had Wegener granulomatosis and died of multiple organ failure (Vallerini et al., 2008). As an isolated case, it is difficult to make any correlation.

By the creation of excessive immunocomplexes, parvovirus B19 can affect the skin and joints. Botto *et al.* compared pathological skin samples from 105 patients with dermatological diseases with healthy skin donations. They found 22% of the diseased skin contained PARV4 DNA, compared to only 8% in healthy donors, however this difference did not reach statistical significance ($p=0.08$)(Botto et al., 2009).

Without larger cohorts to confirm any associations, these data and a number of studies that have detected PARV4 in otherwise healthy individuals, suggest that PARV4 infection may be clinically silent (Fryer et al., 2007b, Fryer et al., 2006, Simmonds et al., 2007, Vallerini et al., 2008, Biagini et al., 2008, Sharp et al., 2009, Lurcharchaiwong et al., 2008, Tuke et al., 2009). The recent high prevalence reported in the general population of central Africa agrees with this (Sharp et al., 2010b).

c. PARV4 and encephalitis

PARV4 has recently been detected in the cerebrospinal fluid (CSF) of two children with a suspected acute central nervous system (CNS) infection. They presented with a febrile illness lasting under two weeks and symptoms including severe headache, altered mental status, seizures and focal neurologic signs (Benjamin, 2011). The authors determined through the detection of viral DNA and IgM that these children were acutely infected with PARV4, which was not present in a control population. They suggest PARV4 may be the causative agent of some cases of encephalitis.

There are many pieces of data concerning PARV4 and disease. However, there is still no clear association between PARV4 and symptoms; the majority of evidence suggests PARV4 is a subclinical infection, although anecdotal evidence suggests a possible role in CNS disease. Most studies suffer from low numbers of study participants hindering the generation of solid statistics. Further investigation in larger cohorts is required to shed light on this issue.

3.5. PARV4 and the immune system

The in-depth study of the humoral response and the high cellular responses to parvovirus B19 suggest a potentially important role for the immune response in PARV4 infections. Until 2009, publications relating to PARV4 were only concerned with detecting viral DNA. Sharp *et al.* then developed an ELISA to detect PARV4 antibodies in human plasma (Sharp et al., 2009). This assay was employed to effectively determine the prevalence of PARV4 in various risk populations, but was not used to study the humoral response to PARV4.

A recent study looked at longitudinal plasma samples in two individuals that were PARV4 IgM positive. The patients seroconverted for PARV4 during the course of the study and showed increasing levels of IgG over time (1 and 18 months) (Lahtinen et al., 2011). Interestingly, IgM levels were persistent up to 21 months in another cohort, suggesting IgM may not be a good indicator of recent infection, or subjects underwent repeated exposure to PARV4 (Yang et al., 2011). Previous studies on parvovirus B19 have shown that this family of viruses can trigger surprisingly strong and long-lived cellular responses, especially to the non-structural protein of B19 (Isa et al., 2005). Moreover, as PARV4 is often seen in individuals co-infected with HCV and HIV-1, where the cellular response is critical, the role of the cellular response to PARV4 is even more interesting. Furthermore, the human body establishes a careful immunoregulatory balance so as to limit immunopathology. This is a concern especially for HCV infection, where persisting virus can lead to chronic liver injury. Thus, determining the immune response to PARV4 may shed light on the course of disease caused by co-infecting viruses.

4. Aims

The aim of this thesis was to characterise the immune response to PARV4, specifically in patients co-infected with HCV or HIV-1. I was also interested in studying the clinical significance of PARV4 co-infection. At the time of starting this project, nothing was known concerning the immune response to PARV4, and data on the clinical significance of PARV4 was also inconclusive.

To this end, this thesis was set up to investigate:

- **Chapter 3:** the prevalence, magnitude, breadth and specificity of the T cell response in PARV4⁺ individuals. Moreover, this study aimed to map and define specific epitopes that could be used to phenotype PARV4-specific T cells.
- **Chapter 4:** the clinical significance of PARV4 by studying HIV⁺ and HCV⁺ cohorts in parallel with disease progression data.
- **Chapter 5:** the cellular and humoral responses during acute PARV4 disease, by characterising responses longitudinally in a cohort of intravenous drug users.

Chapter 2

Materials and Methods

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1. Study subjects

Informed patient consent was obtained from all individuals in several cohorts for different projects. These are outlined below.

1.1. Oxford HCV-infected cohort

88 HCV-infected (HIV-negative) individuals attending clinic as outpatients at the John Radcliffe hospital, provided blood samples for immunological research (COREC 04.OXA.010). Blood samples were taken whilst patients were not undergoing HCV treatment (except for one patient, with no PARV4-specific response). Of those previously treated (43/88), samples were a minimum of 5 months post-treatment (range: 5-77 months, median: 25).

1.2. UK HIV-infected cohort

13 HIV-infected (HCV-negative) individuals attending Northampton General Hospital, the Royal Berkshire Hospital (Reading), Wycombe General Hospital, and the Oxford Radcliffe hospitals in Oxford provided blood samples for immunological research (ethics code 06/Q1604/2).

1.3. Oxford healthy volunteers and B19 cohort

9 healthy laboratory volunteers and 7 acute B19 seropositive (IgM⁺) individuals were tested as control subjects for this study (COREC 04.OXA.010 and CO2.113).

1.4. Nottingham Trent HCV-infected cohort

276 HCV-infected (HIV-negative) individuals from the Trent HCV cohort (MREC/98/3/55) provided plasma samples which were frozen at -80°C until use.

1.5. Swiss HIV-infected and HIV-HCV co-infected cohort

193 HIV-infected individuals from the Swiss HIV cohort study (Rauch et al., 2005) provided plasma samples that were frozen at -80°C until use.

1.6. Baltimore HCV-negative IDU cohort (REACH cohort)

98 HIV- and HCV-negative active IDUs from “The Risk Evaluation Assessment of Community Health prospective study of IDUs” (REACH) in Baltimore, MD consented to have blood drawn for isolation of plasma and PBMCs in a protocol designed for monthly follow-up (Cox et al., 2005).

2. Sample processing

2.1. PBMC

Fresh PBMCs were obtained from heparanised blood over Lymphoprep™ separation medium (Axis Shield Diagnostics) by centrifugation at 2200rpm for 24 mins (no brake). PBMCs were then washed twice in R10 (RPMI 1640 supplemented with 10% FCS, 50,000 U penicillin, 50mg streptomycin and

1mmol L-glutamine (all Sigma)), first at 1700rpm for 7 mins, then at 1500rpm for 10 mins. PBMC were either tested directly or frozen in liquid nitrogen until required.

Frozen PBMCs stored in freezy mix (50% v/v FCS, 10% v/v DMSO, 40% v/v RPMI 1640 medium) in liquid nitrogen were thawed and washed in RPMI 1640 supplemented with CTL wash solution (10% v/v, Cellular Technology Ltd) and DNase I (20U, Roche) and rested overnight at 37°C at 5% CO₂.

These PBMCs were then used in ELISpot and Intracellular Cytokine Staining (ICS) assays.

2.2. Plasma

Whole blood was collected in EDTA tubes and plasma was obtained by centrifugation at 2000rpm for 10mins before being stored at -80°C until use. Plasma from the Trent, Swiss and Baltimore cohorts were processed by each centre before being sent on dry ice for experimentation.

2.3. DNA extraction and HLA-typing

DNA was extracted from whole blood or PBMCs using the Gentra Puregene kit (Qiagen) following the manufacturer's instructions. Briefly, red blood cells were lysed and removed from the sample by centrifugation (4000rpm for 2mins). The remaining cells were lysed and protein precipitation solution was added. The DNA was then washed with isopropanol and 70% ethanol before being re-hydrated. This DNA was used to HLA-type patients from the Oxford cohort using

the Sequence-Specific Primer (SSP) method (Timothy Rostron, WIMM, Oxford UK) (Bunce, 2003).

3. Synthetic peptides

Three hundred and thirty eight peptides of 15 amino acids in length, overlapping by 10 amino acids spanning PARV4 NS, VP, ARF1 and ARF2 proteins of the prototype genotype 1 sequence (AY622943) were synthesized (>70% pure, Kati di Gleria, Fmoc Chemistry, WIMM, Oxford), weighed and dissolved in dimethyl sulfoxide (DMSO, Sigma) to 20mM. NS peptides were combined into 13 pools of 10 peptides (pools 1 to 13), VP into 10 pools of 18 peptides (pools A to K), and 1 pool each for ARF1 and ARF2 (pools A1 and A2) (see Appendix Table AT1).

4. IFN- γ ELISpot assays

ELISpot (enzyme-linked immunosorbent spot) assays were carried out directly *ex vivo* or after short-term culture (see PBMC Short-Term culture). All ELISpots presented here were performed *ex-vivo* unless otherwise indicated, with PARV4 NS, VP and ARF pools and individual peptides. 96-well ELISpot plates (Millipore) were pre-coated with anti-IFN- γ monoclonal antibody (0.5 μ g/well, Mabtech) and incubated at 4°C overnight. Plates were washed twice with PBS before blocking with R10.

200,000 PBMCs were plated per well, with duplicates for each condition, and incubated overnight with peptides at 1 μ M final concentration, at 37°C with 5%

CO₂. Wells containing PBMCs with DMSO or concanavalin A (20 µg/mL [Sigma]) were used as negative and positive controls, respectively. After incubation, plates were washed 7 times with PBS and 0.05% Tween® 20 (Sigma), and incubated at room temperature with biotin mouse anti-human IFN-γ (1:2000, Mabtech) for 3 hours. Plates were washed 4 times and incubated with alkaline phosphatase-conjugated anti-biotin antibody (1:750, Vector Laboratories) for 2 hours at room temperature. Plates were washed an additional 4 times before incubating with NCIP/NBT Substrate Solution (Pierce) for 7 minutes. The plates were left to air-dry overnight, before reading on an automated ELISpot reader (AID). Results were presented as spot-forming units (SFU) per million PBMCs. A well was considered positive if above 55 SFU/million PBMC per pool of peptides after subtraction against the average of duplicate background wells. This cut-off has been extensively evaluated using negative controls in ongoing vaccine trials in our group and in HIV ELISpots (Folgori et al., 2009, Streeck et al., 2009). This value is also above both the mean value + 3 Standard Deviations (=14) and the highest count seen in the controls of this study (healthy and B19⁺ individuals, see Chapter 3).

5. Serological screening

Plasma samples from individuals in each cohort were tested for anti-PARV4 IgG (and additionally IgM in a subset). PARV4 VP capsid protein was used as antigen in an enzyme-linked immunosorbent assay (ELISA) developed by Colin Sharp (University of Edinburgh). Briefly, patient plasma was added to 96 well plates

(Greiner Bio-one) pre-coated with 1µg PARV4 antigen. The plates were washed then incubated with horseradish peroxidase-conjugated goat anti-human IgG antibody (1:1000, Serotec). Finally, after further washes, the substrate for horseradish peroxidase was added (2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid; Liquid Substrate System, Sigma). The resulting reaction was read at 405nm and optical density (OD) scores recorded. Each sample was carried out in duplicate and compared to a viral-like particle control to determine PARV4-specific reactivity (Sharp et al., 2009). Adult attendees of orthopaedic outpatient clinics in Edinburgh were used as negative controls to calculate the cut-off for PARV4 IgG seropositivity (set at 0.5 arbitrary units)(Sharp et al., 2009).

Serology results in this thesis are expressed in arbitrary units. These were calculated using a standard curve plotted with OD data points from a positive sample diluted at several concentrations, where the highest value is 100 units (undiluted), 1:5 dilution of this sample is 20 units, 1:25 dilution of this sample is 4 units and 0 units is based on a negative control OD. This data was analysed using a non-linear regression, and OD data from patient plasma samples was used to interpolate the corresponding unit in GraphPad Prism.

6. PBMC culture

6.1. Short-term PBMC culture

PBMCs were thawed and rested as described above. Samples were set up with 2×10^6 cells per well and were pulsed with 25µM of peptide for 30min before incubating at 37°C, 5% CO₂ for 10 to 14 days. Cells were supplemented every 3

days with natural human T-cell growth factor (10% v/v, Helvetica Healthcare) or recombinant IL-2 (100U/ml, R&D Systems) and peptide if re-stimulation was required. Cells were harvested and assayed by ICS or surface stain.

6.2. Long-term PBMC culture

After 14 days of short-term culture, PBMCs could be maintained with HLA-matched B-cell lines (BCL) pulsed with PARV4 peptide (12.5 μ M for 1 hour at 37°C) and PBMCs from three healthy donors were used as feeders. BCLs and feeders were irradiated with 30 Gray. Cells were resuspended with the individual PBMCs to culture at a 1:1 ratio, and incubated at 37°C, 5% CO₂ until further feeding was required.

7. Intracellular cytokine staining (ICS), surface staining and flow cytometry analysis

Cells were counted and aliquoted at approximately 10⁶ cells per condition. Negative and positive controls were set up with DMSO, and phorbol myristate acetate (PMA, 0.05 μ g/ml, Sigma) and ionomycin (0.5 μ g/ml, Sigma) respectively. Samples were stimulated with 3 μ M peptide and incubated at 37°C, 5% CO₂ for 5-6hrs. Brefeldin A (2 μ g/ml, Sigma) was added to each tube one hour post-stimulation. Cells were surface-stained with fluorochrome-conjugated antibodies for 30mins and processed for analysis; or, for ICS, were permeabilised (BD Cytofix/Cytoperm or eBioscience Fixation/Permeabilisation) then stained for

intracellular markers for 30mins. Appendix Table AT2 lists the fluorochrome-conjugated antibodies used. Wells were washed in permeabilisation buffer before processing by FACS. Cells that were simply surface stained were also fixed with 1% formaldehyde.

All samples were processed on a BD LSR II and analysed using FlowJo software.

Lymphocytes were gated on forward and side scatter, live and CD3⁺ cells. The FACS plots presented in this thesis are gated on CD3⁺ cells unless otherwise indicated. An example of the gating strategy is shown in Figure 2.1.

For more detailed analysis, Boolean gates were set up for samples in the Baltimore REACH IDU cohort (Chapter 5); these were analysed with Pestle software and illustrated with Spice 5.2 software.

8. HLA-A2 RMT tetramer synthesis and PBMC

staining

Protein refolding was carried out for recombinant β_2 microglobulin (β_2m) and HLA-A*0201 heavy chain, containing the BirA recognition sequence at its C-terminus. β_2m and heavy chain solution were refolded around peptide RMTENIVEV by dilution of denaturing conditions. This solution was left to stir overnight then filtered and concentrated using a tangential flow module. The sample was further concentrated with an Amicon Ultra cartridge (Millipore) before biotinylation of the heavy chains using the BirA enzyme (500 μ M d-biotin, 3mg/ml (5000 U/ μ g), Avidity). The biotinylated monomers were then run on Fast Protein Liquid Chromatography (Akta FPLC, Amersham Pharmacia Biotech)

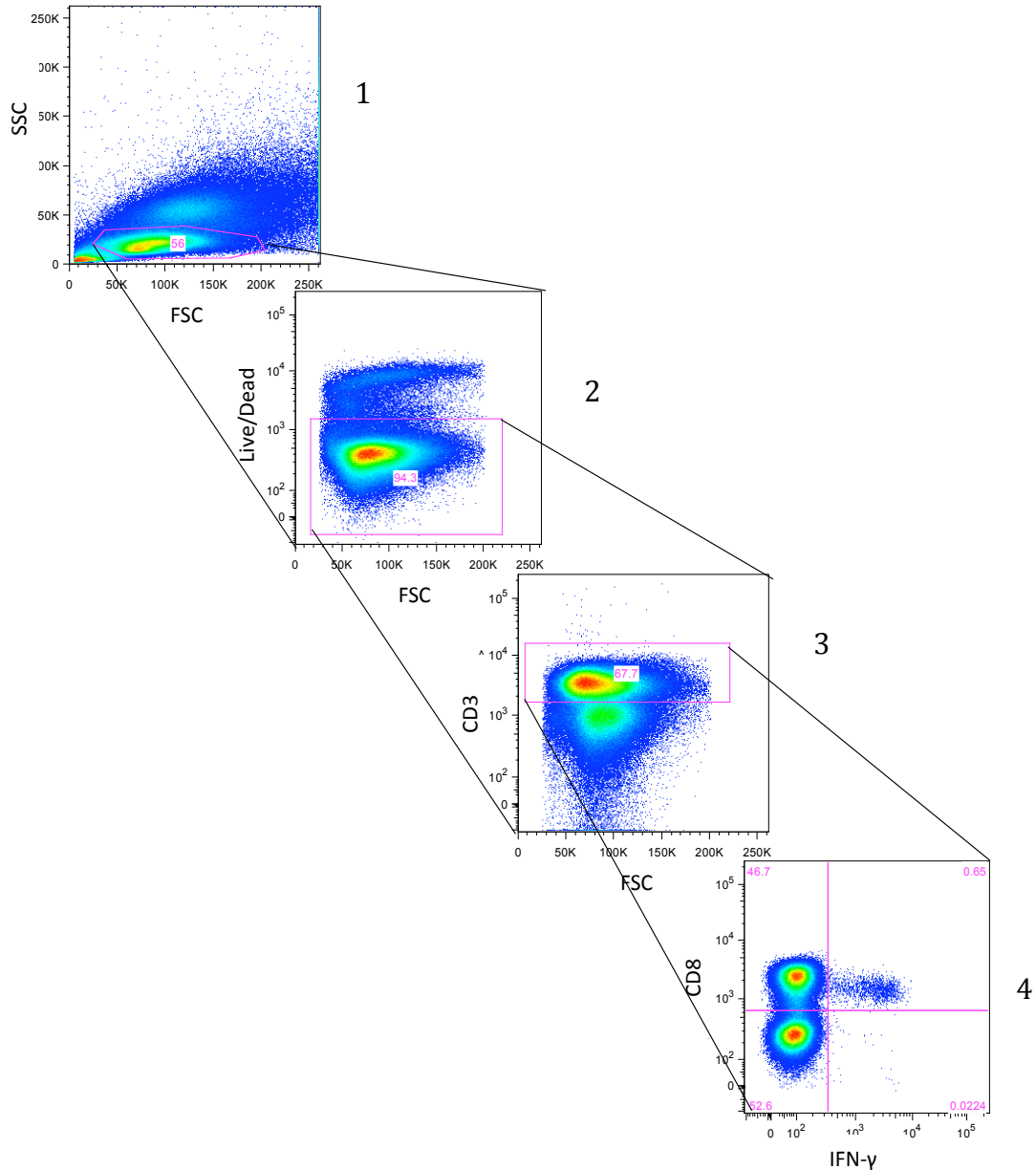


Figure 2.1 Gating strategy for intracellular cytokine staining plots. This example is of PBMC from a healthy individual. Cells were incubated with FEC (Flu, EBV and CMV CD8⁺ epitopes). Cells were first gated on lymphocytes on the SSC vs FSC plot (1), then dead cells were excluded (plot 2), before selecting specifically for CD3⁺ cells (plot 3). The resulting plot (4) is the format of the plots shown in the results chapters.

to check the purity and fold of the sample. These monomers were stored at -80°C until tetramerisation.

When needed for experimentation, the monomers were tetramerised using Extravidin®-R-Phycoerythrin (Sigma) at 1:20 extravidin conjugate:monomer ratio. Half of the final quantity was added and incubated in the cold room for 90mins before adding 1/5th of the remaining volume in 5 steps, leaving 30mins between each addition. This tetramer, hereafter known as the HLA-A2 RMT tetramer, was then stored at 4°C for 3 weeks (shelf-life).

PBMCs were stained with HLA-peptide tetrameric complexes at the optimal dilution (determined at 1:100) for 20mins at 37°C. Cells were then washed with PBS before staining with extracellular markers as described above.

9. Epitope prediction

Bioinformatics programmes Syfpeithi (<http://www.syfpeithi.de>), Bimas (http://www-bimas.cit.nih.gov/molbio/hla_bind/) and NetMHCpan 2.4 server (<http://www.cbs.dtu.dk/services/NetMHCpan/>) were used to predict potential epitopes. Peptide sequences were recorded if their score was above 20 for Syfpeithi, above 50 for Bimas and a weak (WB) or strong binder (SB) for NetMHCpan 2.4 server. Strong binders were defined as having an affinity threshold <50nM, and weak binders were defined as having an affinity threshold <500nM.

10. PCR and agarose gel electrophoresis

The DNA from plasma samples from several HCV-negative IDUs (Baltimore REACH cohort) were screened by nested PCR for PARV4 VP using degenerate primers designed to recognise PARV4, Porcine Hokovirus and Bovine Hokovirus sequences (Hoko/P4). First round reactions were performed using outer primer sets (Ho/P4 Outer Sense: TGAAYCAGACCTTGARCGSCC, Ho/P4 Outer Antisense: CCMCCHAWCCAYTGAGCTTTWACTTT), 5µl of extracted DNA as template and GoTaq reagents (Promega) according to manufacturer's instructions. Second round reactions were performed using inner primer sets (Ho/P4 Inner Sense: TAYAATTATGTTGGTCCTGGTAATCC, Ho/P4 Inner Antisense: GGTARRACCTGYGAWADTTGAACATC) and 1µl of first round product as template. Cycling conditions for both rounds were as follows: 30 cycles of [18 seconds at 94°C, 21 seconds at 50°C and 90 seconds at 72°C] and a final extension of 5 minutes at 72°C (Sharp et al., 2010a). DNA was analysed by electrophoresis in 2% agarose gels containing 0.5µg/ml ethidium bromide in TAE buffer (Tris base, acetic acid, EDTA). Samples of 12µl were mixed with 6x loading buffer (50% w/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v Xylene Cyanol, 0.25% w/v Orange G). Electrophoresis was carried out in horizontal gel tanks containing TAE at 150V for 20 cm wide gels. Samples were compared with 100bp DNA ladder (Promega) for size confirmation. Gels were visualised on a UV transilluminator.

11. IL28B SNP rs8099917 genotyping

DNA was extracted from whole blood from 21 HCV-infected individuals from the Trent HCV cohort (as described above). The alleles for SNP rs8099917 in proximity of the IL-28B gene, were determined by real time PCR using TaqMan® SNP Genotyping Assays kit (Applied Biosystems, C_11710096). Patient DNA added to these reagents were run according to manufacturer's instructions on a Light Cycler 480 (Roche). Cycling parameters were: one cycle at 95°C for 10 min, 45 cycles at 92°C for 15 s, and 60°C for 1 min, followed by one cycle at 40°C for 10 s.

12. Statistics

Statistics were calculated using the Mann Whitney test, Fisher's exact test and log rank (Mantel-Cox test) using GraphPad Prism software. Data sets made up of continuous values were tested for normal distribution using Kolmogorov-Smirnov tests (Eleni Frangou, Department of Statistics). All data sets were found to be non-normally distributed, thereby justifying the application of non-parametric Mann Whitney tests to test for statistical difference. Fisher's exact test was used for contingency analyses as the size of the data sets remained small. More detailed statistical analyses were carried out using "R" software as described in Chapter 4. A test with a p value ≤ 0.05 was considered significant.

Chapter 3

The Immune Response in Chronic PARV4 Infection

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1. Introduction

Most studies on PARV4 to date have looked at the presence of viral DNA in various populations. A recently developed ELISA has allowed to compare DNA detection, which is rarely positive in peripheral blood, with the presence of antibodies to PARV4 (Sharp et al., 2009). The T cell immune response plays a major role in viral clearance as is seen in HCV-infected individuals, where a poor CD8⁺ T cell response correlates with viral persistence (Bertolino P, 2007). Previous studies on parvovirus B19 have shown that this family of viruses can trigger surprisingly strong and long-lived cellular responses especially to the non-structural B19 protein NS1 (Tolfvenstam et al., 2001, Isa et al., 2006).

In view of the importance of cellular immunity in controlling infection and immunopathology, and since the T cell immune response to PARV4 had not been studied previously, this study investigated whether HCV- and HIV-infected individuals made such a response. Stimulated T cells produce IFN- γ as part of the antiviral response, and this cytokine can be detected using ELISpot assays to measure magnitude and specificity. These are the first data on the breadth, magnitude, specificity and phenotype of the T cell responses to PARV4.

Aims:

- To determine the prevalence of PARV4 in cohorts of chronically infected HCV⁺ individuals, HIV⁺ individuals and acutely infected B19⁺ individuals.
- Study the relationship between the cellular and humoral responses to PARV4.
- Investigate the specificity, magnitude and phenotype of the T cell response to PARV4.

2. Methods

2.1. Study subjects and sample collection

Informed patient consent was obtained from four groups of individuals: 88 HCV-infected (HIV-uninfected) individuals (Table 3.2), 13 HIV-1-infected HCV-negative individuals, 7 acute B19 seropositive (IgM⁺) individuals and 9 healthy individuals for control tests. Blood samples were taken whilst patients were not undergoing HCV treatment (except for one patient, with no PARV4-specific response). Of those previously treated (43/88), samples were a minimum of 5 months post-treatment (range: 5-77 months, median: 25 months).

2.2. PBMC assays

ELISpot, ICS and tetramer staining assays were carried out as indicated in Chapter 2. 131 peptides spanning the entire PARV4 NS protein, 181 peptides spanning the entire PARV4 VP protein and 26 peptides spanning the ARF proteins, were tested by IFN- γ ELISpot assay for patient T cell responses. These peptides were 15 amino acids in length, overlapping by 10 amino acids; and were combined into 13 pools of 10 peptides each for NS (pools 1 to 13), 10 pools of 18 peptides for VP (pools A to K) and 2 pools for ARFs (Appendix table AT1). For ELISpot assays, results were considered positive if a well contained over 55 SFU/million PBMC per pool of peptides, after subtraction of the average of background wells. This cut-off has been extensively evaluated using negative controls in ongoing vaccine trials in our group (Folgori A. , 2009) and in HIV-1 ELISpots (Streeck et al., 2009). This value, 55 SFU/million PBMC, is also above

both the mean value + 3 Standard Deviations (=14) and the highest count seen in the negative controls of this study (healthy and B19⁺ individuals, Figure 3.1).

Flow cytometry was carried out as described in Chapter 2, and tetramer HLA-A2 RMT was synthesized by Stuart Sims (Klenerman group). For flow cytometry analysis, lymphocytes were gated on forward and side scatter, then live and CD3⁺ cells. The FACS plots presented in this study are gated on CD3⁺ cells unless indicated.

2.3. Serological screening

Serum samples from 84 out of 88 individuals were tested for anti-PARV4 IgG and additionally IgM in 7 individuals where no anti-PARV4 IgG was detected, as previously described in Chapter 2. These tests were carried out by Colin Sharp (University of Edinburgh).

2.4. Epitope prediction

Bioinformatics programmes Syfpeithi (<http://www.syfpeithi.de>), Bimas (http://www.bimas.cit.nih.gov/molbio/hla_bind/) and NetMHCpan 2.4 server (<http://www.cbs.dtu.dk/services/NetMHCpan/>) were used to predict potential epitopes. These were recorded if their score was above 20 for Syfpeithi, above 50 for Bimas, and a weak or strong binder (WB or SB respectively) for NetMHC pan. Strong binders were defined as having an affinity threshold <50nM, and weak binders were defined as having an affinity threshold <500nM. The peptide RMTENIVEV identified through experiments and epitope prediction was a 9 amino acid epitope that scored 27 in Syfpeithi, 474 in Bimas and a strong binder in NetMHC pan server.

3. Results

3.1. HCV⁺ individuals have strong T cell responses to PARV4 NS peptides which are maintained over time

PBMCs from 88 randomly selected HCV⁺ individuals attending hospital as outpatients, 9 healthy individuals and 7 individuals with acute parvovirus B19 infection were assayed by ELISpot for the production of IFN- γ in response to 13 pools of 10 PARV4 NS peptides. Summed responses to the entire NS protein by HCV⁺ individuals are shown in Figure 3.1A. 23 HCV⁺ individuals (26%) had T cell responses to individual pools higher than the pre-defined cut-off 55 SFU/10⁶ PBMC (see methods) and 18 had T cell responses higher than 250 SFU/10⁶ PBMC, with a maximum of 2045 SFU/10⁶ PBMC per peptide pool (Figure 3.1C-D). Examples of the highest responses seen in this assay are presented in Figure 3.1B. Results from PBMC samples from one time point were also confirmed in other assays such as ICS (see below).

Additionally, a number of responders in the cohort were tested longitudinally: 6/7 individuals with positive responses remained positive across periods of 3 months to 3 years (Table 3.1 and Figure 3.2). The T cell response of the seventh individual was recovered upon PBMC culture with peptide (Figure 3.3). Likewise, 3/3 individuals with no response remained negative over time. Responses to the N-terminal portion of the NS protein were both higher in

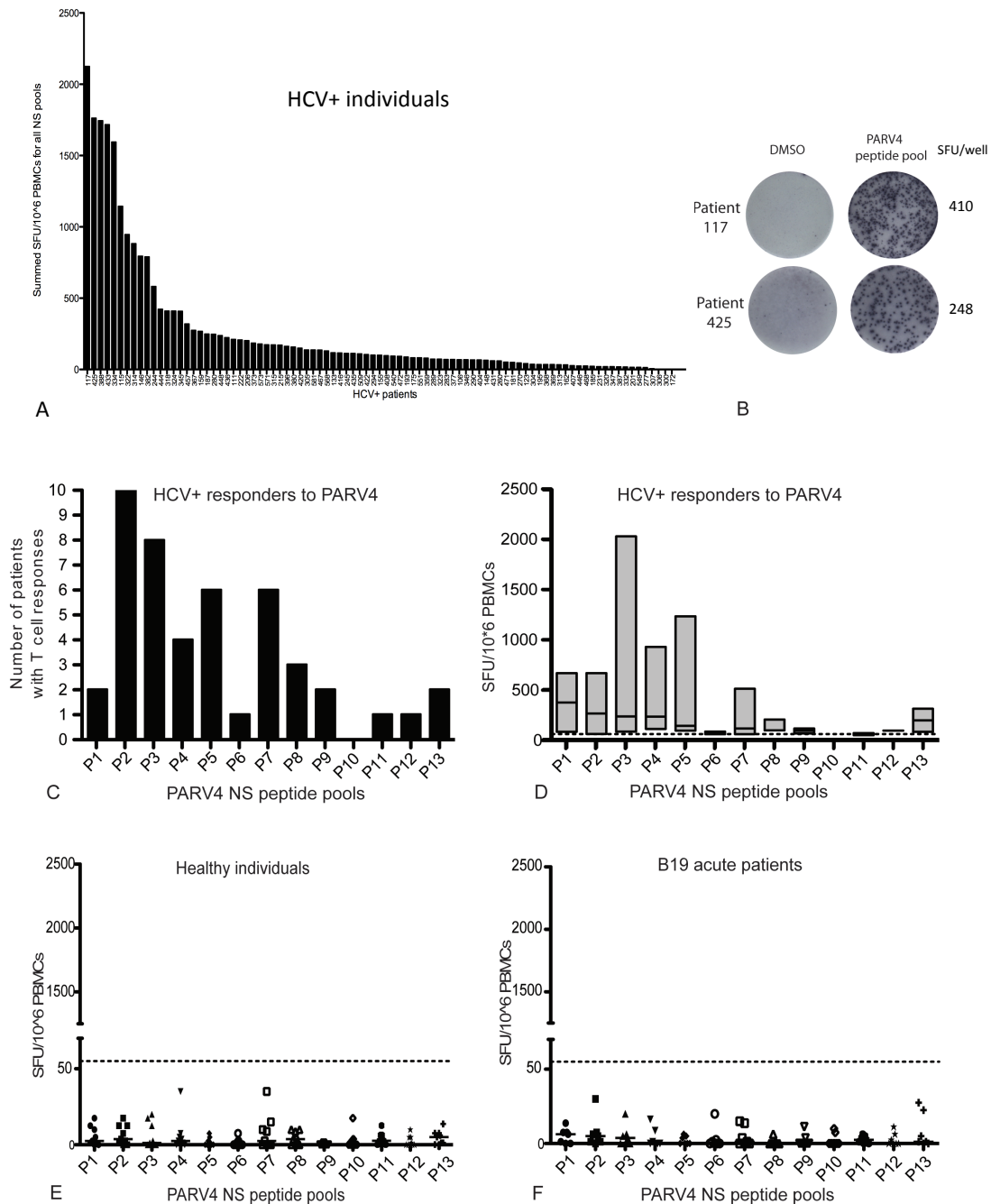


Figure 3.1 Analysis of PARV4 T cell responses in HCV-infected patients and controls. T cell responses were assessed by IFN- γ ELISpot assays with PARV4 NS peptides. **A.** T cells from 88 HCV-infected patients to each peptide pool are summed so that each column represents the response of one patient to the entire NS protein. **B.** These photos of ELISpot wells are examples of the highest levels of responses and their negative control wells (DMSO). **C, D.** These graphs show the distribution and the magnitude of the T cell responses across the NS protein. **C.** Each column represents the number of patients responding to each pool. **D.** Each column represents the range of responses of all responders; the intersecting bars are the median for that peptide pool. T cell responses of 9 healthy individuals (**E**) and 7 B19 IgM⁺ patients (**F**) to PARV4 NS peptide pools are displayed. Each point represents the T cell response of one individual to the peptide pool on the x-axis. The dashed line marks the cut-off for positivity.

Patient number	Sample date 1	Result	Sample date 2	Result
117	Feb-06	+	Feb-09	+
123	Oct-06	-	Nov-09	-
146	Jul-08	+	Oct-09	+
162	Feb-06	-	May-09	-
289	Mar-07	-	May-09	-
324	Oct-06	+	Nov-09	-
382	Apr-07	+	Nov-09	+
422	Apr-08	+	Oct-08	+
433	Feb-08	+	May-08	+
457	Dec-08	+	Feb-10	+

Table 3.1 T cell responses to PARV4 are maintained over time. A positive response (+) is defined by at least one pool eliciting a T cell response over 55 SFU/10⁶ PBMC. Only the response of patient 324 was not detectable *ex vivo* at the second time point; this was recovered after culture (see Figure 3.3). (- represents the absence of response)

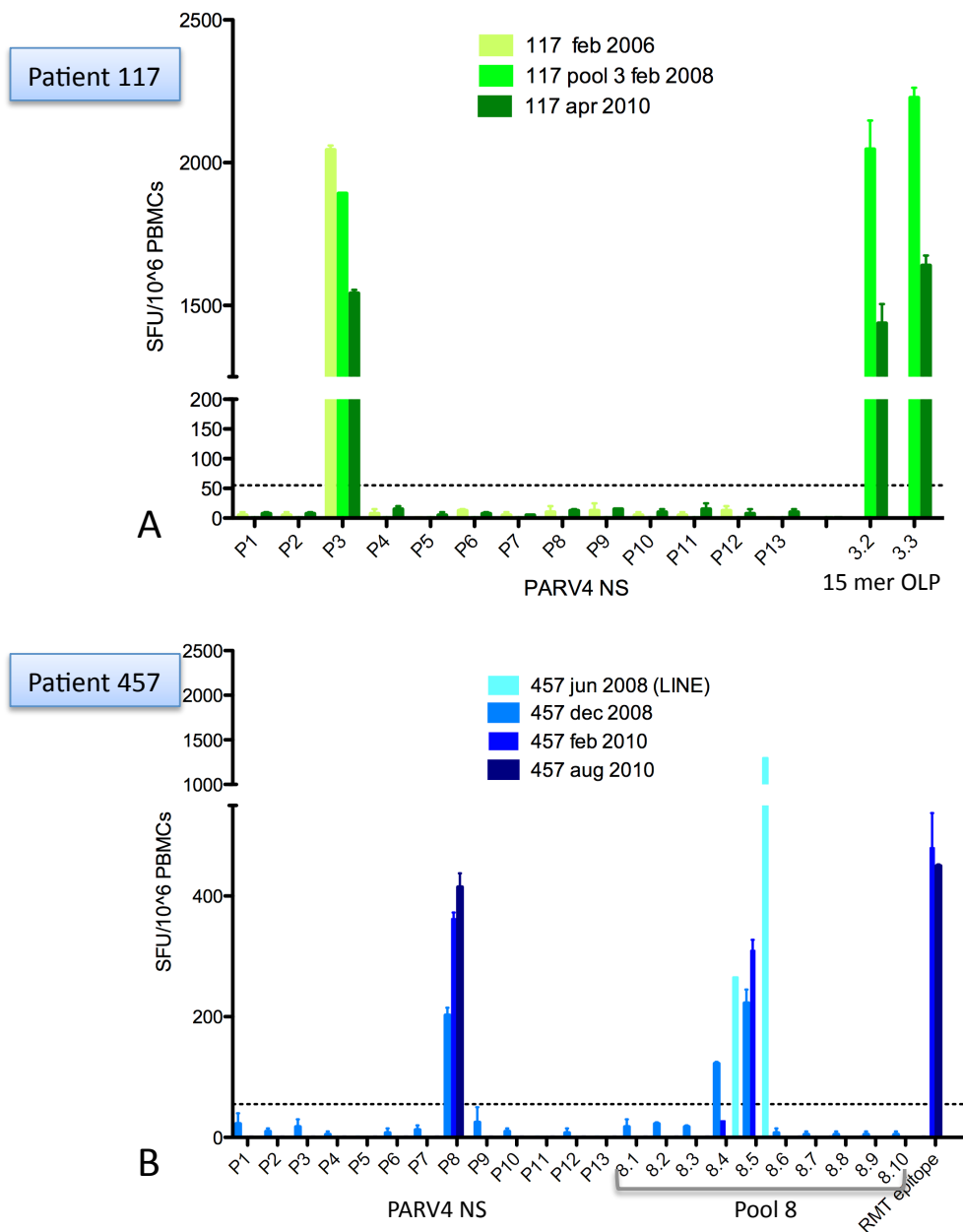


Figure 3.2 Longitudinal analysis of T cell responses to PARV4 NS. T cell responses of patients 117 (**A**) and 457 (**B**) are illustrated here as examples of sustained responses over time. The dates next to the patient numbers represent the sample date. (457 June 2008 sample: these cells were cultured with peptide for 22 days before the assay). OLP = overlapping peptides

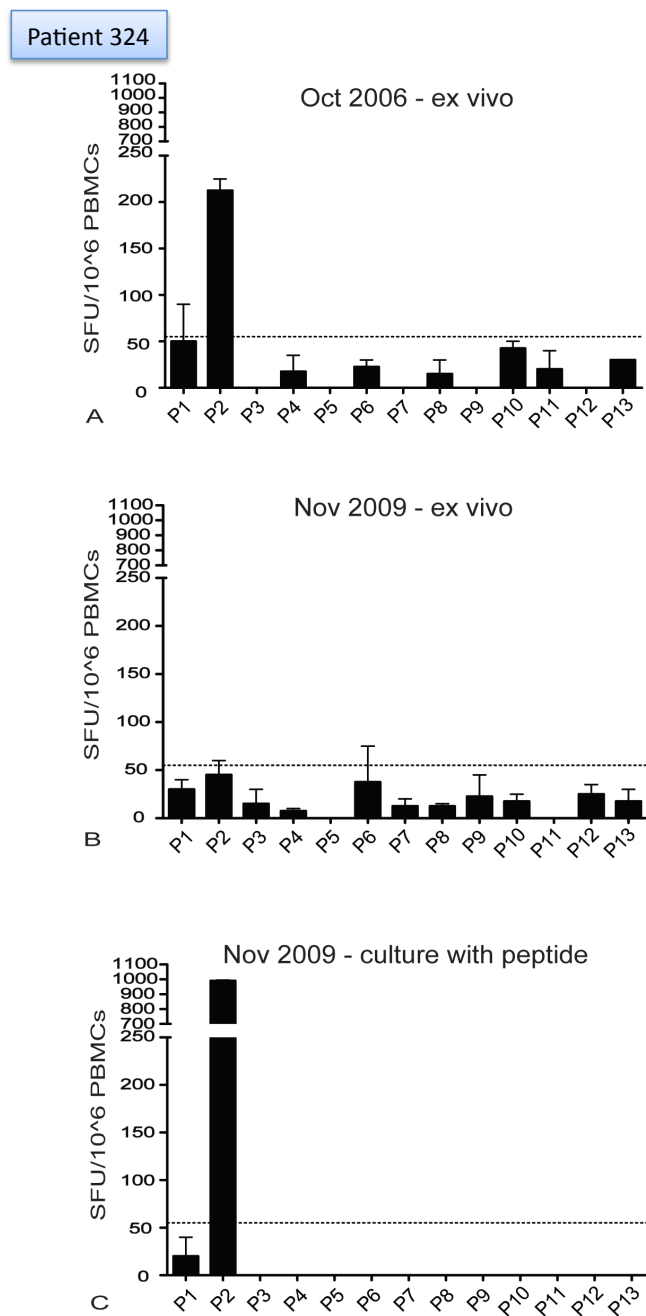


Figure 3.3 Sequential T cell response of patient 324 to PARV4 NS peptide pools. An immunodominant T cell response to pool 2 in 2006 (A) was not detected *ex vivo* in 2009 (B). However, after 10 days T cell culture in the presence of pool 2 peptides, a response to the same pool was detected (C).

magnitude and more frequent (Pools 1-7: 37 responses, median: 6; pools 8-13: 9 responses, median: 1.5; Mann Whitney test $p=0.036$; Figure 3.1C-D).

As controls, 9 healthy individuals at low-risk for HCV infection were tested; the maximum median for one single pool was 3.75 SFU/ 10^6 PBMC, with responses reaching a maximum of 35 SFU/ 10^6 PBMC per peptide pool (i.e. under the predefined cut-off) (Figure 3.1E). Seven individuals with acute parvovirus B19 infection also had low responses with a maximum median for one single pool of 6.25 SFU/ 10^6 PBMC and an individual maximum of 30 SFU/ 10^6 PBMC (Figure 3.1F). The T cell responses of healthy controls and B19 acutely infected individuals did not differ significantly ($p=0.61$, Mann Whitney test).

A random selection of HCV⁺ individuals, where there were sufficient available PBMC, were tested for T cell responses to proteins VP, ARF1 and ARF2. However, none of the responses were above the cut-off (except patient 123; however background was also high, Figure 3.4).

3.2. Analysis of antibody responses in relation to T cell responses

Serum samples from 84 out of 88 HCV⁺ individuals, and 12 out of 16 control individuals (5 B19⁺ and 7 healthy) were available for serological tests. Anti-PARV4 antibodies were found in 22 HCV⁺ individuals (26% seroprevalence), but were not seen in any of the control individuals. Fourteen individuals had both antibodies and T cell responses, 7 showed T cell responses but were seronegative, 8 were seropositive for PARV4 antibodies while 55 had neither (Figure 3.5). To investigate the frequent discrepancies in T cell and serological

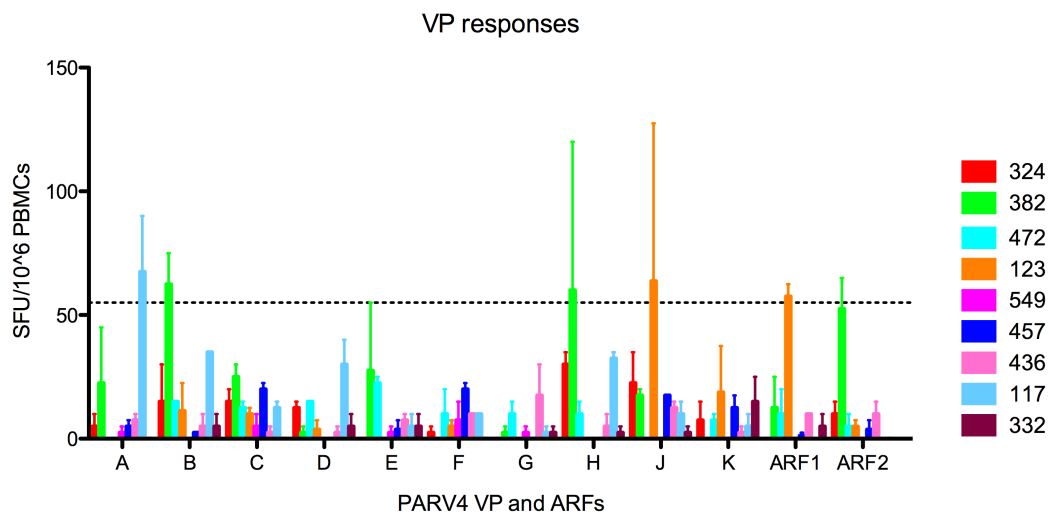


Figure 3.4 T cell responses to PARV4 VP and ARF proteins tested in 9 subjects are below the cut-off. Responses were lower than those elicited by PARV4 NS (Figure 3.1) and it proved harder to obtain consistent duplicates (reflected by the large error bars).

PARV4 serology of HCV⁺ patients

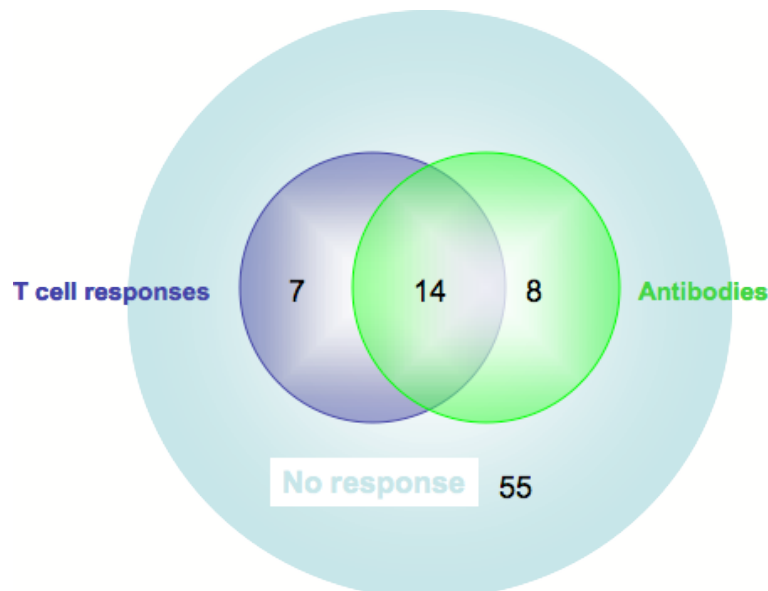


Figure 3.5 Discrepancy in the humoral and cellular responses of HCV⁺ individuals to PARV4. 84 individuals were tested for PARV4 antibodies. 22 were PARV4 seropositive but only 14 of these also had a cellular response. T cell responses were assessed by IFN- γ ELISpot and serology was determined through a PARV4 ELISA. 55 individuals showed neither T cell responses to PARV4 nor PARV4 IgG.

reactivity, antibody and T cell assays were repeated at several time points. For example, individual 117, who had shown a strong T cell response, was tested for antibodies from serum samples from two time points, and both were negative. On the other hand, PBMC samples from these same time points were both positive, and at very high levels (2045 and 1890 SFU/million PBMC per peptide pool, Figure 3.2, antibody data not shown). The seven individuals lacking anti-PARV4 IgG were also negative in an additional ELISA for anti-PARV4 IgM. Overall, the correlation between T cell responses and IgG detection was significant (Fisher's exact test $p < 0.0001$), although clearly individuals may possess T cell responses without detectable antibody and vice versa. No significant differences were found with age, gender, HCV genotype or HCV RNA PCR status at diagnosis between these groups (Table 3.2). There was also no significant difference in the number of antibody or T cell responses between treated and untreated individuals (data not shown, Fisher's exact test, $p = 0.45$ for IgG, $p = 0.61$ for T cell responses).

3.3. HIV⁺ individuals elicit a similar rate of response as the HCV⁺ individuals

I next investigated whether T cell responses to PARV4 could be detected in individuals with another persistent blood-borne infection. 13 HIV⁺ HCV-negative individuals were tested for IFN- γ production in response to PARV4 NS. Figure 3.6A shows that T cell responses were detected at a comparable frequency and level to the HCV cohort; three individuals had responses over 55 SFU/ 10^6 PBMC per peptide pool (N067, N090, R010 with CD4⁺ T cell counts 430, 350, 460

Groups of HCV+ individuals - PARV4 specific responses	n=	Age range in years (median)	Gender distribution	ALT range (median)	HCV RNA PCR status at time of sample	Genotype at diagnosis
T cell response positive	23	27-73 (50)	44% female 56% male	10-156 (41)	78% positive 22% negative	43% genotype 1 39% genotype 3 17% other genotype/unknown
T cell response negative	65	24-78 (58)	45% female 55% male	11-480 (40)	77% positive 23% negative	45% genotype 1 37% genotype 3 18% other genotype/unknown
PARV4 IgG positive	22	27-68 (50)	41% female 59% male	10-375 (38)	77% positive 23% negative	27% genotype 1 55% genotype 3 18% other genotype/unknown
PARV4 IgG negative	62	24-78 (50)	45% female 55% male	12- 480 (41)	79% positive 21% negative	52% genotype 1 34% genotype 3 15% other genotype/unknown

Table 3.2 Population demographics of the HCV⁺ individuals. There was no significant difference between T cell response-positive versus negative groups, nor IgG-positive versus negative groups, with respect to the clinical variables shown.

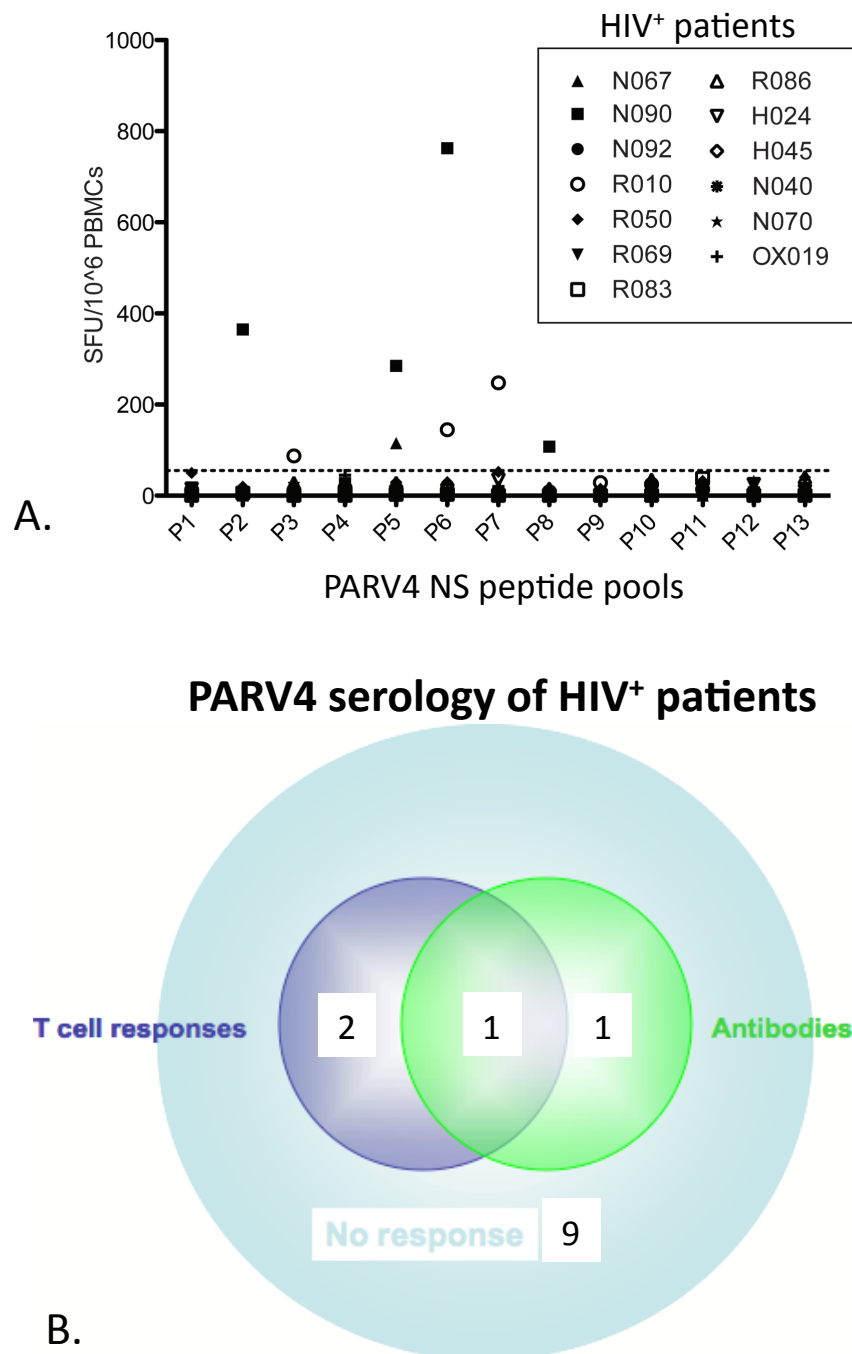


Figure 3.6 Immune responses to PARV4 NS protein in HIV⁺ individuals. A. T cell responses results are displayed as in Figure 3.1E-F. 3/13 HIV⁺ patients had a positive response to PARV4 NS peptide pools (N067, N090, R010) and the distribution of responses across the NS protein is shown. **B.** Discrepancy in the humoral and cellular responses of HIV⁺ individuals to PARV4. 2 were PARV4 seropositive but only 1 of these also had a cellular response. T cell responses were assessed by IFN- γ ELISpot and serology was determined through a PARV4 ELISA.

cells/ μ l respectively). Individuals with no responses had CD4⁺ T cell counts between 130 and 1300 cells/ μ l (median 430 cells/ μ l). This figure shows that, as in HCV-infected individuals, the responses targeted the N terminal half of the NS protein. PARV4 antibodies were detected in 2/13 HIV⁺ individuals, only one of whom also demonstrated a T cell response to PARV4 peptides (Figure 3.6B). Thus an immune response to PARV4 was detectable in HIV⁺ individuals and comparable to that seen in the HCV⁺ cohort. T cell responses were also seen in 1 out of 3 HBV-infected individuals (300 SFU/ 10^6 PBMC to peptide 13.8, data not shown).

3.4. Identification of PARV4-specific CD8⁺ T cells and an HLA-A*0201-restricted PARV4 epitope RMTENIVEV

The nature of the T cell immune response to PARV4 peptides was further characterised through ICS (intracellular cytokine staining) assays. Due to restricted PBMC samples, not all individuals could be tested. Figure 3.7 shows CD8⁺ T cells produced IFN- γ in response to PARV4 NS peptides in two individuals. Individual 314 made a strong CD8⁺ T cell response to NS pools 2 and 3, comprising 2.8% of total CD8⁺ T cells. Individual 457, who was HLA-A*0201 positive, made a similar CD8⁺ T cell response (2.6%) to peptides 8.4 (WWEGRMTENIVEVA) and 8.5 (RMTENIVEVAKAVLG). The overlapping portion of these two peptides contained the HLA-A*0201 anchor residues methionine at P2 and valine at P9 (Table 3.3, Falk et al., 1991), and the epitope RMTENIVEV (hereafter referred to as “RMT”) scored highly using binding algorithms (see methods). This peptide was synthesised and tested in functional assays: Figure

3.7B shows that this epitope RMT triggers a strong production of IFN- γ . Thus, this peptide was synthesised on two separate occasions: first as a 15mer, and secondly as a 9mer, both triggering T cell responses. One other individual responded to overlapping peptides 8.4 and 8.5 (160 SFU/10⁶ PBMC, data not shown); however, no further samples were available for additional characterisation.

To quantify and phenotype the T cell response to the RMT epitope, a fluorescent-labelled tetrameric HLA-A*0201-peptide complex (HLA-A2 RMT tetramer) was synthesised and used to stain PBMC from individual 457. Staining revealed that 0.19% of peripheral CD8⁺ T cells (or 0.06% of CD3⁺ cells) specifically recognised this epitope direct *ex vivo* (Figure 3.8A left panels). *Ex vivo* cells were also stained for CCR7 and CD45RA to identify their memory phenotype. As shown in Figure 3.8A (middle and right panels), cells stained with the HLA-A2 RMT tetramer in this individual were CCR7 and CD45RA negative, suggesting that they were effector memory T cells (T_{EM}). After *in vitro* culture with peptide for 10 days and 6 weeks, 7.0% of CD8⁺ T cells were antigen-specific (Figure 3.8B left and middle panels). A second sample, taken 20 months after the first, was cultured for 23 days and antigen specific cells were still detected (0.17% of CD8⁺ T cells, Figure 3.8B right panels). This provides evidence that the cells were maintained over time *in vivo*.

In total, thirteen peptides were identified through ELISpot assays (Table 3.4). However, there was no apparent immunodominant peptide. The HLA types were not known for all individuals, epitope prediction was not as clear as it had been for RMT, and there was very little overlap between the targets of patient

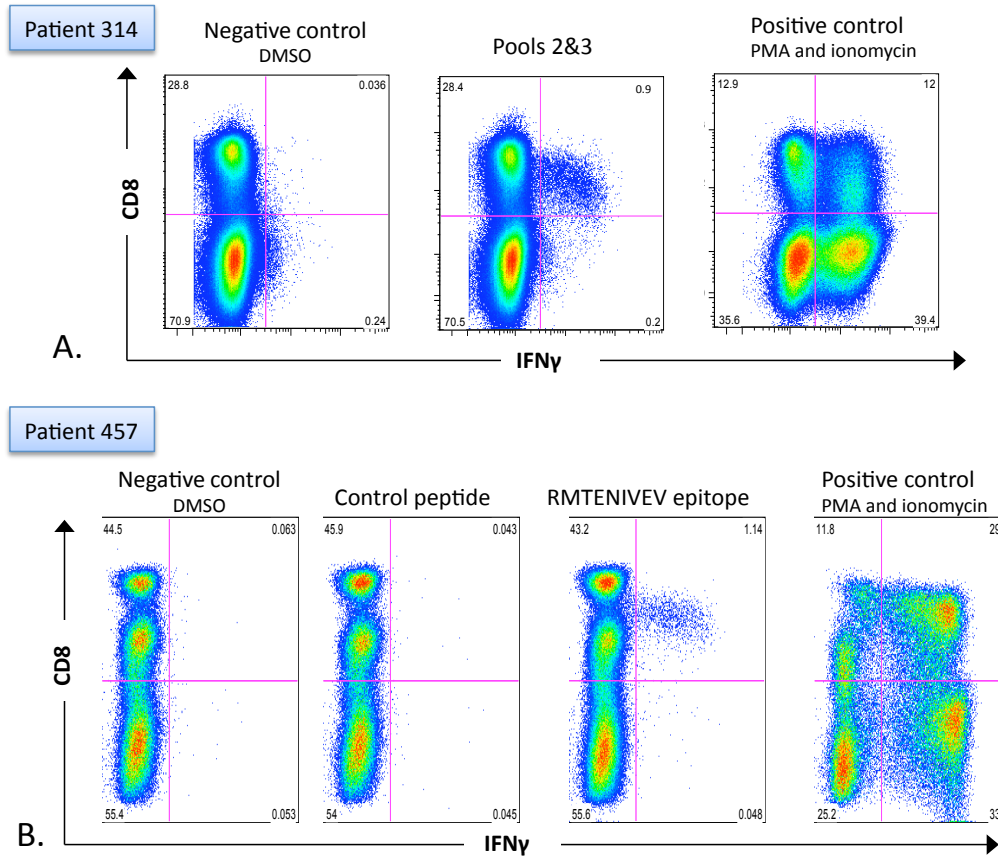


Figure 3.7 Demonstration of PARV4 NS peptides triggering the production of IFN- γ by CD8⁺ T cells. Short-term PBMC lines for patients 314 **(A)** and 457 **(B)** elicit a CD8⁺ T cell response to two pools of peptides and one 9mer epitope respectively. The control peptide is a tetanus toxin peptide (Falugi et al., 2001).

Position in 9mer	1	2	3	4	5	6	7	8	9
Dominant anchor residue		L							V
Strong anchor residue		M K		E		V		K	
Predicted epitope	R	M	T	E	N	I	V	E	V

Table 3.3 Epitope RMTENIVEV is identified from experimental data and anchor residues in the HLA binding site. The overlapping sequences of peptides 8.4 and 8.5 and anchor residues in the HLA binding groove (adapted from Falk et al., 1991) as well as the bioinformatic prediction (methods), led to the epitope prediction seen here.

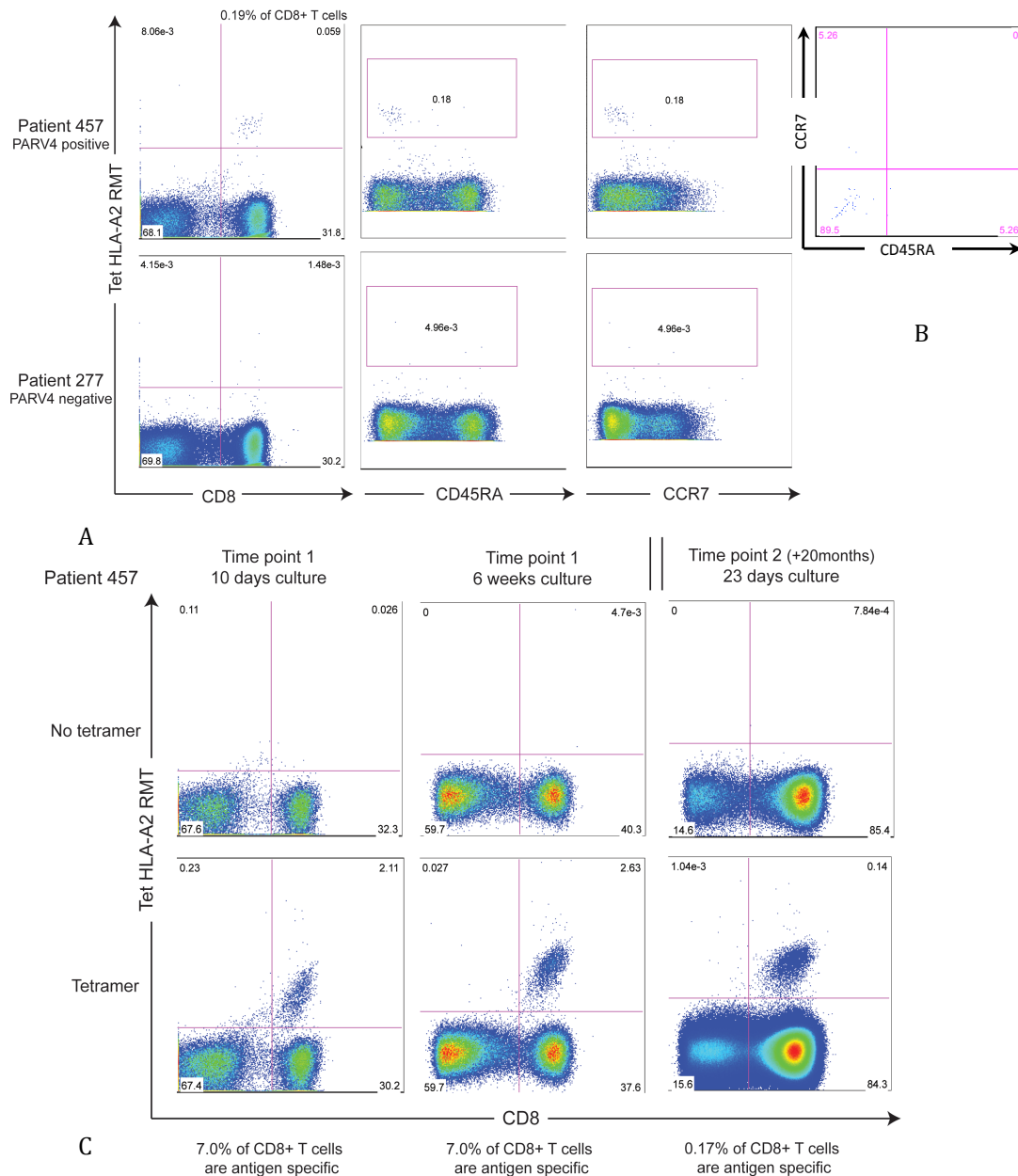


Figure 3.8 HLA-A*0201 restricted PARV4 RMT-specific T cells have an effector memory phenotype and proliferate in culture *in vitro*. **A.** FACS plots for patient 457 (top panel) from whom the epitope was determined (HLA-A*0201 and positive response for peptides 8.4-8.5) and for patient 277 (lower panel, “negative” = no response on ELISpot assays to PARV4 NS). Left hand plots are gated on CD3⁺ cells. The middle and right hand panels looking at CD45RA and CCR7 as memory markers are gated on CD3⁺ CD8⁺ T cells. **B.** This panel shows the level of expression of CD45RA and CCR7 on the same plot. These cells are gated on CD3⁺ CD8⁺ HLA-RMT⁺. **C.** After short-term culture, tetramer HLA-A2 RMT positive cells expanded to 7% of CD8⁺ T cells. This level was unchanged after 6 weeks (middle panels). Tetramer-positive cells were still visible at a second time point (right panels) in the same patient, albeit the percentage decreases. Samples from time points 1 and 2 were taken 20 months apart.

Patient	ELISpot positive pool	ELISpot positive peptides
115	4	4.6, 4.7
117	3	3.2, 3.3
146	13	13.1, 13.7, 13.8
187	2	N/A
206	9	N/A
222	13	N/A
244	2, 3	N/A
314	2, 3	2.3, 2.4, 3.2, 3.3
318	2, 3, 7	N/A
322	2	2.3, 2.4
324	2	2.4., 2.5
334	5	N/A
345	8	8.5
367	7	N/A
382	3, 4, 5, 7, 12	N/A
388	2, 3, 7	2.4, 3.1, 3.2, 3.3, 3.7
396	7	7.3
422	5	N/A
425	5	5.6
433	1, 2, 4	1.10, 2.1, 2.6
444	4	4.1c, 4.6
457	8	8.4, 8.5
563 (HBV ⁺)	13	13.8
571	8	8.4, 8.5

Table 3.4 Individual PARV4 NS peptides targeted by T cells in HCV⁺ patients. All patients in this table were HCV⁺ except 563, who was HBV⁺. N/A = sample not available for further peptide identification.

Patient	Peptide	Peptide sequence	Epitope sequence	HLA restriction	Length of epitope	Syfpeithi score	Bimas score	NetMHCpan 2.2 score	ELISpot responses	
									Peptide	SFU/10 ⁶ PBMC
115	4.6,4.7	NRQLSGPQGAIALPFTDAPP	QLSGPQGAIA	A*03	10mer	20	0.3	30uM	4.6	487.5
			GPQGAIALPF	B*35	10mer	x	20	WB		
117	3.2,3.3	DMVGRECSNWLRWKVFMAG	WLRTWKVF	B*08	8mer	18	0.2	WB	3.2	2047.5
			WLRTWKVFM	B*08	9mer	13	2	WB	3.3	2227.5
146	13.1 13.7,13.8	LHICPPGGPKPYGLF LSRSEAHLINRRFIWAWW	WLRTWKVFM	B*08	10mer	x	8	WB		
			WLRTWKVFM	B*08	10mer	x	8	WB		
324	2.4,2.5	LFASHAVTLPCRVPKPSMFLQ	HICPPGGPKPY	B*15	11mer	x	x	WB	13.1	255
			HLINRRFIWA	A*02	10mer	16	1.8	WB	13.7	125
388	2.4	LFASHAVTLPCRVPK	RSEAHLINR	A*31	9mer	x	0.24	SB	13.8	112.5
			AVTLPCRVPK	A*03	9mer	28	0.3	1.2uM	2.4	85
433	1.10,2.1	RESVPFFDHAVNLGYCILQQ	AVTLPCRVPK	A*1101	9mer	24	0.2	WB		
			ASHAVTLPCR	A*1101	10mer	22	0.004	WB		
444	4.1c 4.6	KLPLNDCCYAWTNID NRQLSGPQGAIALPF	LPCRVPKPSM	B*0702	9mer	17	20	WB	2.5	162.5
			AVTLPCRVPK	A*03	9mer	28	0.3	1.2uM	2.4	85
422	Pool 5	EGVPPTMAGKGTQRFMDLIDWLVENGIAIEK RWLSVNVKLSYRSFLGSSGGVLQATNALQI	ASHAVTLPCR	A*1101	10mer	22	0.004	WB		
			AVTLPCRVPK	A*1101	9mer	24	0.2	WB		
433	1.10,2.1	RESVPFFDHAVNLGYCILQQ	ASHAVTLPCR	A*1101	10mer	22	0.004	WB		
			ASHAVTLPCR	A*1101	10mer	22	0.004	WB		
444	4.1c 4.6	KLPLNDCCYAWTNID NRQLSGPQGAIALPF	ASHAVTLPCR	A*1101	10mer	22	0.004	WB		
			ASHAVTLPCR	A*1101	10mer	22	0.004	WB		
422	Pool 5	EGVPPTMAGKGTQRFMDLIDWLVENGIAIEK RWLSVNVKLSYRSFLGSSGGVLQATNALQI	ASHAVTLPCR	A*1101	10mer	22	0.004	WB		
			ASHAVTLPCR	A*1101	10mer	22	0.004	WB		

Table 3.5 Possible epitopes as determined from ELISpots and bioinformatic prediction software. Epitope predictions for peptides identified to elicit responses through ELISpot were compared in programmes Syfpeithi, BIMAS and NetMHCpan 2.2. These were done using the known HLA types of the patients. Highlighted in blue are peptides eliciting responses in two individuals. Highlighted in red are epitopes with high scores in more than one programme. WB: weak binder, SB: strong binder as defined by NETMHCpan 2.2. “x” suggests no score was available in this programme.

responses. Thus, no further epitopes were determined. The highest scoring predictions, including a potential B*08 epitope, are indicated in Table 3.5.

3.5. $\gamma\delta$ TCR cells are specifically activated in the presence of PARV4 peptides

Upon testing further patients, a second subset of T cells came to attention, in addition to conventional TCR $\alpha\beta$ CD8⁺ T cells. Figure 3.9A shows that these cells were CD3⁺ yet CD4 and CD8 negative and produced IFN- γ in response to peptides 3.2 and 3.3. This suggested that the cells producing IFN- γ were either $\gamma\delta$ TCR or NKT cells. This was identified in the individual with the highest response observed by ELISpot (>2000 SFU/10⁶ PBMC), and the assay was repeated using a $\gamma\delta$ TCR antibody in subject 117 (Figure 3.9B), and in two other patients where the production of IFN- γ by CD8⁺ T cells had not been detected but that had positive ELISpot responses. In total, three individuals showed PARV4-specific $\gamma\delta$ TCR⁺ T cell responses: patients 117, 146 and 422. These three individuals responded to different PARV4 NS peptides, as determined by ELISpot (Table 3.6) and only had HLA-A*02 in common (Table 3.7). No epitope was obvious from potential anchor residues in peptides 3.2, 3.3, 13.1, 13.7 or 13.8. Due to limited samples, responses by 422 to pool 5, and responses by 146 to pools 3 and 5 were not identified at the peptide level.

The $\gamma\delta$ TCR⁺ T cell response for 117 was first identified after culturing the PBMC illustrated in Figure 3.9A with peptides 3.2 and 3.3 for 7 weeks (Figure 3.9B), and represented 1.69% of $\gamma\delta$ ⁺ T cells (0.56% of CD3⁺ T cells). This experiment was carried out simultaneously with an individual known to have a CD8⁺ T cell

response to the RMT epitope (Figure 3.9C). This confirmed the presence of antigen-specific $\gamma\delta^+$ T cells.

Individual 146 elicited a T cell response to several pools. PBMC from a sample from October 2009 elicited a $\gamma\delta^+$ T cell response to peptides 13.1, 13.7 and 13.8 (grouped in one stimulation, Figure 3.10A). This represented 3.13% of $\gamma\delta^+$ T cells (0.1% of CD3⁺ T cells) and interestingly, the cells producing IFN- γ appeared to be $\gamma\delta^+$ bright T cells. This response was seen again in October 2010 with 1.34% of $\gamma\delta^+$ bright T cells producing IFN- γ (1.27% to pool 13, Figure 3.10B). This second sample also showed $\gamma\delta^+$ T cell responses to pools 3 and 5 (0.73% and 1.16% respectively, Figure 3.10C).

The responses in individual 422 were more variable. 8.45% of $\gamma\delta^+$ T cells were stimulated in October 2008 (Figure 3.11A). By June 2010, these had decreased to 0.38% (Figure 3.11B), and a CD8⁺ T cell response became more obvious (Figure 3.11C). By December 2010, no $\gamma\delta^+$ T cell responses could be detected (Figure 3.11D).

Various attempts were made to culture out peptide specific cell lines in order to further define these findings, but we were unable to culture an antigen-specific $\gamma\delta^+$ T cell line. Cells from all three patients were sent to our collaborators in Cardiff for further experimentation, but again without success upon culture. Since the responses were rare, and there were limited cells available from the patients, these findings were not further pursued in this thesis.

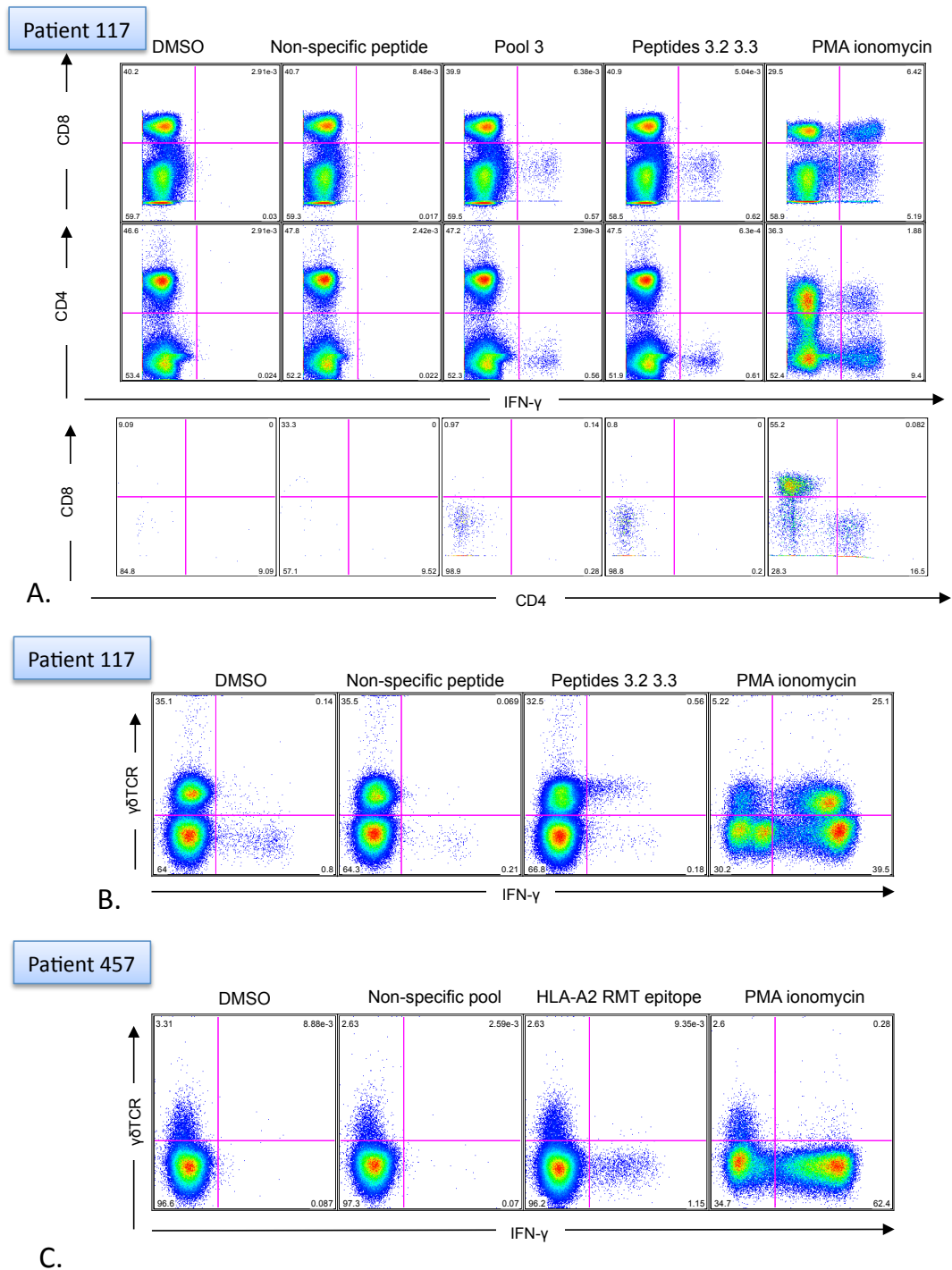


Figure 3.9 $\gamma\delta$ TCR T cells are specifically stimulated by PARV4 NS peptides. **A.** $CD3^+CD8^-$ and $CD3^+CD4^-$ T cells were found to produce IFN- γ in response to PARV4 NS peptides *ex vivo* (top two rows), these were confirmed to be negative for both CD4 and CD8 (bottom row of A, gated on $CD3^+IFN-\gamma^+$). **B.** These cells were cultured for 7 weeks and stained for $\gamma\delta$ TCR T cells, which was confirmed to be the responding subset. This culture had a high background sample (DMSO) but the non-specific peptide pool (pool 5) acts as a clearer negative control. 457 (**C**) and 117 (**B**) were stimulated simultaneously to prevent inter-experimental changes, and confirmed the specific stimulation of $\gamma\delta$ TCR $^+$ T cells by PARV4 peptides.

Patient	Response to:
117	Peptides 3.2, 3.3
146	Peptides 13.1, 13.7, 13.8 Pools 3 & 5
422	Pool 5

Table 3.6 PARV4 NS peptides and peptide pools eliciting a response in individuals with $\gamma\delta$ TCR⁺ T cell response.

146	A0201	A310102*	B4001*	B1501*	C0304	C0304	DRB10401	DRB10404
117	A0101*	A02	B0801	B18	C0701*		DRB10301*	DRB112
422	A*02	A*03	B*44	B*35	Cw*0401/05/08-	Cw*??	DRB1*0101/05/07-13	DRB1*1501/06/09/12/13/

Table 3.7 HLA-types for individuals with $\gamma\delta$ TCR⁺ T cell response.

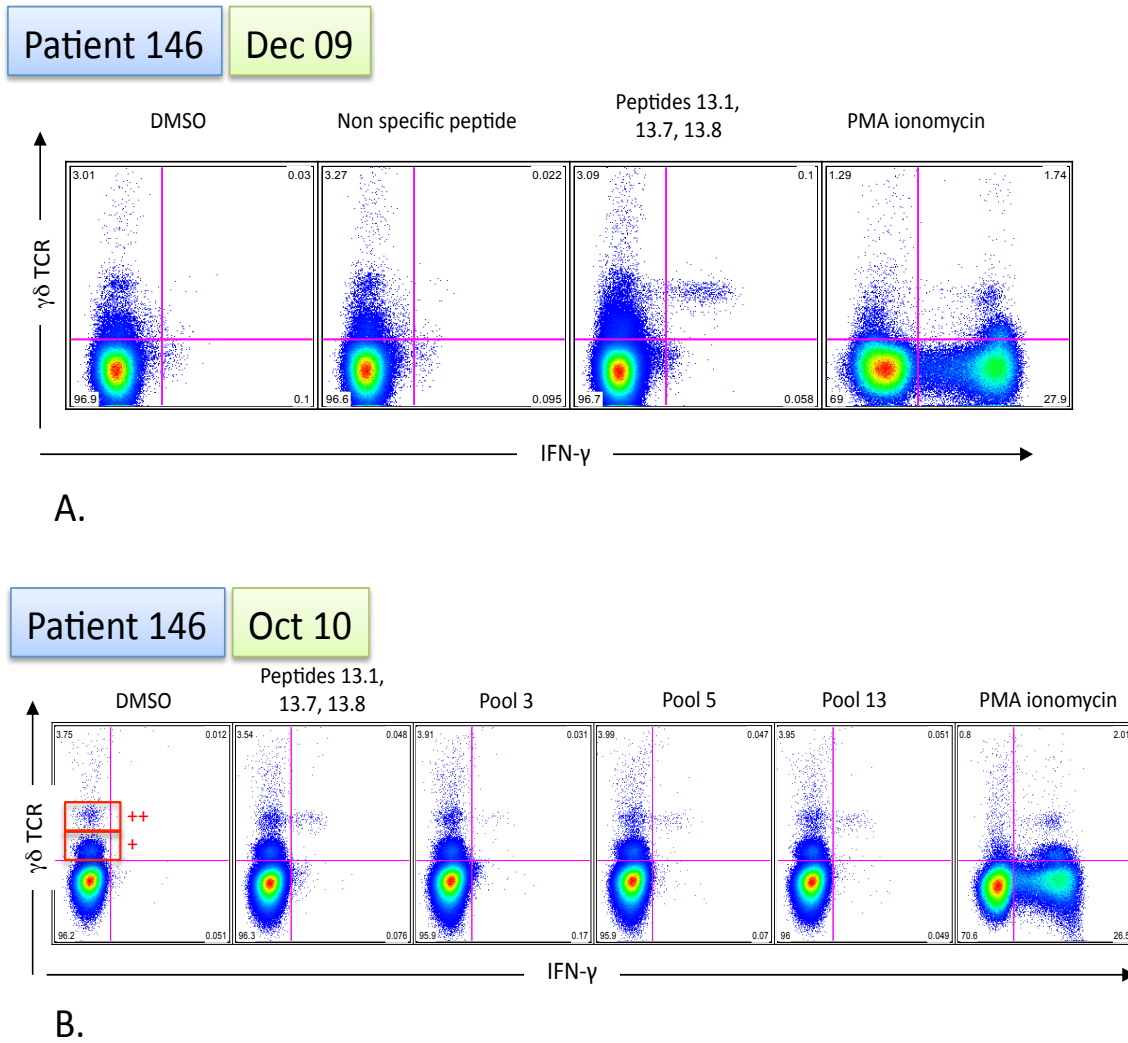


Figure 3.10 $\gamma\delta\text{TCR}^+$ T cell response by patient 146 in October 2009 (A) and October 2010 (B). PBMC in **A.** were tested from frozen, and in **B.** were tested from fresh blood. This patient shows two clearly distinct $\gamma\delta^+$ T cell populations: one bright (++) and one mid (+) represented in the first panel in **B.** The bright cells were specifically stimulated by PARV4 NS individual peptides and peptide pools.

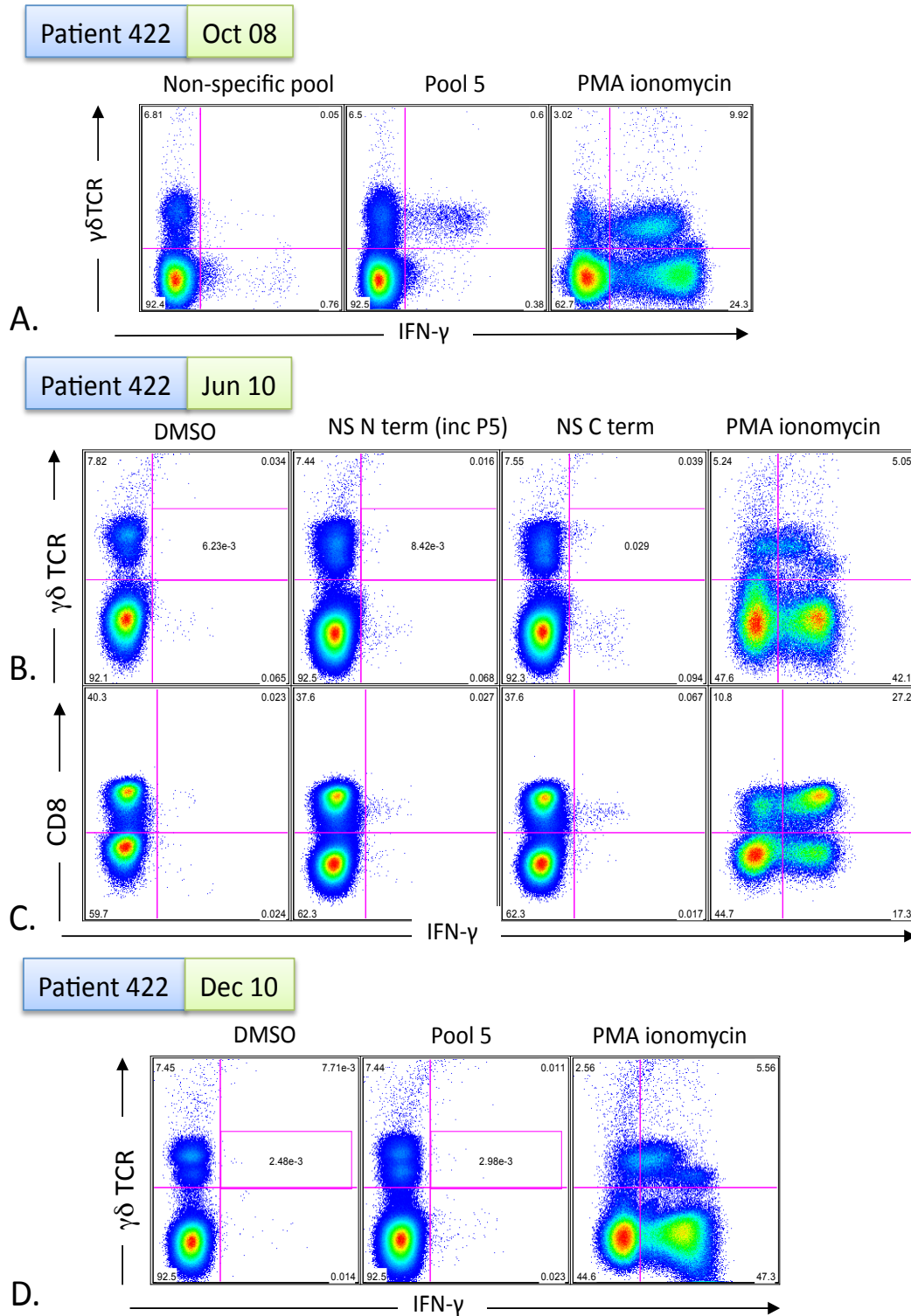


Figure 3.11 $\gamma\delta\text{TCR}^+$ T cell response by patient 422 is inconsistent over two years. PBMC were tested *ex vivo* from frozen. In June 2010 the $\gamma\delta\text{TCR}^+$ T cell response had decreased and a CD8^+ T cell response became apparent (C). In B and C peptide pools were grouped (N term = pools 1-6, C term = pools 7-13). D. The $\gamma\delta\text{TCR}^+$ T cell response had disappeared by December 2010.

4. Discussion

4.1. T cell responses to PARV4 NS are strong, frequent, broad and long-lasting

Strong T cell responses to PARV4 NS were detected in 23/88 HCV-infected individuals (26%), and in 3/13 HIV-infected individuals, two cohorts of individuals exposed to infectious pathogens. These data suggest that PARV4 infection is relatively common in these clinical groups, and may share transmission routes (parenteral or sexual).

The T cell responses were PARV4-specific as there were no T cell responses to PARV4 NS in healthy individuals or in acute B19 seroconverters. Over 60% of the adult population has been exposed to B19 (Heegaard and Brown, 2002), and these data, therefore, exclude the possibility of antigenic cross-reactivity with B19 parvovirus. Crossreactivity with HCV is unlikely for several reasons: responses were also detected in a HCV-negative HIV⁺ group; the key epitope RMTENIVEV does not have a clear homologue in HCV, and T cell responses to HCV-derived peptides in a patient group with persistent HCV infection are of extremely low frequency (Lauer et al., 2004). Additionally, there was a strong association between PARV4 serostatus and PARV4 T cell response (see Results).

T cell responses reached 2000 SFU/million PBMCs to a single 15mer peptide; thus, PARV4 appears to be highly immunogenic, at least in certain individuals. These T cell levels are comparable with other low-load persistent viruses such as CMV and EBV (Gillespie et al., 2000, Callan, 2003).

Most of the responses were concentrated at the N-terminus of the NS protein in both HCV⁺ and HIV⁺ cohorts. This protein is poorly characterised functionally,

although it contains two known motifs: a rolling circle replication motif, found at the N-terminus, and an ATPase domain (Astell et al., 1987, Ding et al., 2002, Fryer et al., 2007a). The latter was represented by pool 7 of the NS peptides, which was shown to frequently elicit T cell responses. Within the N-terminus of the NS protein there was no immunodominant epitope.

T cell responses were seen at a similar frequency to that previously found for PARV4 antibodies (33%) and for PARV4 DNA (30%) in other HCV-infected individuals (Fryer et al., 2007d, Sharp et al., 2009). This corroborates the suggestion that PARV4 is parenterally transmitted. Nonetheless, with similar responses observed in HIV⁺ individuals infected through unprotected sexual exposure, there remains the possibility of sexual transmission, as suggested in other studies (Longhi et al., 2007, Simmonds et al., 2008).

There was no relationship between immune reactivity to PARV4 and age (Mann Whitney, $p=0.71$, Table 3.2). Parvovirus B19 has been shown to persist at very low levels in bone marrow, skin and synovia (Heegaard et al., 2002, Norja et al., 2006, Cassinotti et al., 1997). PARV4 may behave in a similar manner, having been detected in autopsy tissue and at very low viral loads in tissue without viremia (Manning et al., 2007). The T cell responses observed to PARV4 were sustained for periods of up to 3 years, similar to T cell responses to parvovirus B19 (Isa et al., 2005). This continuity suggests that PARV4 is either able to generate good memory responses, or is a low-level persistent virus providing a continuous supply of antigen to stimulate T cells, like parvovirus B19. As discussed in Chapter 1, there is evidence that long-term memory T cells can be maintained even in the absence of antigen (Wherry and Ahmed, 2004). Further

phenotyping will provide information on whether T cells are exposed to antigen or not. This is carried further in Chapter 5 in a cohort followed longitudinally.

The study of HIV⁺ individuals shows that T cell responses to PARV4 NS are detectable even in individuals with a degree of immune suppression (average CD4⁺ T cell count of the three responders was 410 cells/ μ l). In a different study, HCV and HBV infections did not affect the titre of PARV4; the authors suggest this may be due to viral control by the immune system (Touinssi et al., 2011). With the levels of T cell responses detected in my study, this would be very plausible. This may also explain why PARV4 DNA is so infrequently detected: with such a strong immune control, virus levels may be kept very low.

The time of PARV4 infection was unknown for these individuals, but most likely resulted from previous intravenous drug use. It is possible they acquired PARV4 simultaneously with HCV as they share transmission routes. Thus, with the level of T cell responses observed, these individuals are most probably chronically infected with PARV4. The dynamics of acute infection are studied in Chapter 5.

4.2. Both $\alpha\beta$ TCR CD8⁺ and $\gamma\delta$ TCR T cells are stimulated by PARV4

PARV4-specific CD8⁺ T cells have an effector memory phenotype (CCR7⁻CD45RA⁺, T_{EM}, Figure 3.5) suggesting recurrent antigen exposure as seen in CMV (Kern et al., 1999, Stone et al., 2006). However, further ICS assays pointed out that there were several patients with a PARV4-specific production of IFN- γ by CD3⁺ cells that were neither CD4⁺ nor CD8⁺, but $\gamma\delta$ TCR⁺ T cells. $\gamma\delta$ ⁺ T cells recognise a different spectrum of antigens to $\alpha\beta$ TCR T cells, and do not require antigen

processing and MHC presentation allowing direct recognition (Chien et al., 1996). In this sense, their recognition of antigen is similar to immunoglobulins (Allison et al., 2001). $\gamma\delta^+$ T cells are expanded in the peripheral blood of HIV-infected patients (Boullier et al., 1995, De Maria A, 1992), where they may play an essential role as they can respond to infected cells without the threat of infection by HIV-1 (Sciammas and Bluestone, 1999). $\gamma\delta^+$ T cells are also more frequent in the livers of patients with chronic hepatitis (Agrati et al. 2001, Nuti et al, 1998). Moreover, $\gamma\delta^+$ T cells in patients with Herpes Simplex Virus-1 (HSV-1) can lyse HSV-1 infected target cells (Maccario et al., 1993).

In most studies, the antigens that activate $\gamma\delta^+$ T cells appear to be 'generic', such as a heat shock-derived protein or a phospho-antigen (Bukowski et al., 1995) or another host molecule stimulated by viral infection, rather than a viral antigen itself (Ponniah et al., 1996). Sciammas *et al.* did find, however, a $\gamma\delta^+$ T cell clone specific for an unprocessed HSV-1 viral envelope glycoprotein I (gI), and identified the epitope at the N-terminus (Sciammas and Bluestone, 1998, Sciammas et al., 1994). This recognition was independent of MHC class I and II presentation. However, with no successful expansion of these cells from the individuals presented in this chapter, PARV4-specific $\gamma\delta^+$ T cells could not be further characterised.

4.3. Discrepancy between the humoral and cellular responses to PARV4

Out of the 84 HCV⁺ individuals tested in both assays, 21 individuals made T cell responses to PARV4, but only 14 also had antibodies to PARV4. Moreover, 8

more individuals had antibodies to PARV4, but no T cell response. There could be several reasons for this discrepancy. First, the detection thresholds of the two assays are not directly comparable as one detects circulating antibody, and the other, individual cytokine-secreting cells. The lack of IgM in individuals with no IgG suggests they were not acutely infected. There may be an issue of differential specificity since the ELISpot assay identifies immune responses to the PARV4 NS protein, whereas the ELISA detects antibodies to the PARV4 VP protein. As VP is a capsid protein, it is expected it would be a primary target for antibodies, whereas CD8⁺ T cells may preferentially recognise the NS protein, once expressed intracellularly. So, potentially, where antibody is detected but no T cell responses to NS were seen, this may reflect the induction of VP-specific T cells rather than NS-specific T cells. A similar phenomenon has been reported for parvovirus B19 (Modrow and Dorsch, 2002, Norbeck et al., 2005). However, no T cell responses to PARV4 VP could be detected in the individuals tested, although they could be below the threshold of detection. It is also possible that the antibodies having neutralised the infection, antigen is not available to be 'seen' by T cells.

Both assays look at peripheral blood T cells in individuals. While antibodies may be freely circulating, it is possible that T cells activated by PARV4 are compartmentalised to tissues, as seen in HCV infection where CD8⁺ T cells are enriched in the liver (Ward et al., 2004). This is also supported by the loss of CCR7 expression by antigen-specific cells, that therefore no longer home to lymphoid organs. Moreover, PARV4 DNA has also been found in many tissues (reviewed in Appendix Table AT3) (Manning et al., 2007, Longhi et al., 2007, Schneider et al., 2008b). The lack of VP-specific antibodies was surprising, but

consistent, as both assays were repeated for several individuals at multiple time points. There may have been a loss of circulating antibody over time, as occurs with HCV after spontaneous control, whereas T cells may be detected over longer periods. Takaki *et al.* (2000) tested a cohort of women infected by the same HCV strain, and observed that HCV-specific antibodies were undetectable in many patients 18-20yrs after recovery; whereas HCV-specific CD4⁺ and CD8⁺ T cells persisted. These antibodies gradually decreased over time after initial infection. The authors compared it to the loss of antibodies to early EBV antigens after recovery from infection (Takaki *et al.*, 2000, Henle *et al.*, 1970). Sensitive analysis of antigen-specific memory B cells might reveal low frequency populations, as has been previously described after HBV vaccination (Ward *et al.*, 2008). Maintenance of T cell responses in such seronegative persons might then reflect low-level persistence of antigen in tissue or potentially continued limited transcription of NS – analogous to the setting in CMV infection (Simon *et al.*, 2006). Antigenic variation within parvoviruses resulting from distinct circulating genotypes may also impact on the sensitivity of both assays, although this should be limited as there is minimal amino acid sequence variability between the three genotypes (Simmonds *et al.*, 2008).

Discrepancies between the two arms of the adaptive immune response have been observed in exposure to CMV and HIV-1. Zhu *et al.* found that individuals could have a cellular response, or a humoral response to CMV antigen, or both, or neither (Zhu *et al.*, 2001). Individuals with high rates of exposure to HIV-1 but who remained uninfected were able to mount a cellular response, despite the absence of antibodies to HIV-1 (Rowland-Jones *et al.*, 1993, Rowland-Jones *et al.*, 1995, Clerici *et al.*, 1992). It is likely these individuals were immunised by

repeated exposure to the virus. It has been shown that low doses of virus can prime the cellular response whilst the humoral system is not stimulated (Clerici and Shearer, 1993). This issue is further addressed by looking at longitudinal samples in Chapter 5.

This study was the first to identify T cell responses and to define a CD8⁺ T cell epitope for the novel parvovirus PARV4. Until recently this virus was only detected by looking for viral DNA. Through the availability of serological assays (Sharp et al., 2009) and now T cell assays, the prevalence of PARV4 in different populations and its effect on the immune system can be more readily assessed.

5. Summary of findings

- T cell responses to PARV4 NS protein are strong, frequent, broad and long-lasting in HCV⁺ and HIV⁺ individuals
- These T cell responses preferentially target the N terminus of the NS protein
- 26% of HCV⁺ infected individuals are PARV4 seropositive or have T cell responses to PARV4 – this supports parenteral transmission of PARV4
- The presence of humoral and cellular responses only partially overlaps
- The first PARV4 epitope is defined: HLA-A*02-restricted RMTENIVEV
- PARV4 epitope-specific CD8⁺ T cells have an effector memory phenotype
- In certain individuals, $\gamma\delta^+$ T cells are also stimulated by PARV4 NS peptides, and are peptide-specific
- The large responses observed in these individuals, who are most likely chronically infected with PARV4, suggest viral persistence

Chapter 4

Investigating The Clinical Significance Of PARV4

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1. Introduction

As seen in Chapter 3, PARV4 infection is common in individuals at risk of acquiring blood-borne infections. PARV4 has been frequently associated with HIV-1 and HCV infections in the extensive literature reviewed in Chapter 1 and in Appendix Table AT3. These viral infections cause serious disease and are a burden to a significant proportion of the world's population; hence we were interested in investigating the clinical significance of PARV4.

PARV4 has been found in many tissues including the bone marrow of HIV-infected individuals (Manning et al., 2007) and the liver of HCV-infected individuals (Schneider et al., 2008b, Corcioli et al., 2010). Despite the detection of viral DNA in so many organs, PARV4 has rarely been linked to specific symptoms except for a recent study relating PARV4 infection to encephalitis (Benjamin et al., 2011), and the individual in whom this virus was originally discovered. The latter presented with acute infection, with symptoms including fatigue, night sweats, pharyngitis, neck stiffness, vomiting, diarrhoea, arthralgias and confusion (Jones et al., 2005). Determining a pathological association is problematic as PARV4 is so frequently found in individuals burdened with disease related to HCV or HIV-1 infections. However, as a common co-infection with these pathogens it may have an important impact on disease progression or outcome. This is of increased importance in light of the high frequency T cell responses observed against PARV4 NS protein (Chapter 3) and the potential for immunopathology. Since viral antigen can be expressed in both lymphoid and hepatic tissues (Manning et al., 2007, Schneider et al., 2008b), an impact on both HCV and HIV-1 pathogenesis is plausible.

This chapter looks at the impact of PARV4 serostatus on HCV- and HIV-related disease progression in two prospective cohorts of HCV and HIV-infected individuals (mono- and co-infected). HCV viral clearance, genotype, patient gender, age, ALT (alanine amino-transferase) levels, tissue histology (liver fibrosis), CD4 slopes and CDC events were analysed in relation to PARV4 serostatus.

Aims:

- To compare the prevalence of PARV4 in populations with different co-infections and risk factors (HCV and HIV, intravenous drug user (IDU) and men who have sex with men (MSM))
- Determine whether PARV4 infection has any effect on the progression of HIV or HCV disease

2. Methods

2.1. Study subjects and sample collection

Local ethical approval (MREC/98/3/55 for the Trent HCV cohort and <http://www.shcs.ch/30-study-design> for the Swiss HIV cohort) and informed patient consent was obtained from individuals in two cohorts, totaling 469 subjects (Table 4.1). The first cohort was made up of 193 individuals from the Swiss HIV cohort (Rauch et al., 2005). It was divided into 99 HIV⁺ HCV⁻ men who have sex with men (MSM) and 94 HIV-HCV co-infected intravenous drug users (IDUs). The second cohort was made up of 276 individuals from the Trent HCV cohort (Table 4.2). This group was divided into 44 individuals who cleared HCV and 232 who became chronically infected. Biopsy data was also available for 241 individuals, and these were divided into 143 individuals who had persistently mild liver fibrosis (Ishak fibrosis score always 0 or 1, between 1 and 5 biopsies were taken per individual, across 1 to 25 years) and 98 who progressed to severe fibrosis (Ishak fibrosis score 5 or 6). Plasma samples were taken by each centre, and frozen until required. One serum sample per individual was available for serological screening, as described in Chapter 2.

2.2. CD4 slopes and CDC events

CD4⁺ T cell counts before starting antiretroviral therapy were available for 114/193 individuals from the Swiss HIV cohort. CD4 slopes were calculated using linear regression in GraphPad Prism when at least three time points were available, spreading across a minimum of 6 months (number of time points: 3-34

Cohort	Main risk factor ¹	n=	HCV	HIV	Gender % male	HCV genotype at diagnosis	PARV4 serostatus
Swiss HIV	MSM	99	-	+	100%	-	11%
	IDUs	94	+	+	74%	60% genotype 1 26% genotype 3	95%
Trent HCV	66% IDUs	276	+	-	67%	40% genotype 1 48% genotype 3	31%

Table 4.1 Demographic data and PARV4 seroprevalence. HCV genotypes were only available for 58/94 of the Swiss cohort. The percentages shown represent proportions of known genotypes. ¹MSM = men who have sex with men, IDUs = intravenous drug users.

	PARV4 serostatus	n=	PARV4 IgG positive	PARV4 IgG groups	n=	Age range in years (median)	Gender distribution	ALT range (median)	Number of patients with known HCV genotype	HCV genotype at diagnosis	Main risk factor
HCV RNA status	Patients who cleared HCV (always PCR negative)	44	36%	lgG neg	28	31-86 (45)	43% female 57% male	3-69 (31)	0	-	61% IDUs 39% non IDUs
				lgG pos	16	31-71 (51)	0.375 female 0.625 male	15-244 (26)	0	-	75% IDUs 25% non IDUs
	Patients who didn't clear HCV	232	29%	lgG neg	164	21-88 (52)	32% female 68% male	8-822 (51)	159	40% genotype 1 45% genotype 3 15% other	56% IDUs 44% non IDUs
				lgG pos	68	29-84 (56)	31% female 69% male	5-303 (46)	63	40% genotype 1 54% genotype 3 6% other	71% IDUs 29% non IDUs
Fibrosis progression	Always mild (IS score 0 or 1)	143	29%	lgG neg	102	21-83 (48)	33% female 67% male	8-822 (49)	94	49% genotype 1 35% genotype 3 16% other	65% IDUs 35% non IDUs
				lgG pos	41	29-73 (49)	32% female 68% male	5-303 (58)	35	40% genotype 1 51% genotype 3 9% other	73% IDUs 27% non IDUs
	Progressed (IS score 5 or 6)	98	31%	lgG neg	68	32-88 (58)	31% female 69% male	18-253 (79)	65	26% genotype 1 60% genotype 3 14% other	43% IDUs 57% non IDUs
				lgG pos	30	44-84 (60)	33% female 67% male	29-179 (72)	28	39% genotype 1 57% genotype 3 4% other	67% IDUs 33% non IDUs

Table 4.2 The Trent HCV cohort demographics. This cohort was divided into 44 individuals who cleared HCV and 232 who became chronically infected. Biopsy data was available for 241 individuals, and these were divided into 143 individuals who had persistently mild liver fibrosis (Ishak fibrosis score always 0 or 1) and 98 who progressed to severe fibrosis (Ishak fibrosis score 5 or 6). The ALT column represents the closest available ALT value to the plasma sample tested. IDU = intravenous drug user, non-IDU = any other risk factor such as tattoo or blood transfusion

median 7; spreading across 6 to 34 months). CD4⁺ T cell counts were plotted relative to time, where day 0 was the first available sample date (examples in Appendix figure A8). The CD4 slopes of PARV4 seropositive or seronegative individuals were then compared using a Mann Whitney test. Kaplan-Meier survival curves were used to compare time to reach CDC-B, CDC-C events and death, defined by the US Centres of Disease Control and Prevention as hallmarks of HIV-related disease (1992). Symptoms are defined as category B symptomatic conditions (CDC-B) when they are “attributed to HIV infection or indicate a defect in cell-mediated immunity” and/or are “considered to have a clinical course or management that is complicated by HIV infection”. Category C symptoms (CDC-C) are indicators of AIDS and include conditions such as recurrent bacterial pneumonia, candidiasis, HIV-related encephalopathy or Kaposi sarcoma (non-exhaustive list)(AIDS ETC, 2011). Log rank (Mantel-Cox test) was used to calculate the p values of each curve.

2.3. IL28B SNP rs8099917 genotyping

DNA was extracted from whole blood from 21 HCV-infected individuals from the Trent HCV cohort. The alleles for SNP rs8099917, in proximity of the IL-28B gene, were determined by real time PCR using TaqMan® SNP Genotyping Assays kit (Applied Biosystems, C_11710096). Patients DNA added to these reagents were run according to manufacturer’s instructions on a Light Cycler 480 (Roche). Cycling parameters were: one cycle at 95°C for 10 min, 45 cycles at 92°C for 15 s, and 60°C for 1 min, followed by one cycle at 40°C for 10 s. These samples were tested by Cristina Bucci (Barnes group, University of Oxford).

2.4. Statistical analyses

Statistic analyses were carried out using the Mann Whitney test, Fisher's exact test and log rank (Mantel-Cox test) using GraphPad Prism software. For more detailed statistic analyses on the Trent HCV cohort, the open-source R software (www.r-project.org) was used to carry out a logistic regression to analyse the simultaneous relationship of age, gender, main risk factor (IDU or MSM) and ALT on PARV4 serostatus. The HCV genotype parameter was excluded as this information was not available for the individuals that were PCR negative. The log odds of being PARV4 seropositive were modelled as a linear function of all variables potentially affecting this. The formula hence being:

$$\text{Log odds (PARV4 serostatus)} \sim \text{age} + \text{gender} + \text{main risk factor} + \text{ALT}$$

where “~” implies relationship. The coefficients of the model have approximately normal distributions; therefore, p-values were obtained to compare individual coefficients. This analysis was carried out on the 263 individuals of the Trent HCV cohort with complete datasets. This latter analysis was carried out by Eleni Frangou (Department of Statistics, University of Oxford). Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. PARV4 and HIV-1 co-infection

3.1.1. PARV4 strongly correlates with intravenous drug use

To address whether PARV4 has an impact on HIV-1 disease progression, PARV4 serostatus was determined in 193 individuals from the Swiss HIV cohort, using an ELISA to capture PARV4-specific antibodies (Table 4.1). There was a striking association between PARV4 status and clinical group: 95% of the HIV⁺HCV⁺ IDU subjects were PARV4 seropositive compared to only 11% of the HIV-1⁺ MSM group (Figure 4.1, $p < 0.0001$).

3.1.2. PARV4 infection is linked to CDC-B event occurrence

CD4⁺ T cell levels decline as HIV-1 infection progresses. This change is assessed by calculating the slope of decline (for examples, see Appendix Figure A8). The CD4 slopes of the HIV⁺ individuals in this cohort were analysed in relation to PARV4 status using serial CD4 counts available for 114 patients: 49 PARV4 IgG negative individuals with CD4 slopes ranging from -41.3 to 5 CD4⁺ T cells/ μ l/month (median -6.2), and 65 PARV4 IgG positive with CD4 slopes ranging from -32.6 to 7.6 CD4⁺ T cells/ μ l/month (median -6.3, Figure 4.2A). Using this approach we could not detect a difference in CD4⁺ T cell decline between serology groups ($p = 0.89$).

These data were also analysed comparing the clinical groups (IDU or MSM, Figure 4.2B and C), but the presence of PARV4 antibodies was not significantly different either ($p = 0.63$). However, there were only 7 MSMs that were

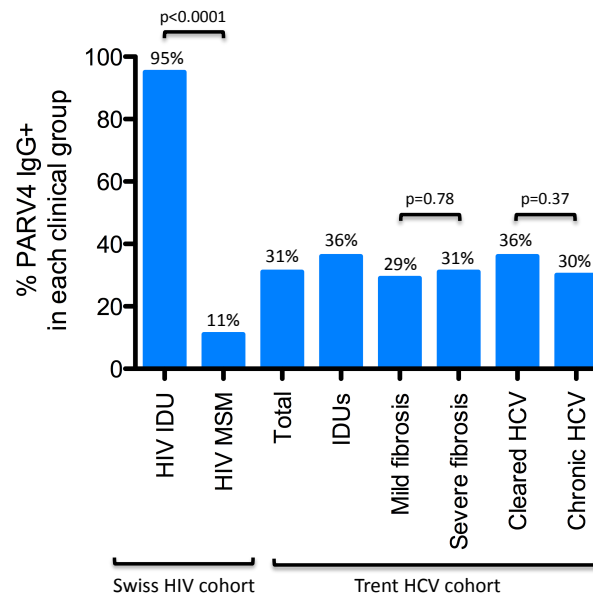


Figure 4.1 Seroprevalence of PARV4 in the HIV and HCV cohorts. This graph represents all the groups of subjects from the Swiss HIV cohort and the Trent HCV cohort tested for PARV4 IgG. IDU = intravenous drug user, MSM = men who have sex with men. The clinical groups in the Trent HCV cohort are defined in the methods section. Fisher's exact tests were carried out to calculate the p values.

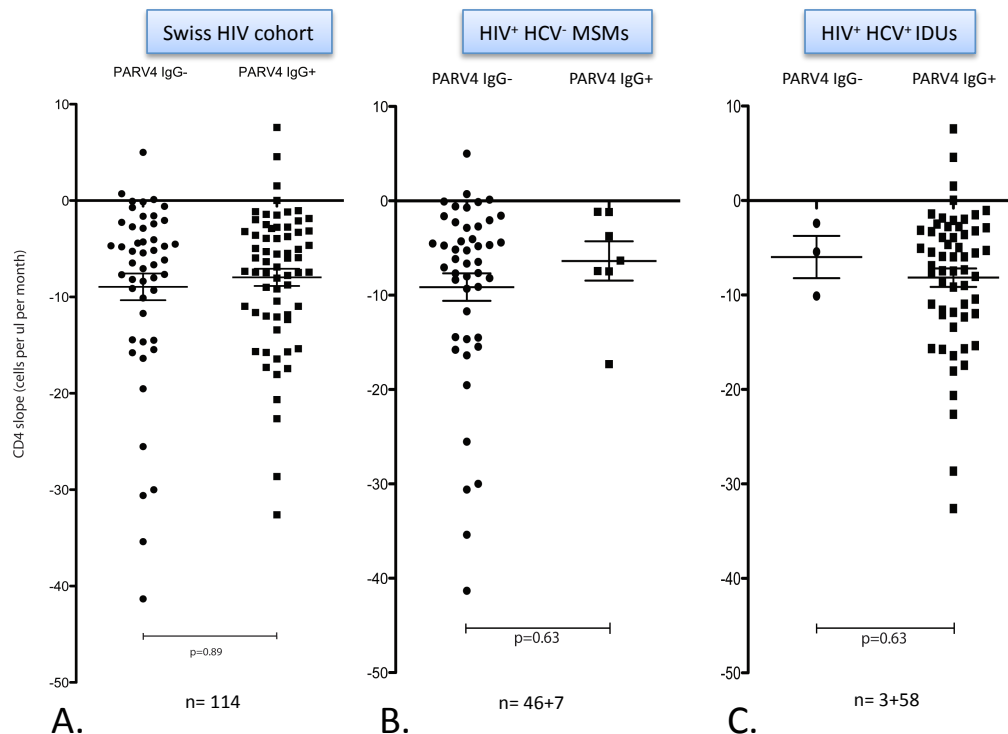


Figure 4.2 CD4⁺ T cell slopes in the Swiss HIV cohort. CD4⁺ T cell slopes were calculated for 114 subjects and plotted according to their PARV4 serostatus. This was done for the entire cohort (A) and for each clinical group: MSM (B) and IDU (C). The number of subjects in each group is indicated beneath each plot. Mann Whitney tests for each group were carried out and p values are indicated in each graph.

PARV4 seropositive, and 3 IDUs that were PARV4 seronegative. Due to this unequal distribution of the individuals by serology, the size of the clinical groups prevented strong statistical analysis.

Limited data points were available for CD4⁺ T cell counts for these individuals which had an important effect on the standard error of the slope and the goodness to fit (examples are given in Appendix Figure A8). A statistical model to take into account the variation of multiple measurements within each individual, the variation in the number of data points between different subjects and the variation in the standard error of CD4 slopes, would have been a more rigorous analysis of this data.

As another robust indicator of HIV-1 disease progression, the occurrence of CDC-B and CDC-C events was analysed (see Methods). The time to reach these hallmarks, as well as the time before death, were compared in PARV4 seropositive and seronegative groups (Figure 4.3A). Keeping in mind that HCV infection was a confounder in half of this population, a correlation was seen between PARV4 serology and time to progress to CDC-B events from the time of seroconversion for HIV, where PARV4 seropositive individuals were faster at presenting CDC-B symptoms ($p=0.02$, top left panel). However, the association was not significant for CDC-C or death ($p=0.39$ and $p=0.11$ respectively).

The time to progress to these hallmarks was also compared within the clinical groups (MSM, Figure 4.3B and IDU Figure 4.3C). No significant difference was found. It should be noted that the sizes of some of the groups of individuals, when sorted by clinical and PARV4 serology, were very small (e.g.: data concerning death was only available for 1 individual that was MSM and PARV4 seropositive), thereby limiting the power of the analysis. Thus, in this analysis

we did observe an association between PARV4 serostatus and early HIV-1 disease progression, but the close association between PARV4 serostatus, risk factor for HIV-1 transmission and HCV serostatus made the individual components of risk difficult to further dissect.

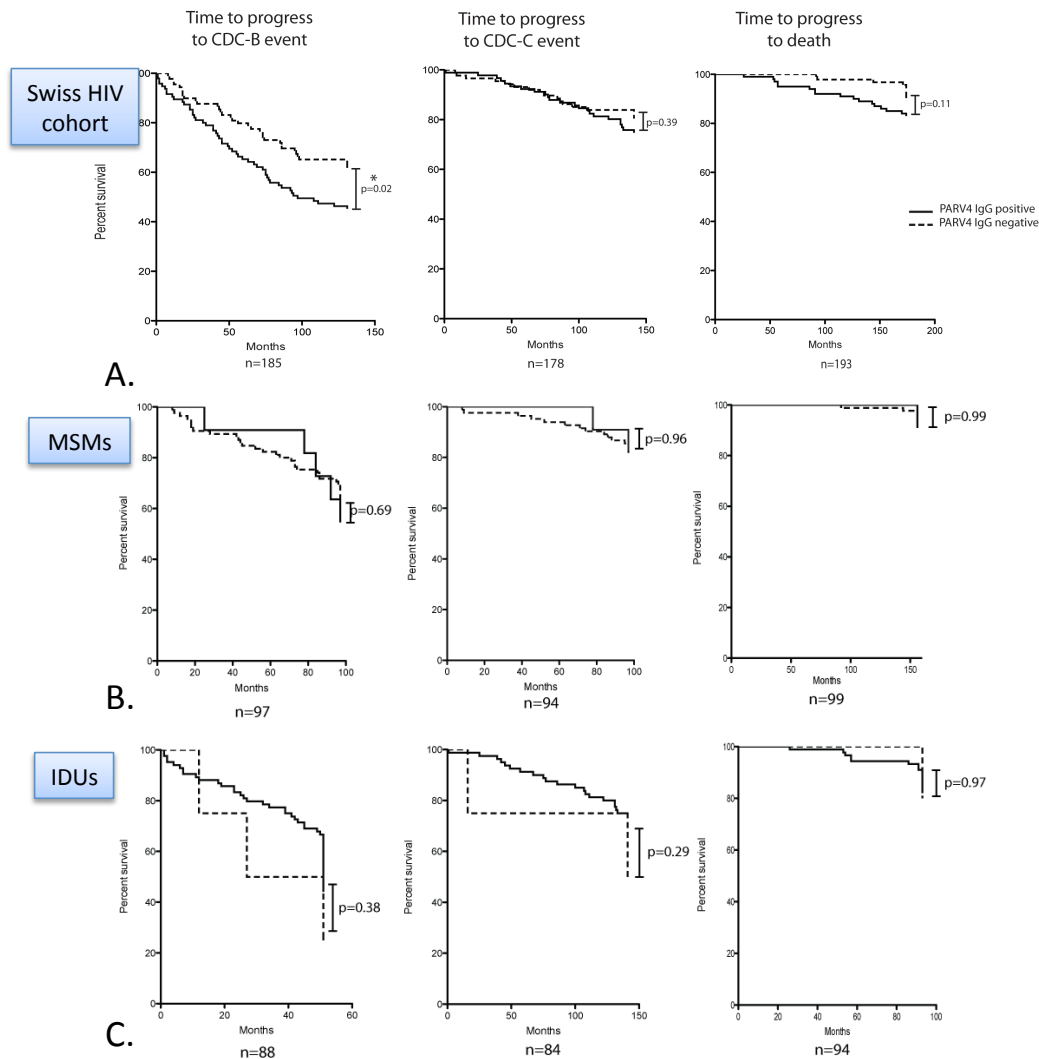


Figure 4.3 Time to progress to CDC-B, CDC-C events and death in each PARV4 serology group. Data was available for a limited number of individuals for each set of events. Individuals from the Swiss HIV cohort were grouped in **A** and split by clinical group in **B** and **C**. Time is expressed in months since HIV seroconversion. Each survival curve was compared using log-rank (Mantel-Cox test) and p-values are indicated adjacent to each graph. * represents statistical significance, which was achieved only when all subjects were grouped and for the time to progress to CDC-B event (A, left panel).

3.2. PARV4 and HCV co-infection

3.2.1. PARV4 infection is also common in HCV mono-infected individuals

The prevalence of PARV4 was also studied in 276 HCV-infected HIV-negative individuals from the Trent HCV cohort. 31% of individuals were PARV4 seropositive. Looking at IDUs only, this reached 36% (Figure 4.1).

To determine whether PARV4 serostatus affected clinical outcome in HCV⁺ individuals, age, gender, HCV genotype, ALT levels and main risk factor (IDU or non-IDU) were compared in the PARV4 IgG⁺ group versus the PARV4 IgG⁻ group (Table 4.2 and Figure 4.4). Only individuals infected with genotypes 1 and 3 were compared in this analysis, as the number of those with other HCV genotypes was too small to be representative. No significant difference was seen between PARV4 seropositive and seronegative individuals in gender, HCV genotype, age or ALT levels ($p=0.89$, 0.64 , 0.26 and 0.34 respectively, Table 4.2 and Figure 4.4). We did find a strong correlation between PARV4 seropositive individuals and intravenous drug use as a risk factor ($p=0.01$).

Furthermore, the parameters of age, gender, ALT and risk factor were analysed together with PARV4 serology using a logistic regression framework (see methods). The results of this analysis show that only the risk factor significantly correlated with the presence of PARV4 IgG, with IDUs being mainly PARV4 seropositive (Table 4.3, $p=0.01$).

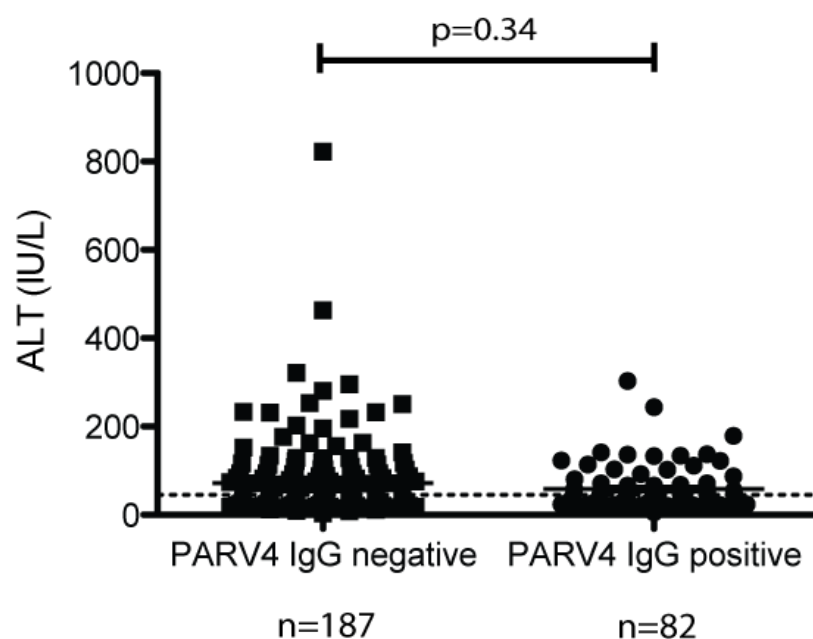


Figure 4.4 ALT levels in the Trent HCV cohort according to PARV4 serology. ALT levels were available for 269/276 individuals in this cohort. This test was carried out to determine any effect PARV4 infection may have on the progression of HCV-related disease, as assessed by liver damage reflected in ALT levels. These were compared with a Mann Whitney test, for which the p value is indicated above the data.

	Estimate	Standard Error	P value
Age	0.020	0.012	0.108
Gender	0.123	0.299	0.682
Main risk factor (IDU or non-IDU)	0.771	0.311	0.013
ALT	-0.003	0.002	0.206
(Intercept)	-2.26	0.757	0.003

Table 4.3 Results of the logistic regression model looking at the effect of PARV4 infection on age, gender, main risk factor and ALT in the Trent HCV cohort.

3.2.2. PARV4 does not affect the progression of HCV-related disease

To assess any impact of PARV4 serostatus on HCV disease progression, 276 individuals from the Trent HCV cohort were grouped by clinical outcome into a) spontaneous resolution versus chronic carriage and b) fibrosis progression. To examine this, these individuals were divided by PCR status: either persistently positive (n=232) or always negative (n=44), as an indication of spontaneous HCV clearance. 36% of individuals who cleared HCV were PARV4 seropositive versus 29% of individuals who did not clear. Fisher's exact test showed no significant difference in PARV4 serology between these two groups (p=0.37, Table 4.2, Figure 4.1).

Biopsy data was available for 241 of these individuals. They were divided into those who maintained a mild fibrosis score (Ishak score 0 or 1 over time, n=143) and those who progressed to severe liver fibrosis (Ishak score 5 or 6, n=98). 29% of the individuals who remained mild were PARV4 seropositive versus 31% of those who had progressive disease (Figure 4.1). Fisher's exact test showed no significant difference in PARV4 serology between these two groups (Table 4.2, p=0.78).

Additionally, the treatment outcome of 131 individuals from the Trent HCV cohort was compared with their PARV4 serostatus. Treatment outcome was grouped into successful (sustained virological response, SVR) and unsuccessful (non-responder or relapser) responses. No significant difference was seen between patients in these two groups (p=0.45, Figure 4.5).

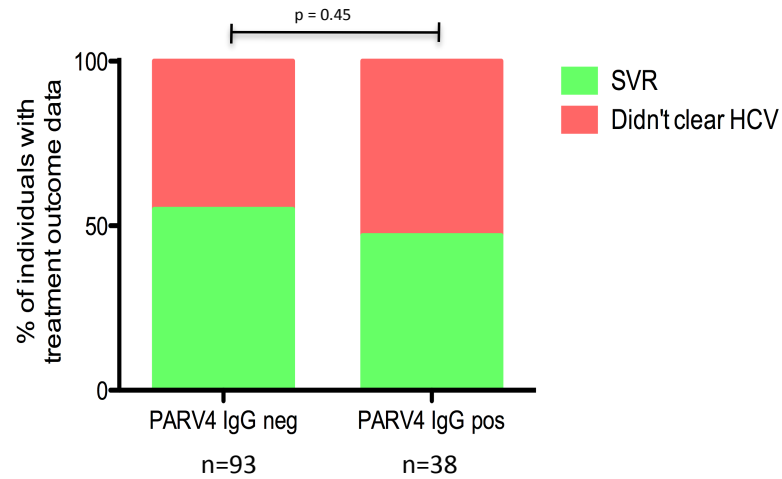


Figure 4.5 HCV treatment outcome relative to PARV4 serology. The treatment outcome for 131 individuals from the Trent HCV cohort was analysed to assess whether PARV4 serostatus had any affect on the likelihood of clearing HCV infection. SVR = sustained virological response. “Didn’t clear HCV” includes non-responders to treatment and individuals who cleared during treatment but then relapsed.

3.2.3. No propensity for PARV4 infection in different IL-28B SNP genotypes

It is likely that a combination of multiple factors influences the outcome of infections such as HCV. These may include other co-infections such as HIV or even PARV4, and will include host genetics such as HLA-type and single nucleotide polymorphisms (SNPs), for example in the proximity of the IL-28B gene.

Variations in the IL-28B SNP rs8099917, specifically the TT allele, have been shown to be associated with improved treatment outcome (Aparicio et al., 2010, Rauch et al., 2010, Suppiah et al., 2009, Tanaka et al., 2009). To determine whether PARV4 may contribute to the effects seen, the distribution of IL-28B SNP rs8099917 genotype was tested according to PARV4 serology. As a pilot study, 21/276 individuals were genotyped by real time PCR. Results are shown in Table 4.4. 3/9 individuals with GG/GT genotype were PARV4 seropositive, compared to 4/12 with TT genotype, thus there was no difference between the two groups. Due to this result, this study was not extended to the rest of the cohort.

IL-28B SNP genotype	n =	Number of PARV4 seropositive individuals (%)
GG/GT	9	3 (33)
TT	12	4 (33)

Table 4.4 Results of IL-28B SNP rs8099917 genotyping pilot study in the Trent HCV cohort, relative to their PARV4 serology.

4. Discussion

This study confirms that PARV4 infection is common in HCV-infected individuals, and almost universal in IDUs co-infected with HIV and HCV. These data suggest that PARV4 does not influence HCV disease progression but HIV-coinfected subjects progress faster to CDC-B symptoms related to HIV-1 infection, however this was confounded by HCV co-infection.

4.1. The prevalence of PARV4 in high-risk populations

31% of the HCV-infected individuals of the Trent HCV cohort were PARV4 seropositive. This is in accordance with the results of Chapter 3 that found 26% seropositivity in 84 HCV-infected individuals, and other studies that tested for PARV4 IgG (Sharp et al., 2009, Lahtinen et al., 2011) or directly for viral DNA in this risk group (Fryer et al., 2007d, Touinssi et al., 2011). PARV4 has been frequently found at very low viral loads (Fryer et al., 2007d, Tuke et al., 2009, Touinssi et al., 2011) and this may lead to a relative inefficiency of transmission compared to HCV.

In the Swiss HIV-1 cohort, a striking 95% of the HIV⁺ HCV⁺ IDU individuals were PARV4 seropositive. This is the highest published figure for this clinical group, with other studies reporting between 55% and 85% (Simmonds et al., 2007, Sharp et al., 2009). Together with only 11% PARV4 seropositivity of the HIV⁺ HCV⁻ MSMs in this cohort, these data strongly support parenteral transmission of PARV4. This is consistent with the analysis of the Trent HCV cohort where the presence of PARV4 IgG was strongly associated with injecting drug use as the

main risk factor for acquiring HCV. PARV4 seroprevalence in HCV⁺ HIV⁻ IDUs was still only 36%. This suggests that HIV-1 HCV co-infection may be a marker for more severe intravenous drug use and increased exposure. As PARV4 specific antibodies were also present in non-IDU MSMs, albeit in fewer individuals, it is possible infection may have occurred through unreported intravenous drug use or other transmission routes, as all these individuals were also HCV-negative. Other studies on HIV⁺ MSMs support an alternative transmission route (Sharp et al., 2009), along with the reports of the presence of PARV4 in the general population in Central Africa (Sharp et al., 2010b). It is unclear how PARV4 is being transmitted in these cases but high rates of seroprevalence of PARV4-like viruses have also been found in non-human apes, suggesting a possible environmental risk factor (Sharp et al., 2010b). It is possible that there are multiple routes of transmission depending on the geographical region, and that, similar to HIV-1, PARV4 originated from non-human primates in Africa. However, its transmission is also not always associated with HIV-1 in central Africa (Sharp et al., 2010b), suggesting a different epidemiological pattern.

4.2. PARV4 infection does not affect HCV-related disease progression but is correlated with earlier occurrence of CDC-B HIV-related symptoms

These two cohorts have allowed us to study the relationship between PARV4 serostatus and age, gender, ALT levels, HCV genotype, HCV treatment outcome and HCV or HIV-1 disease progression. Whilst we found no correlation between PARV4 seropositivity and HCV disease, significant correlations were found

between PARV4 and both IDU and early HIV-1 disease related symptoms (CDC-B events). However, this latter association is tightly linked to both HCV status and clinical group (IDU). There was no association with the time before death; though death of individuals in this cohort was not usually a direct cause of physical degeneration due to HIV-1 related disease.

HIV-1 immunosuppression can impact on HCV disease progression, a robust clinical association linked to increased viral load, and potentially to gut translocation and local immune activation in the liver (Klenerman and Kim, 2007, Balagopal et al., 2008, Daar et al., 2001). In the case of increased mortality in HCV⁺ HIV⁺ cohorts, it is clearly very hard to distinguish the impact of HCV infection *per se*, and the impact of the associated risk factor of IDU, or even another co-infection such as PARV4. As IDUs, these individuals are also less likely to be treated or to comply with treatment, thereby allowing the disease to progress faster. It would be important to study a cohort of IDU individuals that are HCV and HIV-1 uninfected to assess the incidence and prevalence of PARV4 independently of these common infections. Such is the cohort studied in Chapter 5. However, the appropriate control group of HIV⁺HCV⁺ IDUs who lack PARV4 infection is very unlikely to exist, given the very high prevalence of this infection in this specific risk group.

There was also no effect of PARV4 co-infection on HCV disease progression, reflected by the lack of difference in ALT levels, fibrosis progression or treatment outcome in those seropositive vs seronegative. This was reminiscent of the frequent co-infection of GB virus C (GBV-C) with HCV, which has no apparent effect on disease progression (Oshita et al., 1998, Pawlotsky et al., 1998). It

therefore remains an open question as to how PARV4 affects disease progression and whether this virus could be having an independent impact on this process.

In the case of acute resolution of infection there are a number of critical factors which are defined, including host genetics such as IL-28B SNP polymorphisms and HLA type (Rauch et al., 2010, Thomas et al., 2009, Neumann-Haefelin et al., 2006). As IL-28B polymorphisms do not exclusively explain treatment outcome, other factors must contribute to the propensity to clear HCV. From our pilot study, we saw no correlation between IL-28B SNP rs8099917 genotype and PARV4 infection. The factors which determine HCV disease progression in those chronically infected are more complex and poorly defined, although they do include environmental factors such as alcohol use, in addition to gender and age (Poynard et al., 1997).

It does not appear that PARV4 serostatus is having a detectable impact in our relatively small, although well-defined cohort, but larger prospective studies in this area could address this further. Notably, a recent study has linked PARV4 infection with encephalitis in two children in India. Hence it is possible that PARV4 may have a pathological effect in specific conditions. Further investigation into the presence of PARV4 in the general population in central Africa may reveal its role in disease as this presents an opportunity to study PARV4 separate from HCV and HIV-1 infections.

5. Summary of findings

- 95% of HCV-HIV co-infected individuals of the Swiss HIV cohort were PARV4 seropositive.
- PARV4 infection appeared to have no effect on HCV disease progression or treatment outcome.
- PARV4-seropositive individuals progressed faster to HIV CDC-B symptoms; however, this was tightly linked to their HCV and IDU status.
- The clinical significance of PARV4 is still unclear as PARV4 mono-infected individuals are rare.

Chapter 5

The Immune Response To PARV4 In Acute Infection

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1. Introduction

Chapter 3 of this thesis defined the immune responses to PARV4 peptides in a cohort of individuals attending a HCV clinic for treatment. There was no data on when each individual had acquired PARV4. Most of these individuals were previous IDUs, hence, if it is assumed this was the route of PARV4 transmission, it can be speculated that PARV4 infection occurred many years prior to PARV4 testing. With the levels of T cell responses observed, and the hypothesis that they may be due to persisting antigen, it could be assumed that these patients were chronically infected. The dynamics of T cell responses to PARV4 in acute infection, on the other hand, have never been studied.

Chapter 4 demonstrated the difficulties involved in dissecting the clinical significance of PARV4 infection in individuals co-infected with HCV and HIV-1. Thus, it was important to study a cohort of individuals that were HCV and HIV-1 negative, but had a risk of acquiring PARV4. Such a cohort existed in Baltimore: the REACH cohort consists of a group of active IDUs that attended clinic over several years at the Johns Hopkins Medical Institutions, Baltimore, MD. A collaboration was set up with Andrea Cox's group, who carried out the collection of samples studied in this cohort. Colin Sharp (University of Edinburgh) and I then tested these samples for PARV4 infection.

T cell and serology assays carried out on the Oxford cohort (Chapter 3) had shown there was often a discrepancy between the presence of anti-PARV4 IgG and T cell responses to PARV4. A study of longitudinal humoral and cellular responses would help shed light on how this is occurring. Longitudinal plasma and PBMC samples from 98 HCV-negative patients from the REACH cohort were

sent over to the UK for the study of PARV4. Because of the monthly follow-up over several years, it was possible to identify several patients who seroconverted during the time they attended clinic. This allowed the antibody, T cell levels, T cell phenotype and duration of viremia to be studied during acute infection.

Aims:

- To study the immune response to PARV4 in acute infection
- Investigate the infection rate of PARV4 in an active IDU cohort
- Compare T cell responses with antibody levels over the course of infection
- Study the phenotype of PARV4-specific T cells in acute and persistent infection

2. Materials and methods

2.1. Study samples

98 HCV-negative, active IDUs from “The Risk Evaluation Assessment of Community Health prospective study of IDUs” (REACH) in Baltimore, MD, consented to have blood drawn for isolation of plasma and PBMCs in a protocol designed for monthly follow-up. This cohort will hereafter be named the REACH cohort. These individuals were between 15 and 30 years of age, and acknowledged use of injection drugs (Cox et al., 2005). At enrolment, the time of intravenous drug use was less than 5 years, and for 90% of individuals was less than 2 years (A. Cox, personal communication).

At each clinic visit, individuals were tested for HCV serology. HCV-negative individuals were not tested systematically for HIV-1; however, the rate in the cohort was very low (1.7%, A. Cox, personal communication). Recruitment, HCV testing, plasma and PBMC collection were carried out at the Johns Hopkins Medical Institutions. A summary of patient demographics can be found in Table 5.1.

2.2. Study design

The study design is illustrated in Figure 5.1. The most recent available plasma samples for 98 individuals were initially screened for anti-PARV4 IgG. For those that tested IgG⁺ at this time point, the earliest plasma sample available was then tested. Plasma samples around the time of seroconversion were also tested for

	Patient n=	Demographic data on n=	HIV	Gender	Race	Enrolment	
						between	and
Total cohort	98	62	Negative	53% male	54% White	25/07/2002	30/07/2009
					44% Black		
					2% White/Hispanic		
PARV4 seropositive	35	25	Negative	64% male	71% White	25/07/2002	23/02/2009
					29% Black		

Table 5.1 Patient demographics for the REACH IDU cohort. All these individuals were between 15 and 30 years old at enrolment. Data on some individuals was missing, as indicated by the third column.

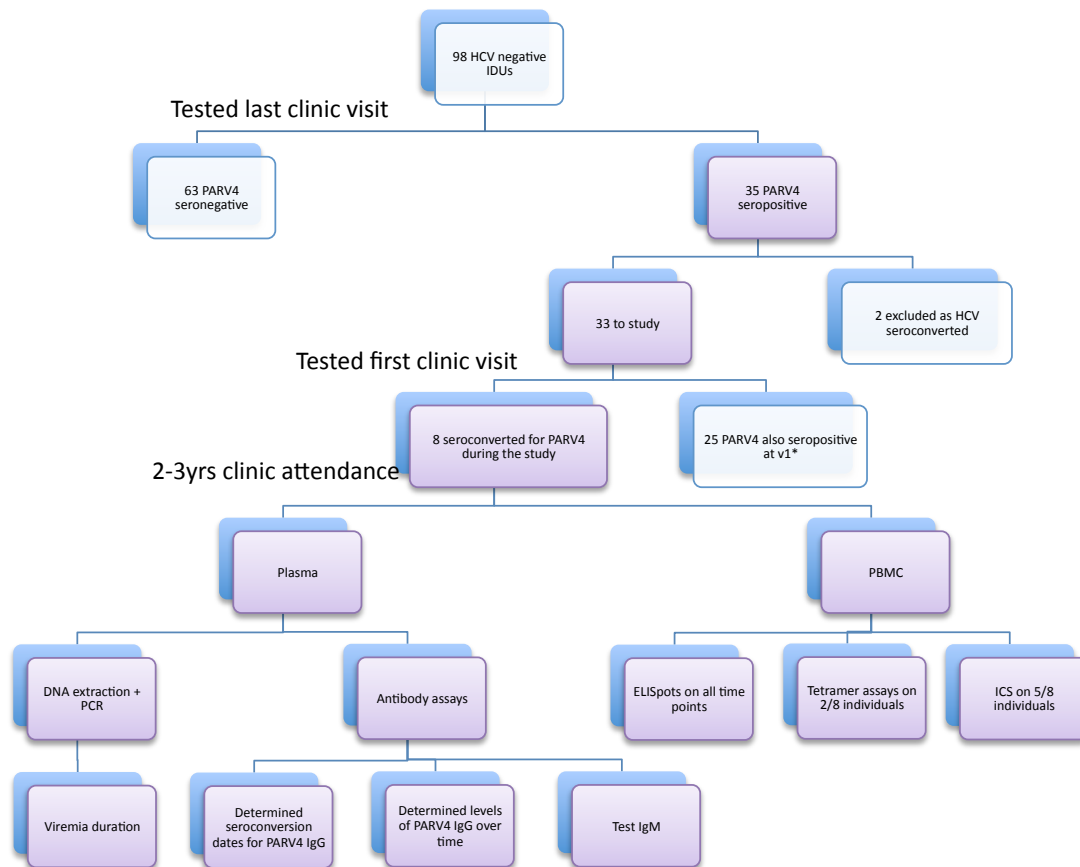


Figure 5.1 Study design of PARV4 infection in the REACH IDU cohort. These were the steps carried out to study acute PARV4 infection, and the humoral and cellular immune responses to PARV4 peptides. *v1= first clinic visit

PARV4 DNA to establish the length of viremia. PBMC samples for 5 seroconverters were available for ICS assays, and 2 of these were studied using HLA-A2 RMT tetramer (defined in Chapter 3).

2.3. Serological screening

Plasma samples from the last clinic visit of 98 individuals were screened for anti-PARV4 IgG as described in Chapter 2 (Materials and Methods). Control subjects (adult attendees of an orthopaedic outpatient clinics) had been tested previously to define the cut-off for PARV4 IgG seropositivity (set at 0.5 arbitrary units)(Sharp et al., 2009). We use the term seroconversion in this study as the time when a subject goes from having no detectable (or below the cut-off) anti-PARV4 IgG in their plasma, to having detectable levels above the cut-off, i.e. being IgG seropositive. The 8 individuals who seroconverted during the time of the study were tested longitudinally for IgG (median time span of 33 months, range: 14-63 months), as well as for anti-PARV4 IgM. IgM tests were carried out by Colin Sharp (University of Edinburgh).

2.4. DNA extraction and PCR amplification

DNA was extracted from plasma samples from the 8 individuals who seroconverted during the time of the study, using AllPrep DNA/RNA kit (Qiagen) following the manufacturer's instructions. Nested PCR for PARV4 VP was carried out and observed by gel electrophoresis as described in Chapter 2. The DNA testing was carried out by Colin Sharp (University of Edinburgh).

2.5. T cell assays

IFN- γ ELISpot assays were carried out on all 8 PARV4 IgG seroconverters as described in Chapter 2, using PARV4 NS and VP peptide pools and individual peptides. All PBMC samples post PARV4 seroconversion were tested (including the time point prior to the first IgG⁺ sample). This represented a median time span of 11 months, range: 4-63 months. DMSO was used as a negative control; ConA and FEC (Flu, EBV, CMV epitopes) were used as positive controls.

Further PBMC samples were available for 2 individuals (11067 and 50117) who responded to the RMTENIVEV epitope defined in Chapter 3. Phenotypic analysis using fluorescent-conjugated tetrameric complex HLA-A2 RMT (Chapter 3) was carried out on seven samples from patient 11067, spanning 22 months, and four samples from patient 50117, spanning 15 months. Cells were stained with live-dead marker, HLA-A2 RMT tetramer, CD3, CD8, CCR7, CD45-RA, CD57, CD127, CD38, perforin, granzymes A and B, CD161, PD-1, CCR2, CCR6, CXCR4 and CX3CR1 antibodies (for fluorochromes see Appendix Table AT2).

Additional PBMC samples were available from 5 PARV4-seroconverters (11067, 50114, 50117, 70110 and 70184) for intracellular cytokine staining (ICS) assays, as described in Chapter 2. All 5 patients were tested at one time point only, except 70184 who had two time points (6 months apart) and 11067 who had five time points (spanning 22 months). Samples were stimulated with PARV4 NS peptide pools as shown in Table 5.2. Cells were incubated for 5-6 hours, brefeldin A was added after one hour stimulation, and cells were stained for live-dead marker, CD3, CD4, CD8, IFN- γ , MIP1- β , TNF α , CD107a and IL-2 (for fluorochromes see Appendix Table AT2).

Patient	PARV4 NS pools/peptides used for ICS stimulation
11067	Peptides 2.3, 3.6, RMTENIVEV
50114	Pools 2, 7, 11
50117	Pool 3, RMTENIVEV
70110	Pools 2, 7
70184	Pools 2, 7, 11

Table 5.2 PARV4 peptides and peptide pools used for each subject in intracellular cytokine staining (ICS) assays. Responses to these peptides were identified by ELISpot assays. RMTENIVEV is the PARV4 epitope identified in Chapter 3.

3. Results

3.1. PARV4 antibodies and viral DNA detected in the plasma of REACH cohort subjects

3.1.1. Anti-PARV4 IgG frequently detected in the REACH cohort

Plasma samples from the last clinic visit of 98 HCV-negative active IDUs were screened for PARV4 antibodies (IgG). 35/98 (36%) were PARV4 seropositive. Two individuals were excluded from the cohort at this point, as they later seroconverted for HCV and were retained for study by the group in Baltimore.

3.1.2. Identification of subjects that seroconverted for PARV4 IgG during the study

33 individuals with anti-PARV4 IgG at their last time point were tested for these antibodies at the first sample taken (v1). 8 individuals seroconverted for PARV4 IgG during the study (as they were seronegative at v1, Figure 5.2). Serum samples before, at and after seroconversion were tested for PARV4 IgM. IgM was detected in 6 out of 8 of these individuals either prior to IgG seroconversion or at the same sample time point, providing additional support for acute infection. IgM was only detected in one time point for each individual, except 50114 where IgM persisted over 2 months (monthly samples). Longitudinal samples available for these 8 individuals are summarised in Table 5.3. Seroconversion dates for PARV4 IgG were determined using all available samples (Table 5.3).

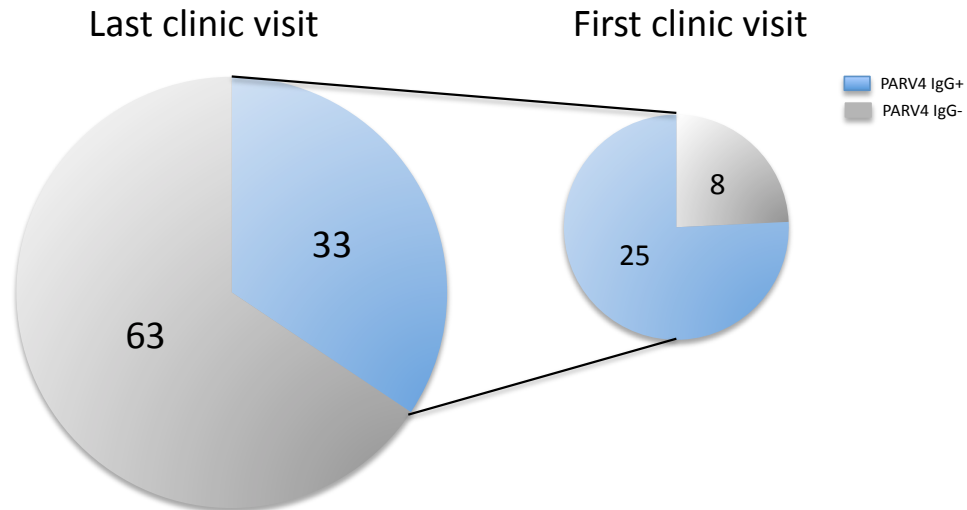


Figure 5.2 Subjects from the REACH cohort that acquired PARV4 infection during the study were determined by the absence and presence of PARV4 IgG at the first and last time points available, respectively. These pie charts represent the number of individuals that were PARV4 seropositive at their last clinic visit (left pie chart), and from these, those that were seronegative at the start of the study (right pie chart). From this, it was determined that 8 individuals seroconverted during the study. The numbers in the pie charts represent the number of individuals in each group.

	Date of enrolment ¹	1st clinic visit (v1)	Last clinic visit	Time attended clinic (months)	Number of clinic visits	Period of IgG seroconversion ²	IgM ⁺ date ³	Time to seroconversion (months) ⁴	PARV4 pools (P) eliciting a T cell response	Tetramer ⁵ study	Tetramer time points	Tetramer time span	Tested by ICS ⁶	ICS time points	ICS time span
11067	30/09/2003	26/05/2004	02/09/2009	71	24	26/05/04 - 19/07/07	26/05/2004	33	P2, P3, P8	✓	7	22 months	✓	5	22 months
11161	12/12/2003	09/12/2004	25/01/2008	49	24	22/12/05 - 24/01/06	01/03/2006	49	P1, P2, P6, P13						
50114	27/10/2006	27/10/2006	30/04/2009	30	24	01/05/08 - 02/06/08	2/06/08 - 2/07/08	43	P7, P13				✓	1	N/A
50117	02/11/2006	02/11/2006	08/01/2008	14	7	02/11/06 - 08/01/07	IgM negative	25	P1, P3, P5, P8	✓	4	15 months	✓	2	6 months
70110	-	24/01/2000	03/10/2002	32	21	16/04/02 - 16/05/02	16/05/2002	51	P2, P3, P7, P8				✓	1	N/A
70184	-	07/06/2000	22/01/2003	31	22	05/03/02 - 09/04/02	09/04/2002	45	P2, P3, P7, P8, P11				✓	1	N/A
70418	-	12/04/2001	12/01/2004	33	24	08/09/03 - 10/11/03	10/11/2003	54	P6, P8						
70429	-	04/04/2001	12/08/2004	40	12	12/04/04 - 12/08/04	IgM negative	62	P1, P4						

Table 5.3 Seroconversion and T cell response data of 8 individuals from the REACH cohort who acquired PARV4 infection during the prospective study. ¹Date of enrolment: this data was not available for all individuals; in this case v1 (column 2) was used as the study start date; ²Period of seroconversion is defined as the date of the last sample that was PARV4 IgG negative, to the date of the first sample that was PARV4 IgG positive. ³IgM⁺ date = date(s) of samples where IgM was detected. ⁴Time to seroconversion: this was calculated with the assumption that individuals started using intravenous drugs 2 years prior to enrolment. The time in months from this start point to the time of seroconversion (the middle point within the period of seroconversion) represents the amount of time it took that individual to acquire anti-PARV4 IgG. ⁵Tetramer = PE-conjugated tetrameric complex specific for PARV4 epitope RMT T cells. ⁶ICS= intracellular cytokine staining assay

3.1.3. Estimation of PARV4 infection rate

The rate of PARV4 infection can be deduced from the rates of IgG seroconversion. These calculations are based on the assumption that no PARV4 seropositive individual reverted to seronegativity.

35/98 individuals were seropositive at the last clinic visit. These individuals attended clinic for a median time of 33 months (2.75 years, range: 6-71 months, based on data available for 62/98 individuals) and had been using intravenous drugs for under 5 years, and for most under 2 years. This translates as a total time of illicit drug use for 7.75 years, and for most 4.75 years. If it is assumed that these individuals acquired PARV4 through intravenous drug use, then 35/98 individuals were infected with PARV4 over 4.75-7.75 years. This translates as an incidence rate between $(35 \div 7.75)/98$ and $(35 \div 4.75)/98$ per year, i.e. 4.5% to 7.5% per year.

Additionally, the rate of infection per individual can be calculated from the time it took each of the 8 subjects to seroconvert during the course of the study. To calculate this, several assumptions were made: the time of seroconversion was defined as the mean between the two dates of the period of seroconversion (Table 5.3). The subjects had been using intravenous drugs for under 5 years, and for most under 2 years, thus the time from start of intravenous drug use to the date of seroconversion for PARV4 IgG could be determined. This is presented in Table 5.3 (column "Time to seroconversion"). For drug use under 2 years, the median time of an individual to seroconvert for PARV4, and therefore to be infected with this virus, was 47 months (range: 25-62 months), or 3.9 years. Alternatively, for drug use under 5 years, the median time increases to 6.9 years.

These are conservative estimates of the lowest and slowest infection rates as they are based on the maximum time of intravenous drug use. If, in fact, individuals acquired infection after a shorter time of drug use, rates per year would increase and time until infection would decrease.

3.1.4. PARV4 viremia is short-lived

DNA extracted from plasma samples was amplified by nested PCR and run on a gel (Figure 5.3). Several time points around the date of seroconversion for PARV4 IgG were tested to determine the length of viremia, defined as the presence of viral DNA in peripheral blood. This was calculated using the amount of time viral DNA could be detected in the samples.

Patient 11067 was PARV4 DNA-positive at the first time point, but not the second, taken three years after the first (v1: 26/05/04, v2: 19/07/07) due to a lack of clinic attendance. Hence, 11067 was acutely infected with PARV4 in 2004 but no longer in 2007. As all but one sample were dated post 2007 for this subject, 11067 will therefore be used as an example of a persistently infected individual. No viral DNA was detected in patient 50117 suggesting viremia had cleared at the time of seroconversion or was below the level of detection, and no earlier time points were available for analysis. Patient 70429 was viremic at the last time point available; hence, we could not use this data to calculate maximum length of viremia. Results from 70110 could not be replicated. Thus, 11067, 50117, 70429 and 70110 were excluded from calculating the length of viremia.

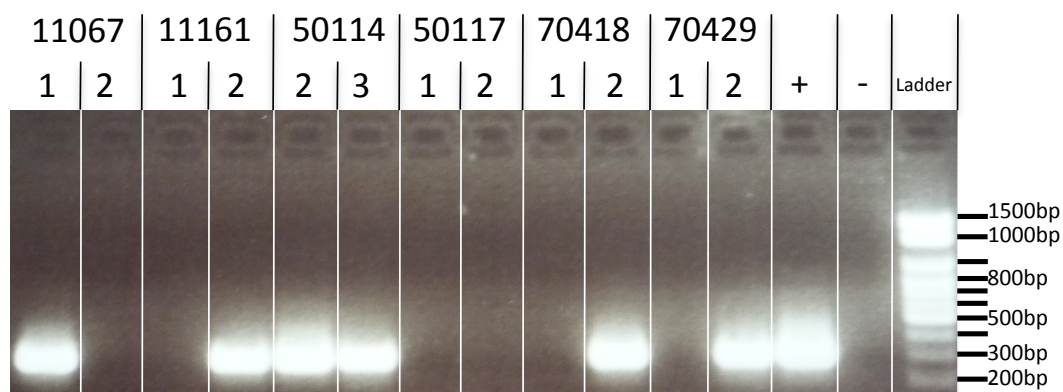


Figure 5.3 Detection of PARV4 VP DNA in 6 individuals who seroconverted for PARV4 IgG during the study. Nested PCR was carried out at several time points around seroconversion to determine the length of viremia. 12 DNA samples from 6 individuals are represented here. The patient numbers are indicated above the gel, and the numbers below them represent the order of the samples in time. + and – columns represent positive and negative controls for the experiment, respectively. DNA amplification by PCR followed by gel electrophoresis.

The minimum length of viremia was defined as the time from the first to last day where PARV4 DNA was detected. The maximum duration of viremia was defined as the time from the day after the first DNA negative time point until one day before the second DNA negative time point (where viral DNA is detected between the first and second negative time points, Figure 5.4). From these calculations, viremia was determined to last between 32 and 104 days (range: 30-125 days, Table 5.4). Viral loads were between 5×10^2 and 10^5 per ml of plasma (median 4.7×10^3 , Table 5.4).

3.1.5. Anti-PARV4 IgG levels are maintained at varying levels over time

Longitudinal samples for each PARV4-patient who seroconverted for PARV4 IgG during the course of this study were tested for anti-PARV4 IgG and levels were plotted against time (Figure 5.5). IgG levels are expressed in arbitrary units (see Methods). These levels can all be compared when the time of seroconversion is normalised (Figure 5.6). IgG levels rose very rapidly to maximum levels in patients 11161, 70418 and 50114, whereas others remained below 50 arb. units. IgG was detected up to 24 months after seroconversion.

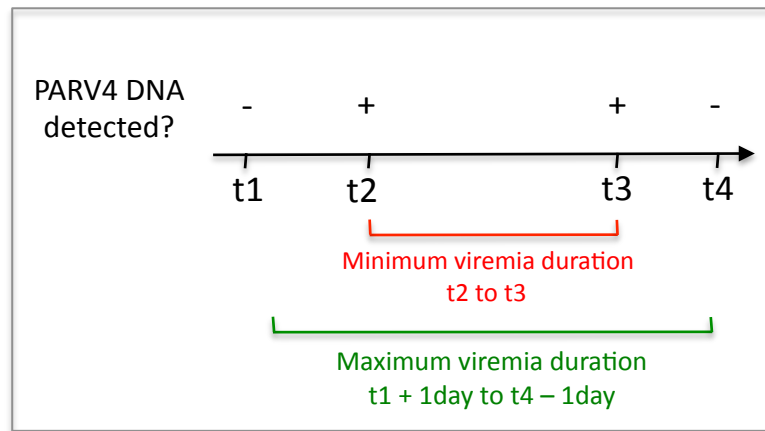


Figure 5.4 Schematic representation of how the length of PARV4 viremia was calculated. The top line indicates the presence (+) or absence (-) of viral DNA as detected by PCR. The arrow represents time with several arbitrary time points (t1 to t4).

Patient	Viremia		Viral load per ml
	Minimum duration (days)	Maximum duration (days)	
11161	33	109	10^3
50114	32	98	10^3 to 10^5
70184	-	63	4.7×10^4
70418	30	124	10^4
11067	Excluded		4.7×10^3
50117	Excluded		None
70110	Excluded		$<5 \times 10^2$
70429	Excluded		10^3
Median	32	104	4.7×10^3

Table 5.4 The duration of viremia in each individual that seroconverted for PARV4 IgG during the study. Four patients were excluded as start or end dates of viremia were unknown. The minimum duration of viremia could not be calculated for 70184 as only one time point was PCR positive. Viral loads are expressed per ml of plasma.

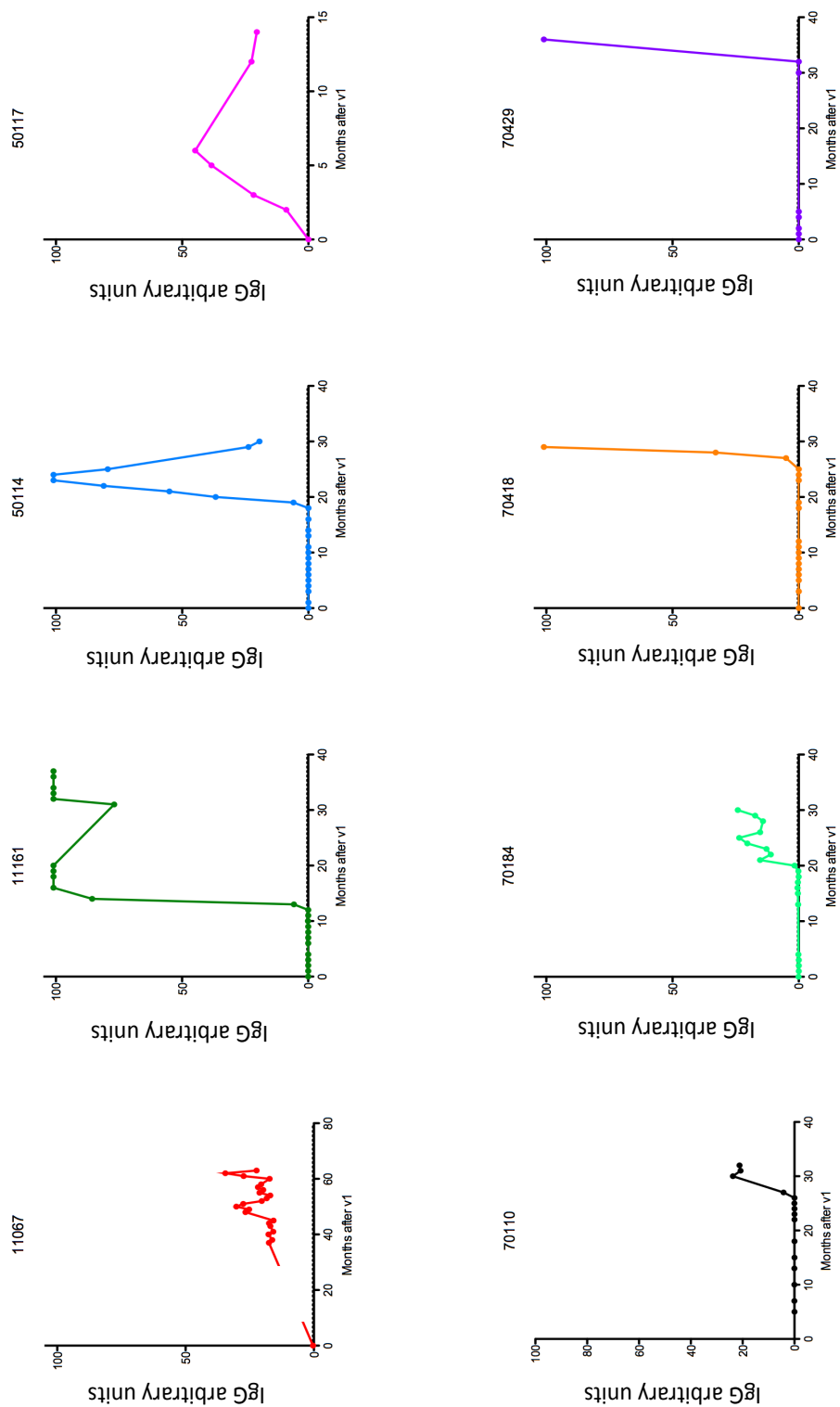


Figure 5.5 Levels of anti-PARV4 IgG increase over time before occasionally dropping in the 8 IDUs who seroconverted for PARV4. These are expressed in arbitrary units (see methods) and time is expressed in months after the first sample (v1).

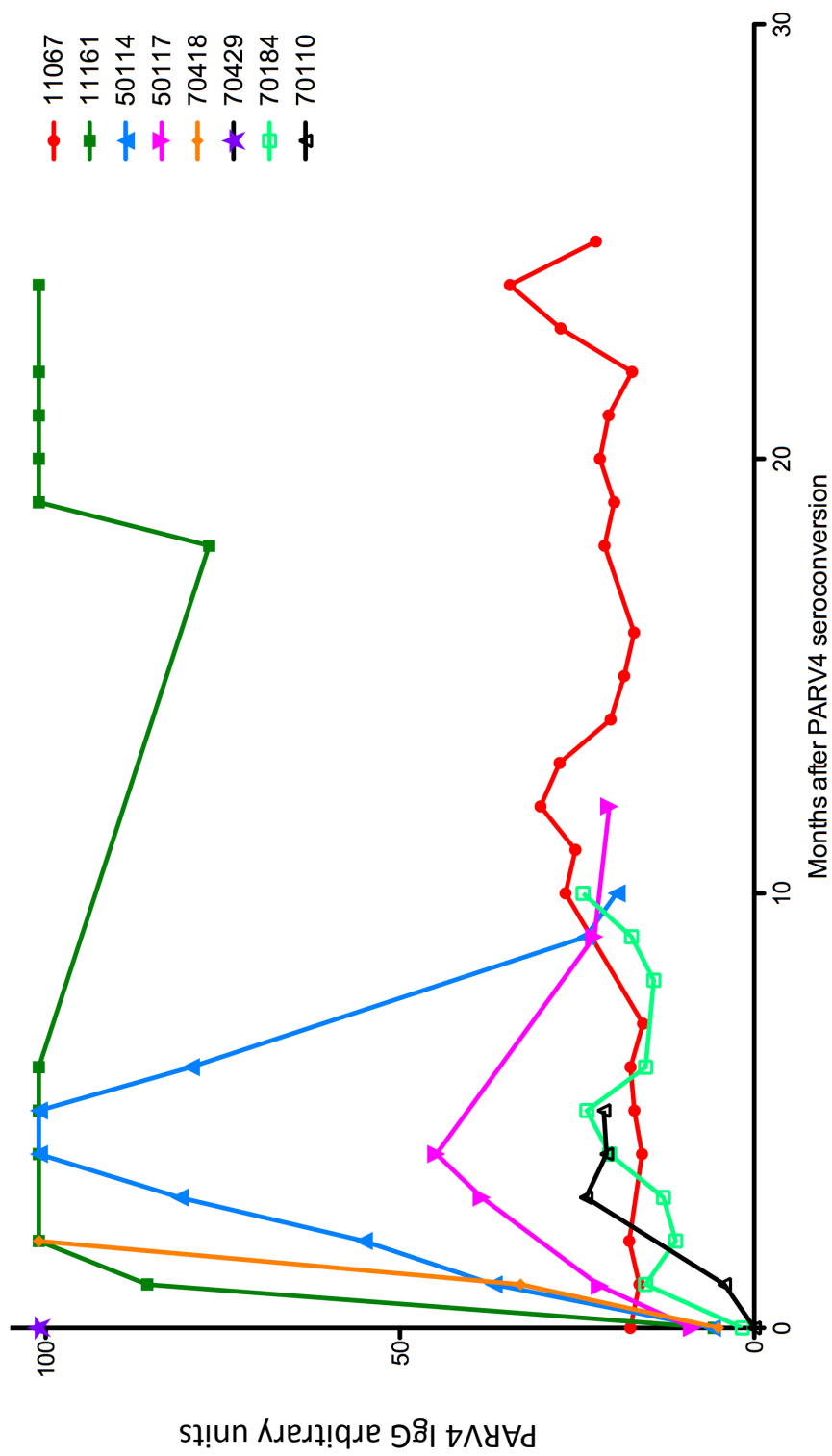


Figure 5.6 Levels of anti-PARV4 IgG increase steeply or remain below 50 arbitrary units. The data was normalised to the date of seroconversion of each subject. This may not be an accurate estimate of seroconversion time for 11067 as there was a 3-year gap between seronegative and seropositive time points. Cut-off for seropositivity was set at 0.5 arbitrary units (see methods).

3.2. Functional T cell responses to PARV4 are maintained over time

3.2.1. PARV4 peptides elicit the production of IFN- γ

a. Strong T cell responses to PARV4 NS peptides

All time points available for the 8 PARV4 IgG seroconverters were tested by ELISpot with PARV4 NS peptide pools. 4/8 individuals were also tested with VP peptide pools; however, responses were infrequent and very low (see Appendix Figure A1). Strong and frequent T cell responses to NS reached over 1,000 SFU/ 10^6 PBMC for several individuals (Figure 5.7A). Responses were distributed across 10 out of 13 NS pools, although there was a preference for the N-terminus of NS: pools 1 to 8: 24 responses, median 3; pools 9 to 13: 3 responses, median 0 (Mann Whitney, $p=0.02$) (Figure 5.7B). Individual patient results are described below.

b. PARV4 elicits broad T cell responses

11 time points spanning 10 months were tested for patient 70184. These revealed increasing T cell responses over time, with a notable increase 5 months post seroconversion (Figure 5.8, for responses to all pools see Appendix Figure A2). 70184 showed broad T cell responses were elicited to PARV4 NS peptide pools with responses to pools 2, 3, 4, 7, 8, 11 and 13, with a preference for pool 11. IgG levels also increased over time, but were relatively low in comparison to T cell responses, as illustrated in Figure 5.8B. Broad T cell responses were also seen in patients 70110, 11161 and 70429 (Appendix Figures A3-A5).

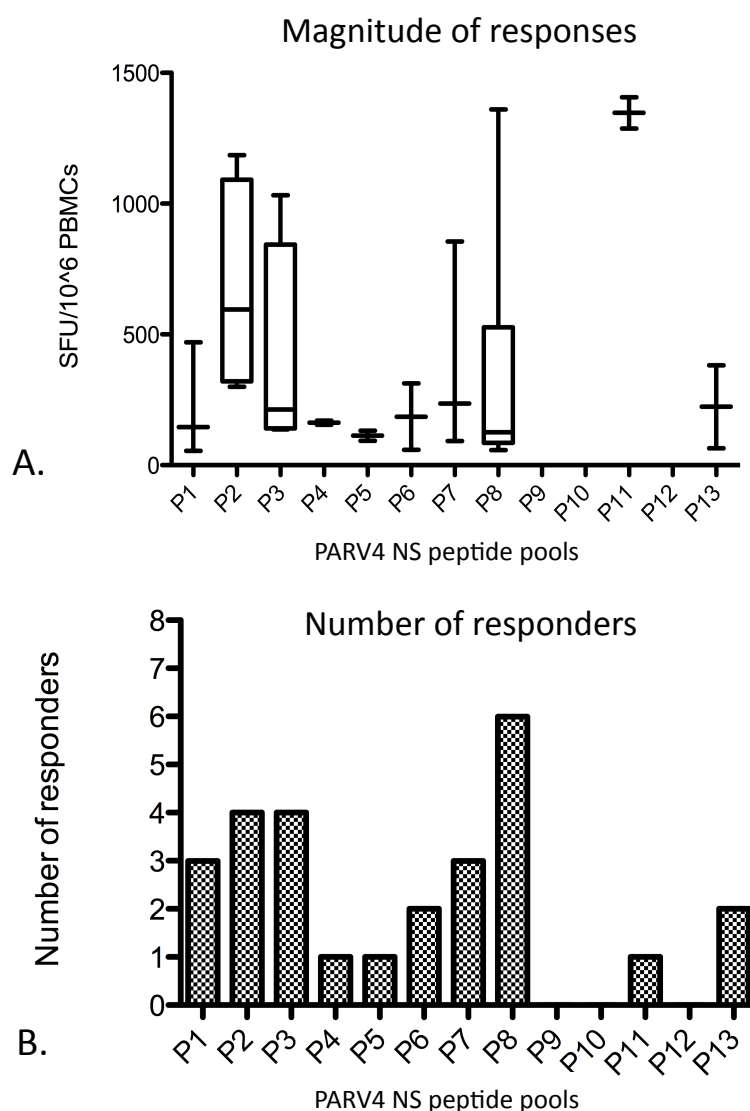


Figure 5.7 Broad T cell responses elicited by PARV4 NS as measured by IFN- γ ELISpot assay. **A.** The magnitude of T cell responses by PARV4 IgG seroconverters was plotted using the time point with the highest value, when several were available. **B.** The number of responders per PARV4 NS pool. A “responder” is defined as a subject with observed T cell responses to any PARV4 NS peptide pool above the pre-defined cut-off (see methods). The peptide pools are defined in Appendix Table AT1.

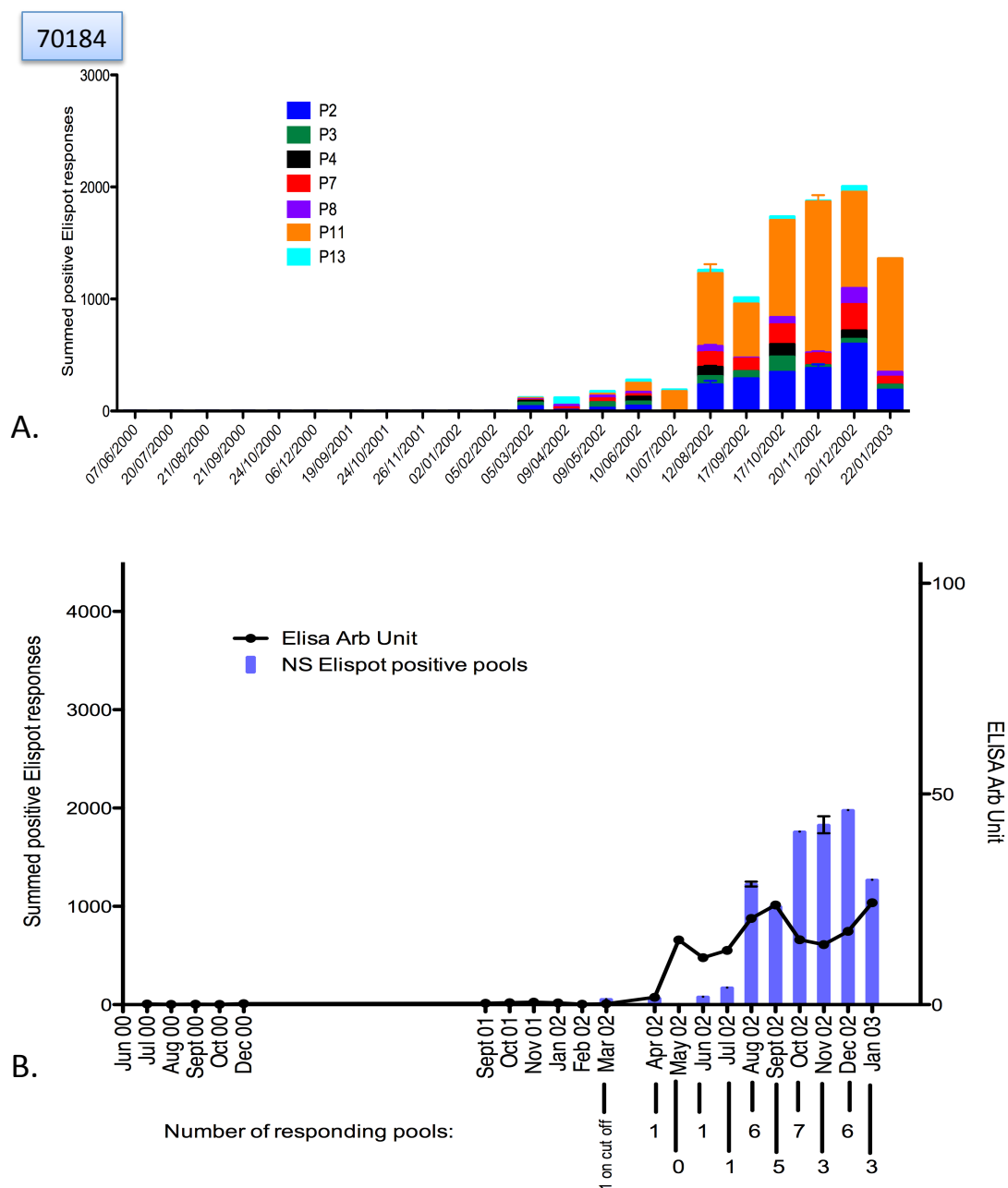


Figure 5.8 Broad T cell responses elicited in patient 70184. A. Broad responses were seen to PARV4 NS peptide pools over time, with a preference for pool 11 (orange). B. T cell responses (left axis) and IgG levels (right axis) gradually increased over time but IgG levels remained below 30 arbitrary units. ELISpot results for patient 70184 for all time points to all NS pools can be found in Appendix Figure A2.

c. T cell responses to PARV4 were not detectable at all time points

Seven time points spanning 14 months were tested for patient 50117. T cell responses were lower than in patient 70184, reaching 300 SFU/10⁶ PBMC (Figure 5.9). The T cell response to pool 8 was directed to epitope RMTENIVEV (defined in Chapter 3). Although T cell responses were observed at 5 months post seroconversion (April 2007), they had disappeared by 12 months (November 2007). However, they did reappear 3 months later (January 2008). This was also visible for patient 70110 where responses dropped for 2 months before reappearing (Appendix Figure A2B). IgG levels for 50117 peaked 7 months post seroconversion, but were also lower when T cell responses were absent in November 2007 (Figure 5.9B).

d. Strong PARV4 IgG levels but no T cell responses detected

10 time points spanning 11 months were tested for patient 50114. T cell responses to PARV4 NS were rare during the course of this study (Figure 5.10). Pool 13 elicited a response just above the cut-off at 1 month post-seroconversion, while pool 7 elicited similar levels of IFN- γ secretion at 2, 7 and 10 months post-seroconversion. Conversely, PARV4 IgG reached maximum levels of detection within 5 months (October 2008, Figure 5.10C). This was also evident for patient 70418, studied over 5 months, where high IgG levels were observed with very few T cell responses (Appendix Figure A6).

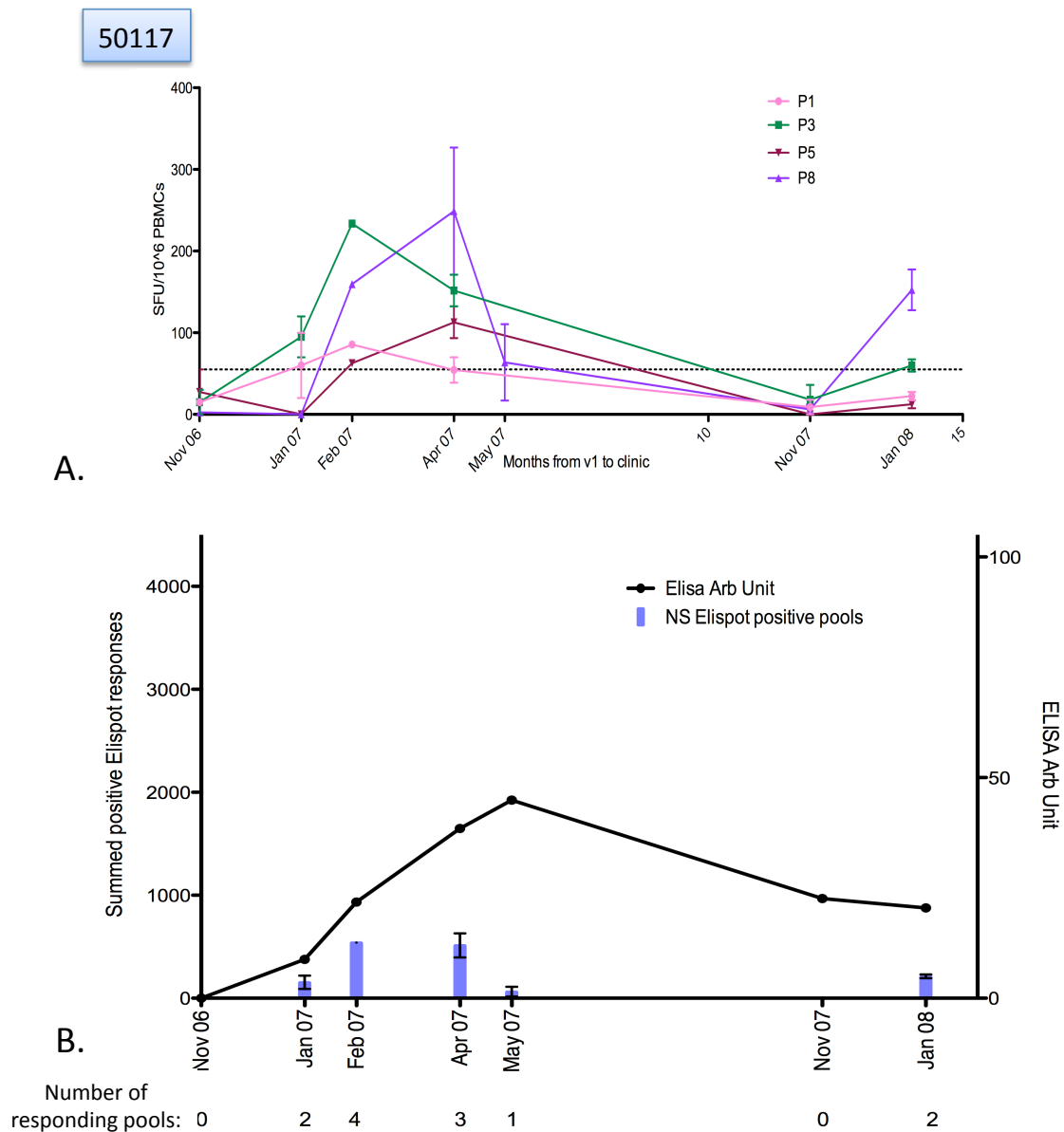


Figure 5.9 T cell responses were not detected at all time points in patient 50117.

A. Changes in responses to PARV4 NS peptide pools 1, 3, 5 and 8 over time. The dashed line represents the pre-defined cut-off for a positive response (see methods). **B.** T cell responses (left axis) remained low over the course of infection, and IgG levels (right axis) peaked before falling again. ELISpot results for patient 50117 for all time points to all NS pools can be found in Appendix Figure A2B.

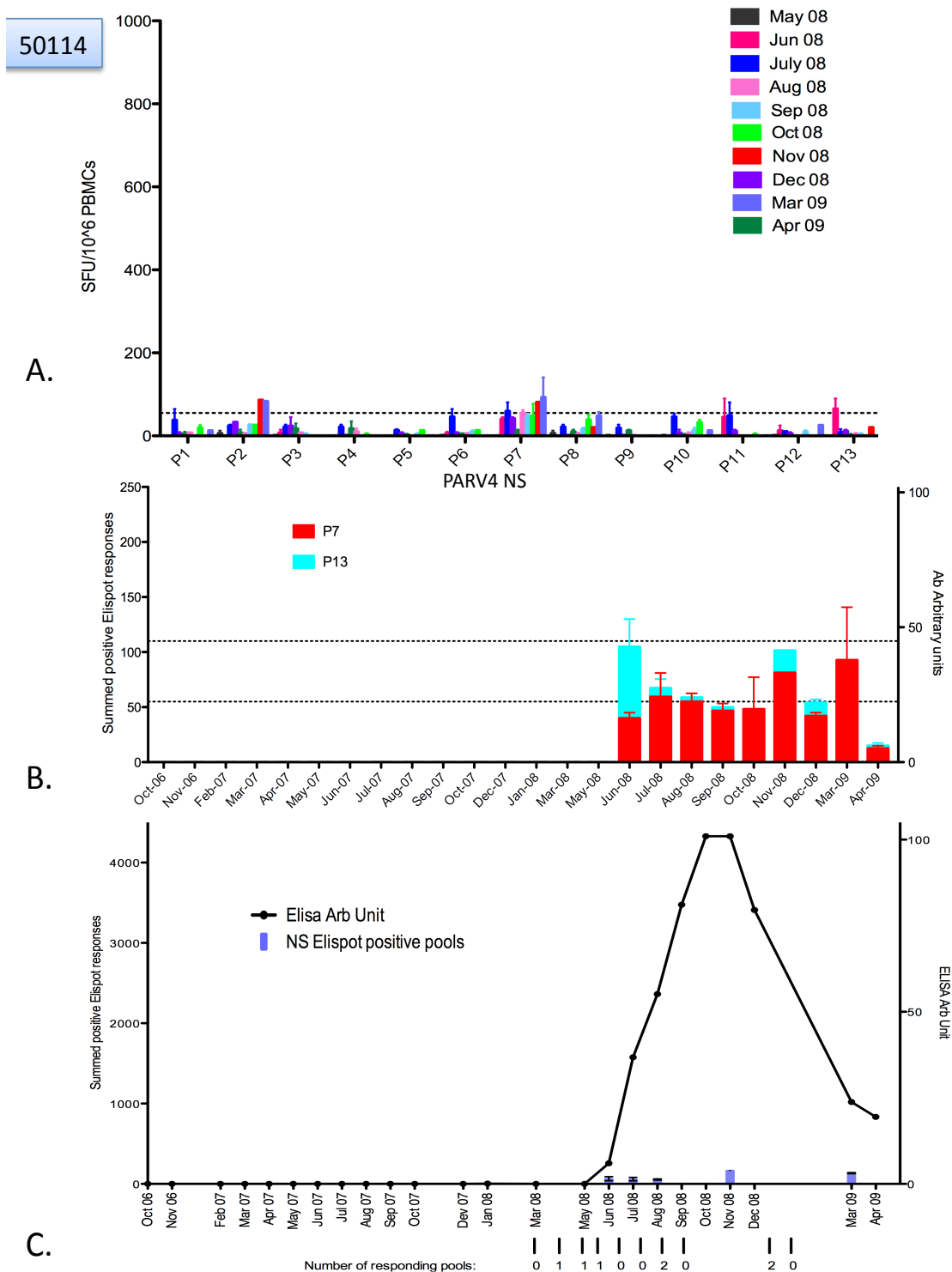


Figure 5.10 High levels of anti-PARV4 IgG but very low T cell responses in patient 50114. **A.** T cell responses remained under the pre-defined cut-off save for 4/10 time points (May 08 is the last seronegative sample). **B.** Responses to two PARV4 NS peptide pools over time occasionally exceeded the pre-defined cut-off for a positive response (dashed lines; 55 for 1 pool, 110 for 2 pools; see methods). **C.** T cell responses (left axis) remained low while IgG levels (right axis) reached the upper limits of detection by ELISA.

e. T cell responses to PARV4 consistently elicited several years after infection in patient 11067

Samples from 24 time points spanning 63 months were tested for patient 11067. This patient was acutely infected at entry, however all but one sample were taken three years after primary infection, thus they are an example of persistent PARV4 infection. T cell responses were consistently observed to three pools: pools 2, 3 and 8 (Figure 5.11A). Responses frequently reached over 1000 SFU/10⁶ PBMCs for pool 8. The number of samples available for this patient meant it was possible to identify individual peptides eliciting the T cell response. Overlapping peptides 2.3 (CILQQLFASHAVTLP) and 2.4 (LFASHAVTLPCRVKP) in pool 2 were positive, with a higher response for peptide 2.3. Overlapping peptides 3.5 (VFMAGYLVAPVWTLN) and 3.6 (YLVAPVWTLNWNIRE) in pool 3 were positive, with a higher response for peptide 3.6. Peptides 8.4 and 8.5 from pool 8 were positive, which includes the previously identified epitope RMTENIVEV (Chapter 3); when tested, this epitope triggered the highest T cell response for this patient (Figure 5.11B). No patient HLA data was available to determine epitopes or HLA-restriction for peptides 2.3 and 3.6 (further detailed in the discussion).

IgG antibody levels were detectable but low compared to T cell responses, remaining below 50 arbitrary units throughout the course of study (Figure 5.11C).

11067

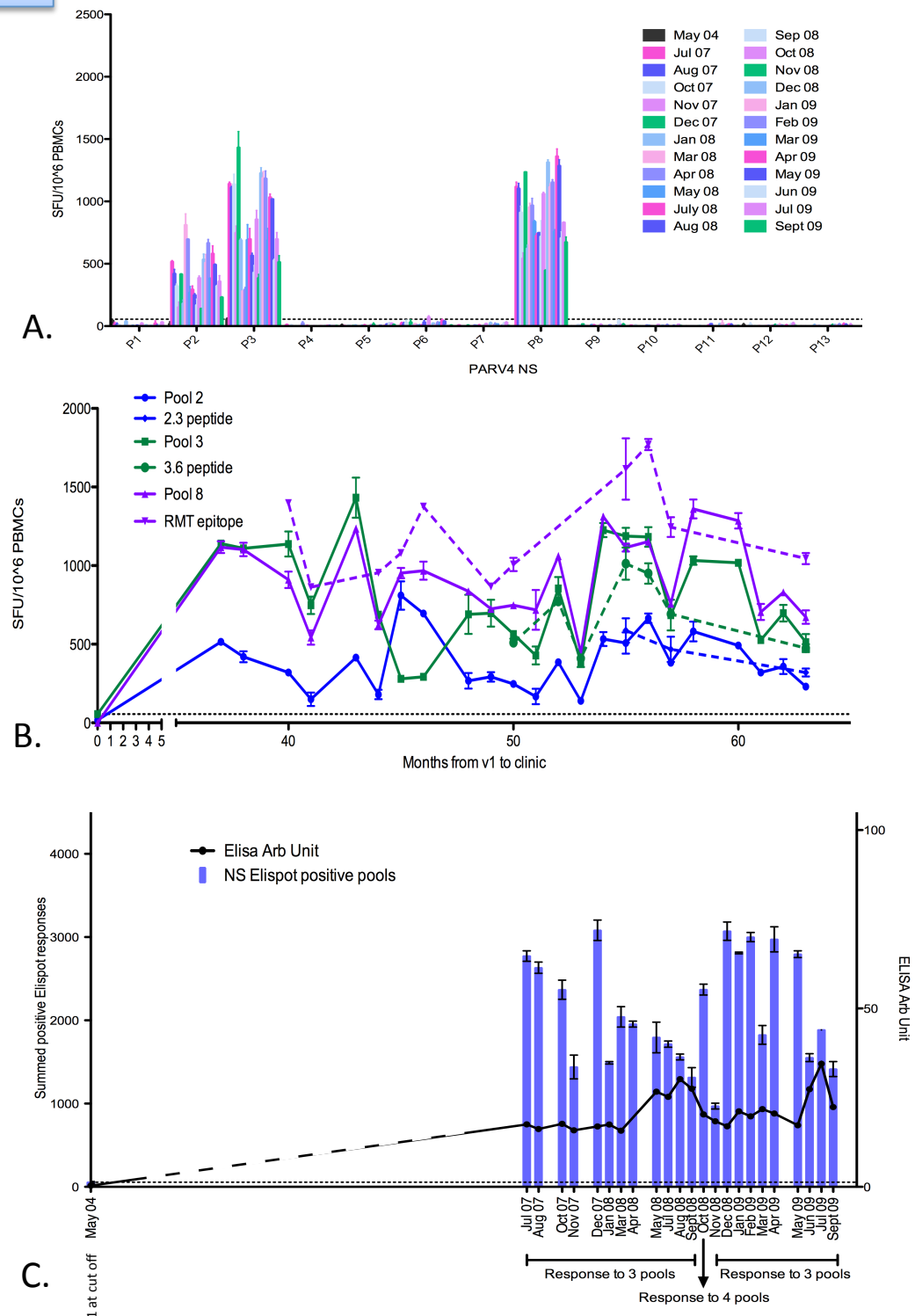


Figure 5.11 T cells to PARV4 were persistently high several years after infection in patient 11067. **A.** ELISpot results over 24 time points (May 04 is the last seronegative sample) show very high T cell responses focussed on pools 2, 3 and 8. **B.** Responses over time to PARV4 NS peptide pools 2, 3 and 8 (solid lines) and peptides 2.3, 3.6 and epitope RMT (dashed lines). **C.** T cell responses (left axis) remained high over the course of infection while IgG levels (right axis) remained below 50 arbitrary units. No samples were available between 2004 and 2007 (represented by the dashed area of the IgG line).

3.2.2. Phenotypic analysis shows evidence of mature and activated PARV4-specific T cells

Patients 11067 and 50117 had T cell responses to the epitope RMTENIVEV defined in Chapter 3. Using the fluorescent-conjugated tetrameric complex HLA-A2 RMT synthesised in house, the phenotype of epitope-specific cells was studied. Seven time points spanning 22 months were studied for patient 11067 and 4 time points spanning 15 months were studied for patient 50117.

a. Antigen-specific cells are maintained over time

PBMC from patients 11067 and 50117 were stained with HLA-A2 RMT tetramer to detect the frequency of RMTENIVEV-specific cells (hereafter described as RMT-specific cells, Figure 5.12A and B). This experiment was performed using two panels both containing the tetramer, but combined with other antibodies described below. Hence, RMT-specific cells were tested in duplicate experiments represented in Figure 5.12D and E.

RMT-specific cells represented approximately 1% of 11067 CD8⁺ T cells. These levels peaked at 1.5% 57 months after v1 (March 2009), before dropping to 0.5% then recovering back to 1% CD8⁺ T cells. 50117 RMT-specific cells represented lower frequencies of 0.1-0.2% of CD8⁺ T cells. In section 3.2.1 c., I described that T cell responses detected by ELISpot in subject 50117 disappeared 12 months post-seroconversion (November 2007). This was confirmed in this experiment, as RMT-specific cells were absent at this time point, but recovered to higher levels 2 months later (Figure 5.12E). As T cell responses were only detected in 2 out of 4 time points, the following sections will concern just these two samples (April 2007 and January 2008).

T cell responses by 11067 were substantially higher than those observed in patient 50117. These can be compared to the levels of T cell responses detected by IFN- γ ELISpot assay (Figure 5.13). If the approximation is made that CD8⁺ T cells represent 20% of PBMCs (as calculated from FACS plots), the number of antigen-specific cells per million PBMC can also be calculated from the levels of tetramer-positive cells. For example, 1% of antigen-specific CD8⁺ T cells would represent 0.2% antigen-specific PBMCs (if 1% of CD8⁺ T cells are antigen-specific out of 20% PBMCs), which, if all produced IFN- γ would translate as 2,000 SFU/10⁶ PBMC in an IFN- γ ELISpot assay. The results of these calculations at each time point are presented in Figure 5.13C. The number of antigen-specific cells was always higher than the number of cells producing IFN- γ .

b. RMT-specific T cells have an effector memory phenotype

The memory phenotype of the RMT-specific cells was determined using antibodies for CCR7 and CD45RA. CD57 and CD127 were also labelled for additional information on cell senescence and antigen exposure. RMT-specific cells from patient 11067 were 65% to 76% CD45RA⁺ CCR7⁻ (effector memory CD45RA⁺ cells, T_{EMRA}, also known as ‘revertants’), throughout the course of study (Figure 5.14A). Conversely, RMT-specific cells from patient 50117 were 54% and 62% CD45RA⁻ CCR7⁻ (effector memory cells, T_{EM}, Figure 5.14B). Antigen-specific cells for both 50117 and 11067 were predominantly CD127⁻. 0.6% to 20% of 11067 antigen-specific cells were CD127⁺ (Figure 5.15A), and 2% of 50117 cells were CD127⁺ (Figure 5.15B). Due to limited sample availability, CD57 staining was only carried out on PBMC from 11067. Antigen-specific cells were 80% to 85% CD57⁺ throughout the duration of the study (Figure 5.15C).

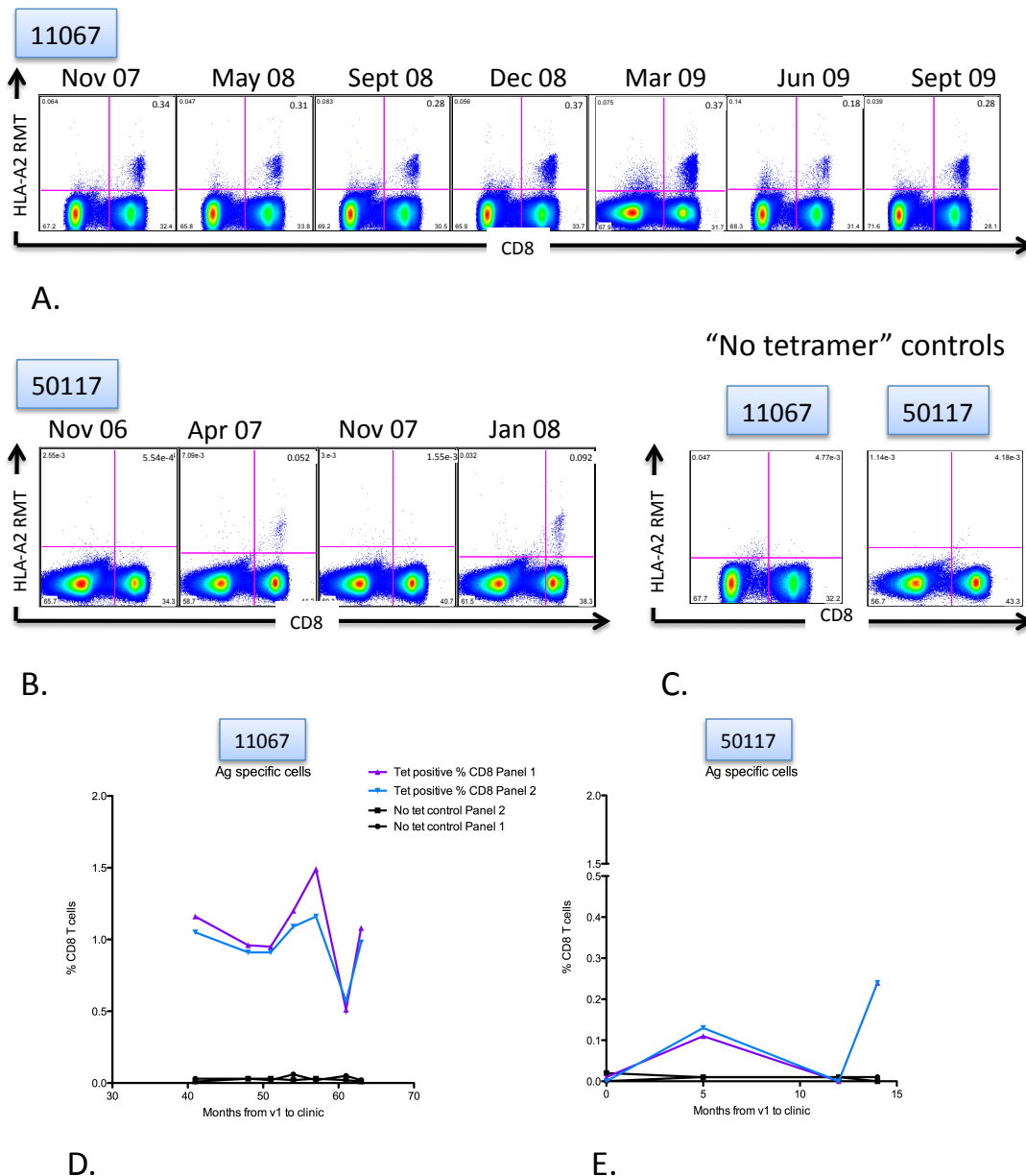


Figure 5.12 Antigen-specific T cells were detected over time in subject 11067 but dropped and reappeared in subject 50117. A, B. Epitope-specific T cells were detected using HLA-A2 RMT tetramer for 11067 (A) and 50117 (B). **C.** For each sample, a control with no tetramer was tested. An example for each patient is given here. These FACS plots are gated on CD3⁺ cells. **D, E.** Tetramer-positive and tetramer-negative cells were plotted against time for patient 11067 (D) and 50117 (E). Each sample was effectively tested in duplicate as tetramer-positive cells were detected using two different antibody panels. In E, the values for the last two time points are identical: only one curve is visible because they are superimposed. Black lines represent the control samples where no tetramer was added.

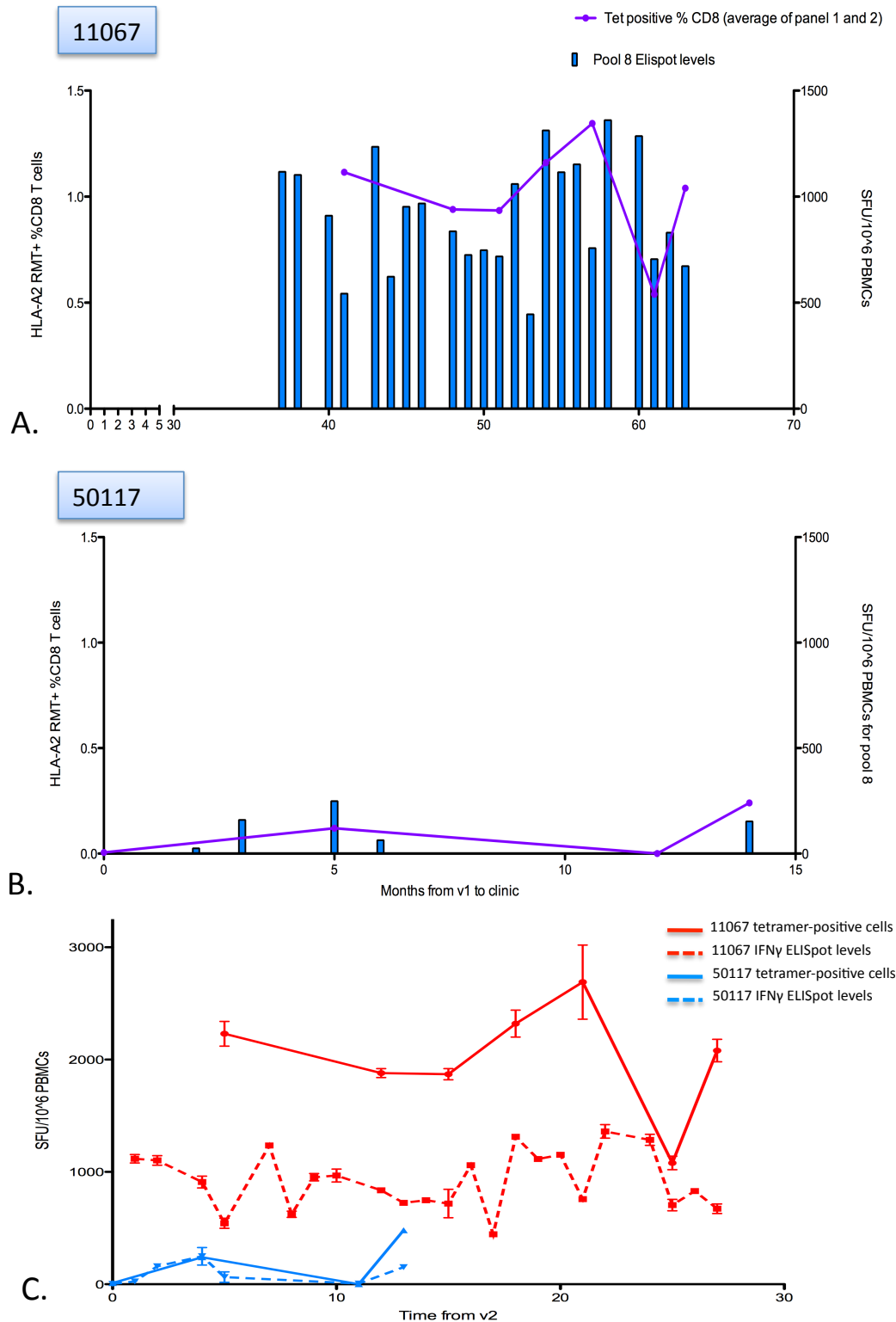


Figure 5.13 Comparison of HLA-A2 RMT tetramer-positive CD8⁺ T cells and levels of T cells producing IFN- γ . **A, B.** The percentage of CD8⁺ T cells that were tetramer-positive (as assessed by FACS analysis, left axes) in relation to the T cells producing IFN- γ (as assessed by ELISpot, right axes) are shown for patients 11067 (**A**) and 50117 (**B**). The columns represent ELISpot results for pool 8 which contains the epitope RMTENIVEV. **C.** shows the same values but estimating the number of antigen-specific cells per 10⁶ PBMC from tetramer-positive CD8⁺ T cells, so as to directly compare cell numbers with functional cells.

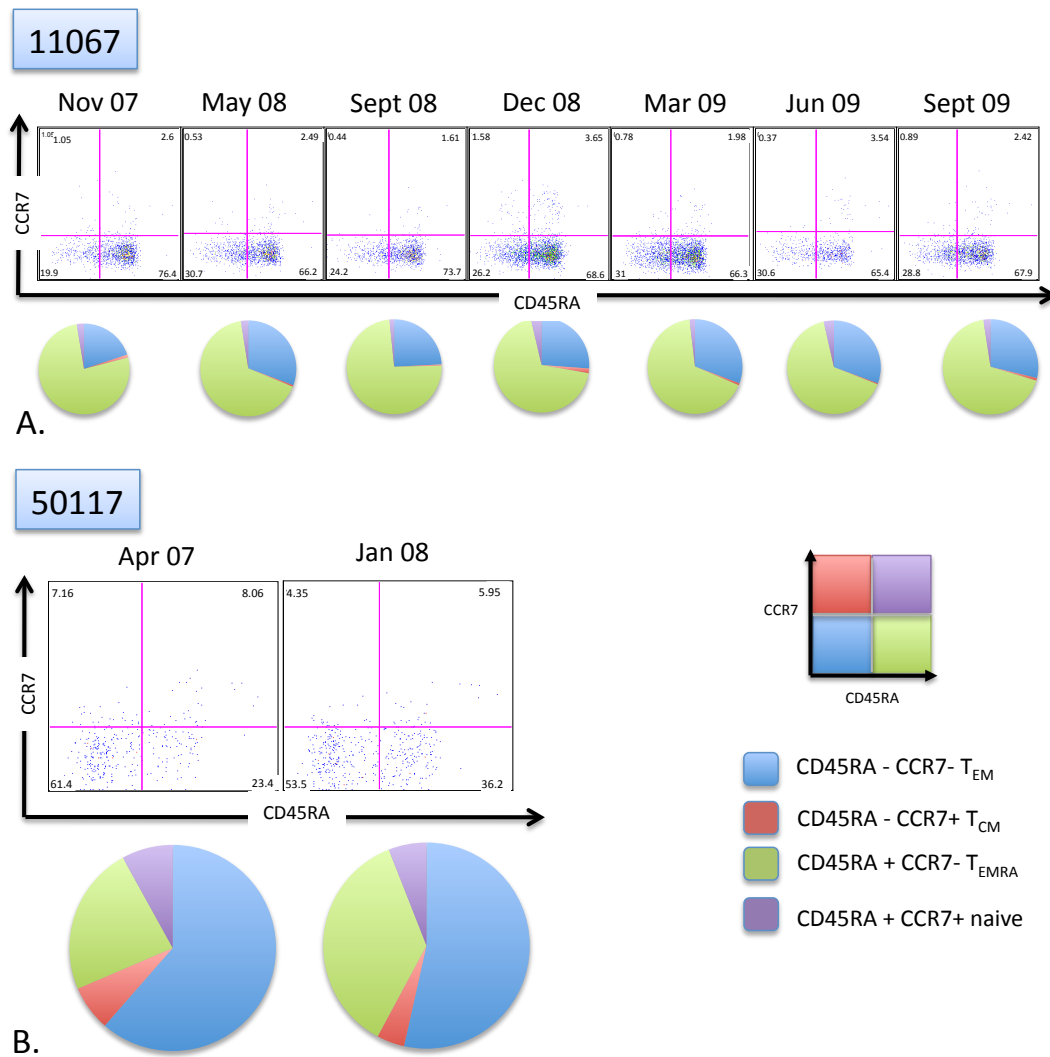


Figure 5.14 RMT-specific T cells have an effector memory phenotype (T_{EM} or T_{EMRA}). FACS plots and corresponding pie charts indicate the presence or absence of CCR7 and CD45RA on the T cell surface in patients 11067 (A) and 50117 (B) over time. FACS plots are gated on $CD8^+$ HLA-A2 RMT $^+$ cells. T_{EM} = effector memory T cells, T_{CM} = central memory T cells, T_{EMRA} = effector memory CD45RA $^+$ T cells

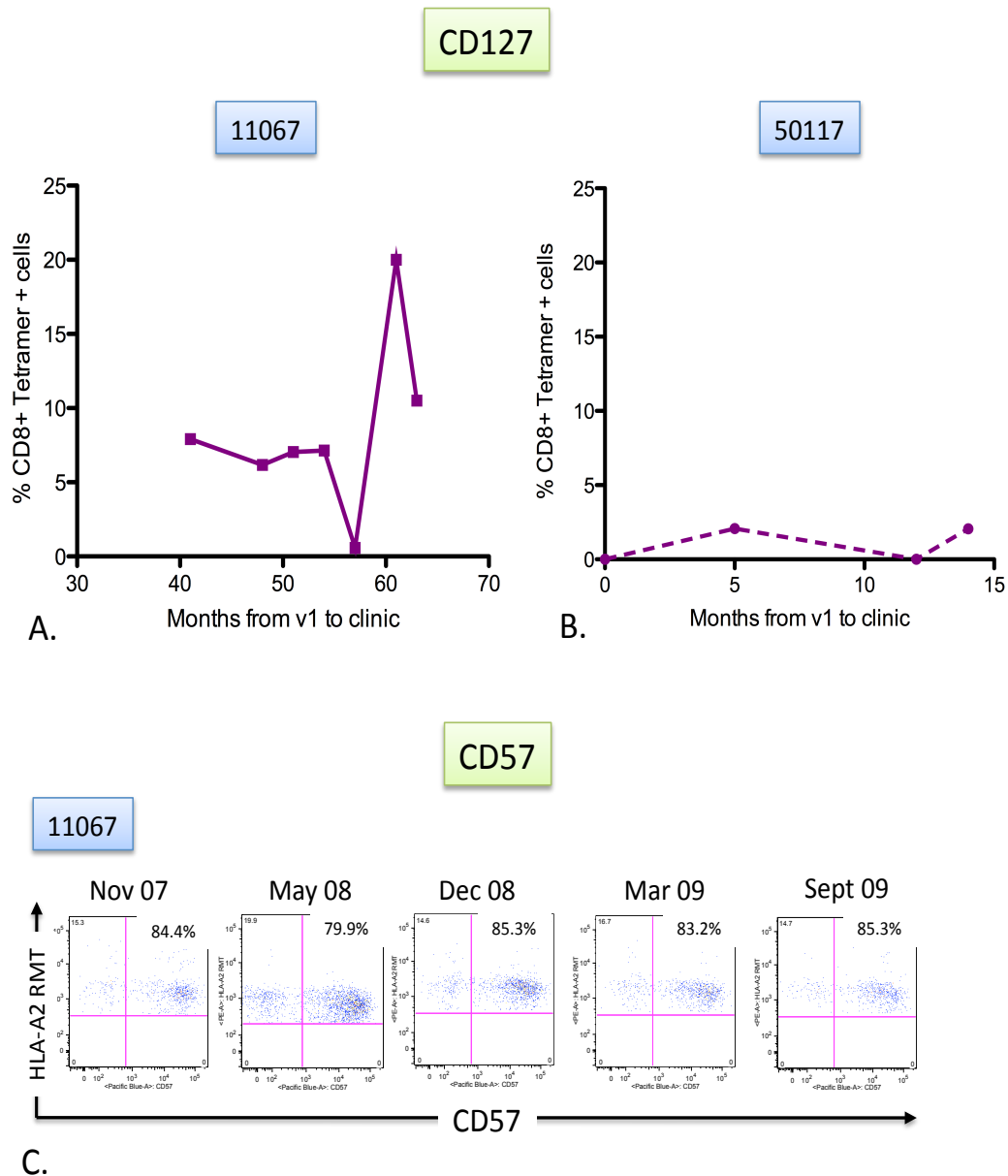


Figure 5.15 PARV4-specific T cells are principally CD127⁺ and CD57⁺. The percentage of CD127⁺ cells is indicated for patients 11067 (A) and 50117 (B). The connecting line for 50117 is dashed as previous experiments showed that at 12 months, RMT-specific T cells were absent (rather than simply being CD127⁺ as this graph might imply). C. 11067 T cells were principally CD57⁺. The percentage of CD57⁺ cells is indicated in the top right corner of each plot. These cells are gated on CD8⁺ HLA-RMT⁺ T cells.

c. RMT-specific T cells are activated

11067 RMT-specific T cells were over 80% PD-1⁺, and between 40% and 60% CD38⁺ (median: 52%, Figure 5.16A). 50117 RMT-specific T cells were 58-78% PD-1⁺ and 44-52% CD38⁺ (Figure 5.16A). Hence, PARV4 antigen-specific T cells appear to have an activated phenotype. PD-1 can be a marker of cellular exhaustion or activation, which was determined through the ICS assays described below.

d. RMT-specific T cells contain cytolytic proteins

The RMT-specific cells of patients 11067 and 50117 carry the activation surface marker CD38, thus it was interesting to look at the presence of granzymes A and B, and perforin, to assess the T cells' ability to kill infected cells. Between 60% and 89% of 11067 RMT-specific cells expressed granzymes A and B, as well as perforin intracellularly (Figure 5.16B). Conversely, fewer RMT-specific T cells from patient 50117 were granzyme B⁺ or perforin⁺ (14-36% for granzyme B⁺ and 3-14% for perforin⁺, Figure 5.16B). 58% and 66% of 50117 RMT-specific cells were granzyme A⁺ at each time point respectively.

e. CD161 and chemokine receptors

5-8% and 13-21% of 50117 and 11067 RMT-specific cells respectively were CD161⁺ (Appendix Figure A7 for 50117 and Figure 5.17A for 11067). There were no RMT-specific CD161 bright T cells (Figure 5.17D, left panel). 11067 T cells were further stained for several chemokine receptors. RMT-specific cells were predominantly CCR2⁻, CCR6⁻, CXCR4⁺ and CX3CR1⁺ (Figure 5.17B and C). Gating strategies for CD161, CCR2 and CCR6, which proved more challenging, are shown in Figure 5.17D.

f. RMT-specific T cell phenotype summary

Though samples from patient 11067 spanned 22 months, the phenotype of PARV4 antigen-specific T cells did not change significantly over time in either patient. Thus the CD8⁺ T cell phenotype can be summarised in Table 5.5.

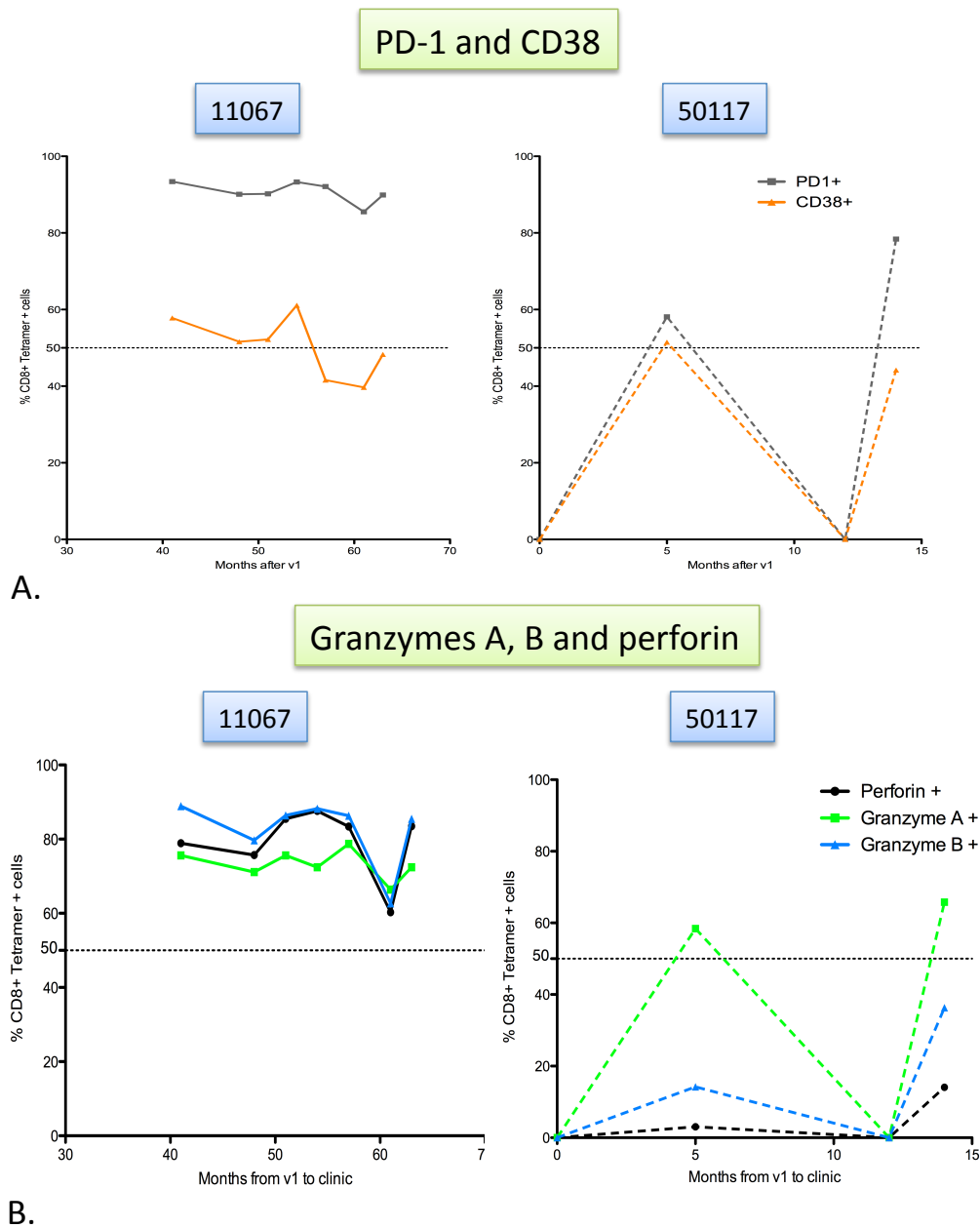


Figure 5.16 Antigen-specific T cells are activated and contain cytolytic proteins. The activation status of PARV4-specific CD8⁺ T cells was assessed by markers PD-1 and CD38 **(A)** and granzymes A, B and perforin **(B)** for patients 11067 (left panels) and 50117 (right panels). Each data point represents the percentage of tetramer-positive cells that are positive for that marker.

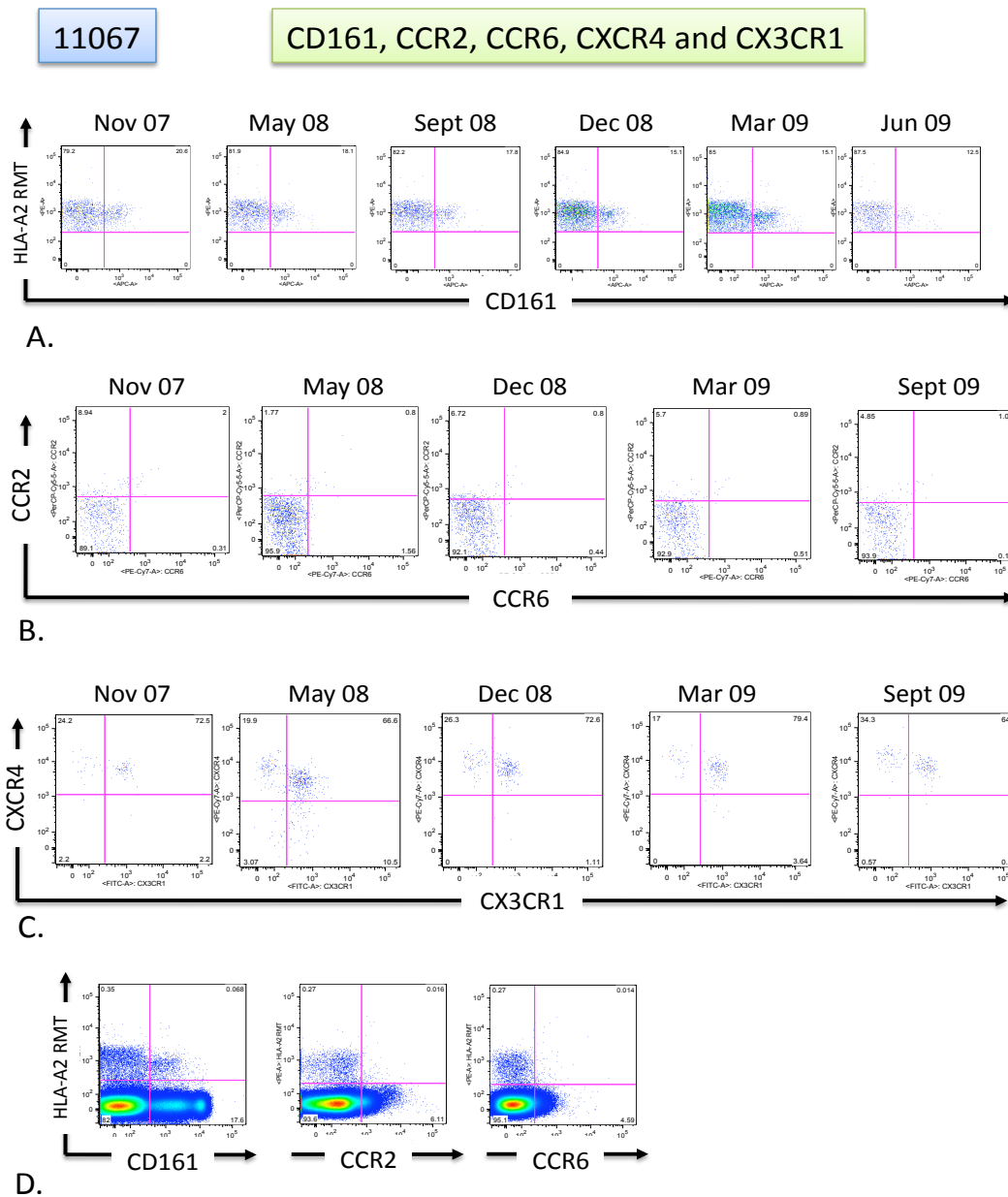


Figure 5.17 PARV4-specific T cells are predominantly CD161⁻, CCR2⁻, CCR6⁻, CXCR4⁺ and CX3CR1⁺. PBMC from patient 11067 were stained for CD161 (A), CCR2 and CCR6 (B), CXCR4 and CX3CR1 (C). FACS plots in A, B and C are gated on CD8⁺ HLA-A2 RMT⁺ T cells. For CD161, CCR2 and CCR6 staining, the gates are shown in D. (gated on CD3⁺ cells) as negative and positive populations were not obvious from CD8⁺ T cell plots.

	11067	50117
CCR7 CD45RA	T _{EMRA}	T _{EM}
CD57	+	N/A
CD127	-	-
CD38	+	+
Perforin	+	-
Granzyme A	+	+
Granzyme B	+	-
CD161	-	-
PD-1	+	+
CCR2	-	N/A
CCR6	-	N/A
CXCR4	+	N/A
CX3CR1	+	N/A

Table 5.5 Summary of the phenotype of HLA-A2 RMT tetramer-positive T cells in patients 11067 and 50117. N/A: data was not available due to limited samples available to test.

3.2.3. PARV4-specific T cells are polyfunctional

PBMC samples from patients 11067, 50114, 50117, 70110 and 70184 were available for intracellular cytokine staining (ICS) and were stimulated with peptides defined by ELISpot (Table 5.2). The production of IFN- γ , MIP1- β , TNF α , CD107a and IL-2 by CD8 $^{+}$ and CD4 $^{+}$ T cells is plotted in Figure 5.18A and B. Each data point represents the production of cytokine at one time point by one individual in response to one PARV4 peptide or pool.

11067 T cells showed broad polyfunctionality at most time points (Figure 5.18, symbols in red); more so by CD8 $^{+}$ T cells than by CD4 $^{+}$ T cells. 70110 and 50117 showed T cells producing IFN- γ , MIP1- β and some IL-2 by both CD8 $^{+}$ and CD4 $^{+}$ T cells. Pie charts were drawn using SPICE software to assess the polyfunctionality of T cells stimulated by PARV4 at a single-cell level. Representative FACS plots and SPICE pie charts are shown in Figure 5.19 for 11067 responding to peptides 2.3, 3.6 and epitope RMT, Figures 5.20 and 5.21 for 70110 responding to pools 2 and 7, and Figure 5.22 for 50117 responding to pool 3 and epitope RMT.

PARV4-specific CD8 $^{+}$ T cells from 11067 produced predominantly IFN- γ , MIP1- β and TNF α (Figure 5.19A and B), as well as IL-2 and some were CD107a $^{+}$. These cells were often polyfunctional over several time points, in response to peptide stimulation as shown in Figure 5.19C and D.

CD8 $^{+}$ T cells from patient 70110 expressed IFN- γ in response to pool 7 especially (Figure 5.20A), but fewer cells were stimulated as well as fewer being polyfunctional (Figure 5.20B and C). On the other hand, more CD4 $^{+}$ T cells produced MIP1- β and IFN- γ (Figure 5.21A), and many of these cells were polyfunctional (Figure 5.21B and C). CD4 $^{+}$ T cells stimulated from patient 50117

are shown in Figure 5.22A, these cells were also polyfunctional (Figure 5.22B and C). High background levels in patients 50114 and 70110 made it difficult to distinguish any PARV4-specific stimulation. The T cells stimulated by different individuals produced different cytokines and in different combinations. These are summarised in Table 5.6.

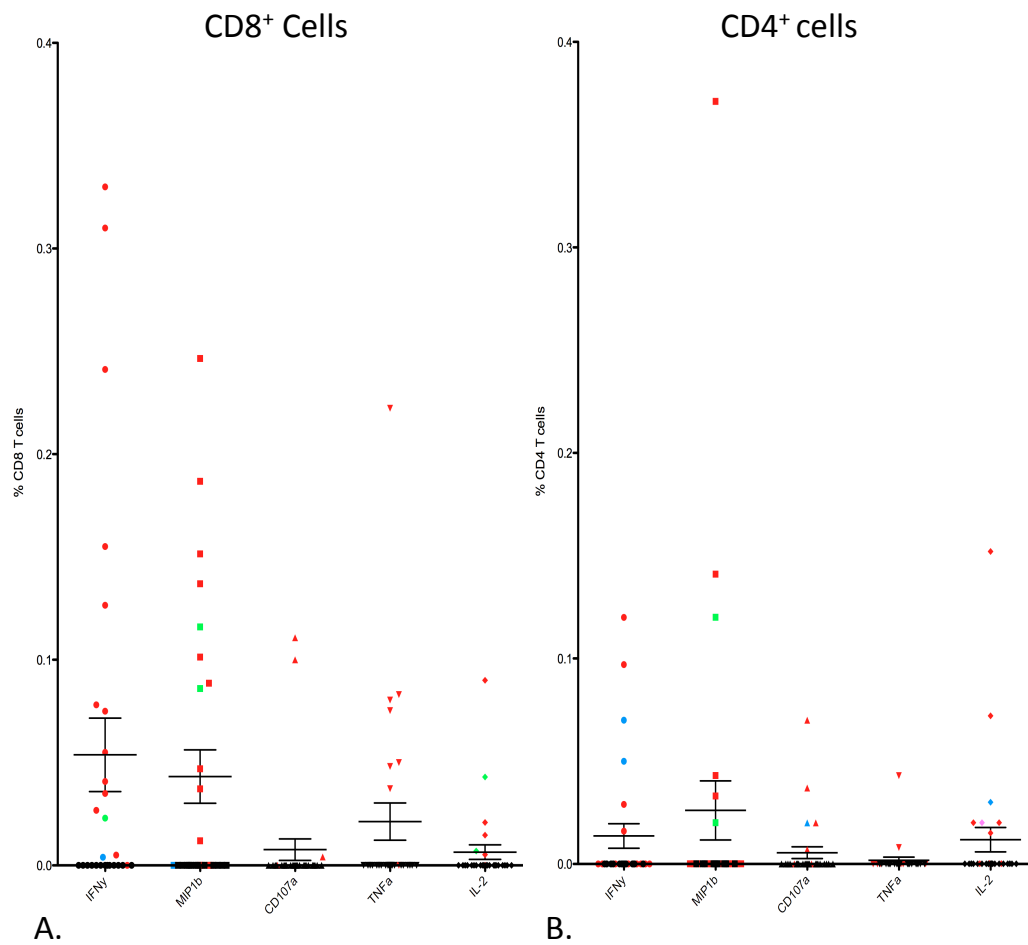
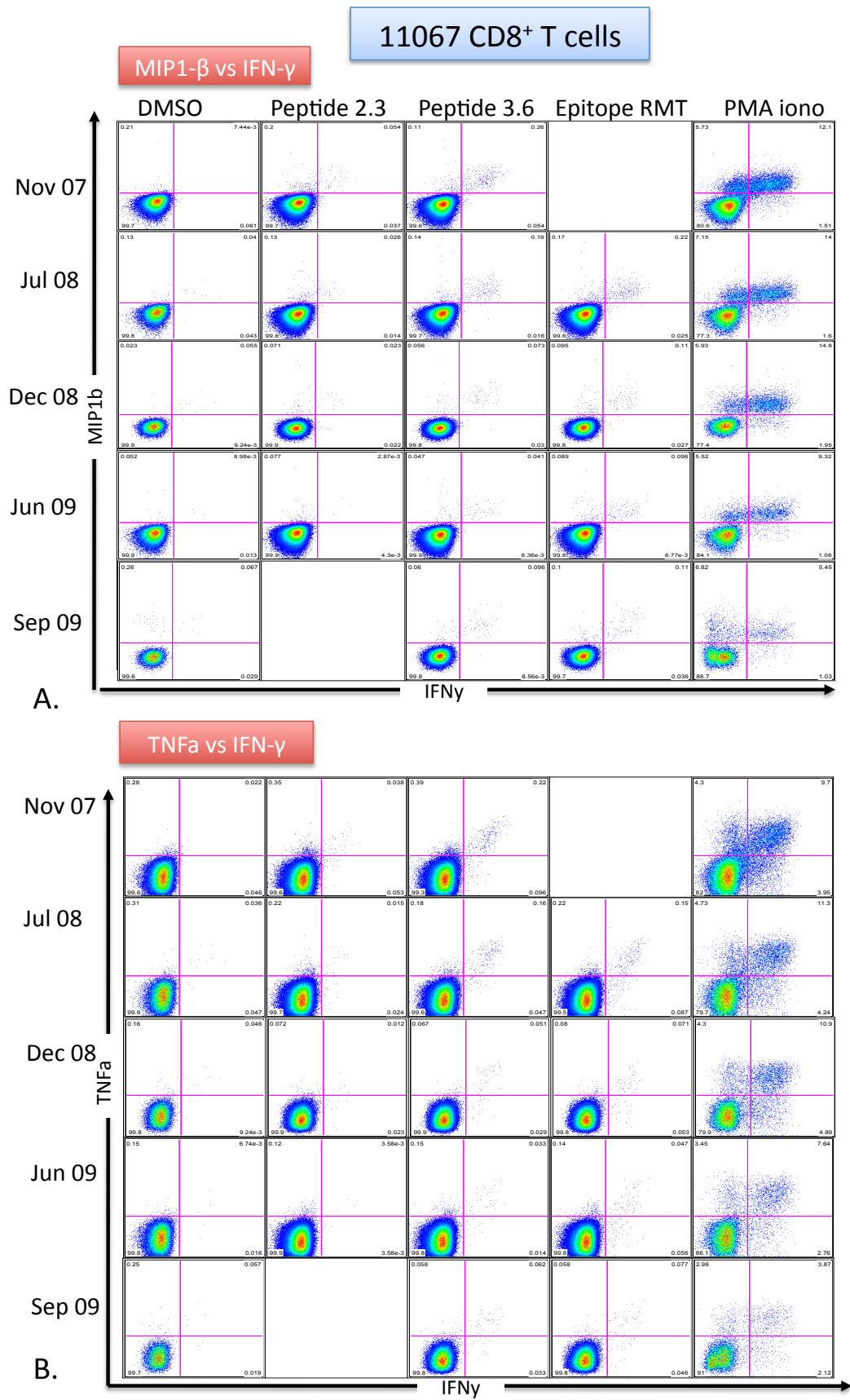


Figure 5.18 PARV4-specific T cells produce multiple cytokines and express degranulation marker CD107a. ICS assays identified the cytokines produced in response to stimulation by PARV4 NS peptides by CD8⁺ T cells (A) and CD4⁺ T cells (B). Each data point represents the production of cytokine at one time point by one patient. Only responses by patients 11067, 70110 and 50117 were visible and these are in red, green and blue respectively. For each time point a sample stimulated with DMSO only was used as a control and this value was subtracted to the peptide-stimulated sample values.



See next page for Figure legend.

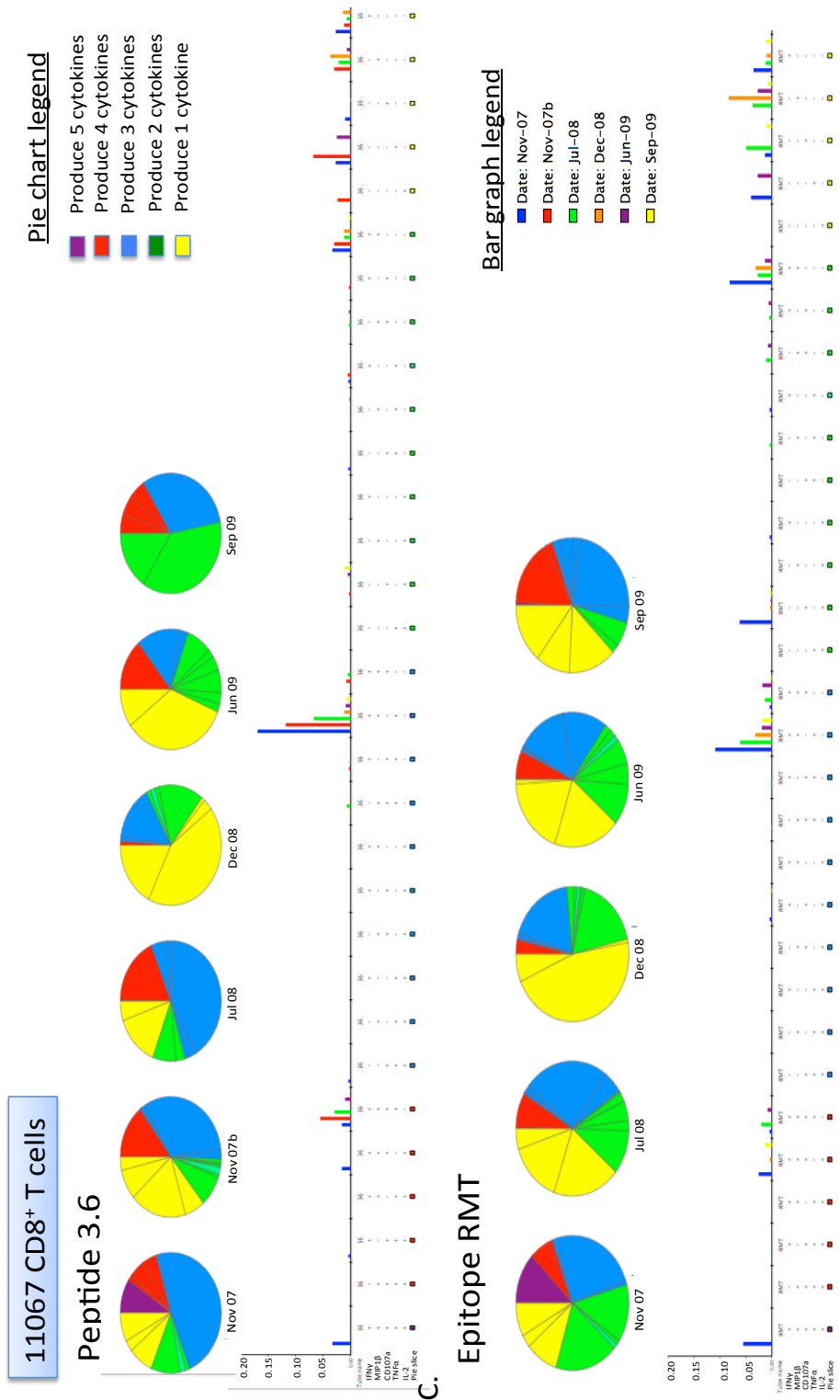
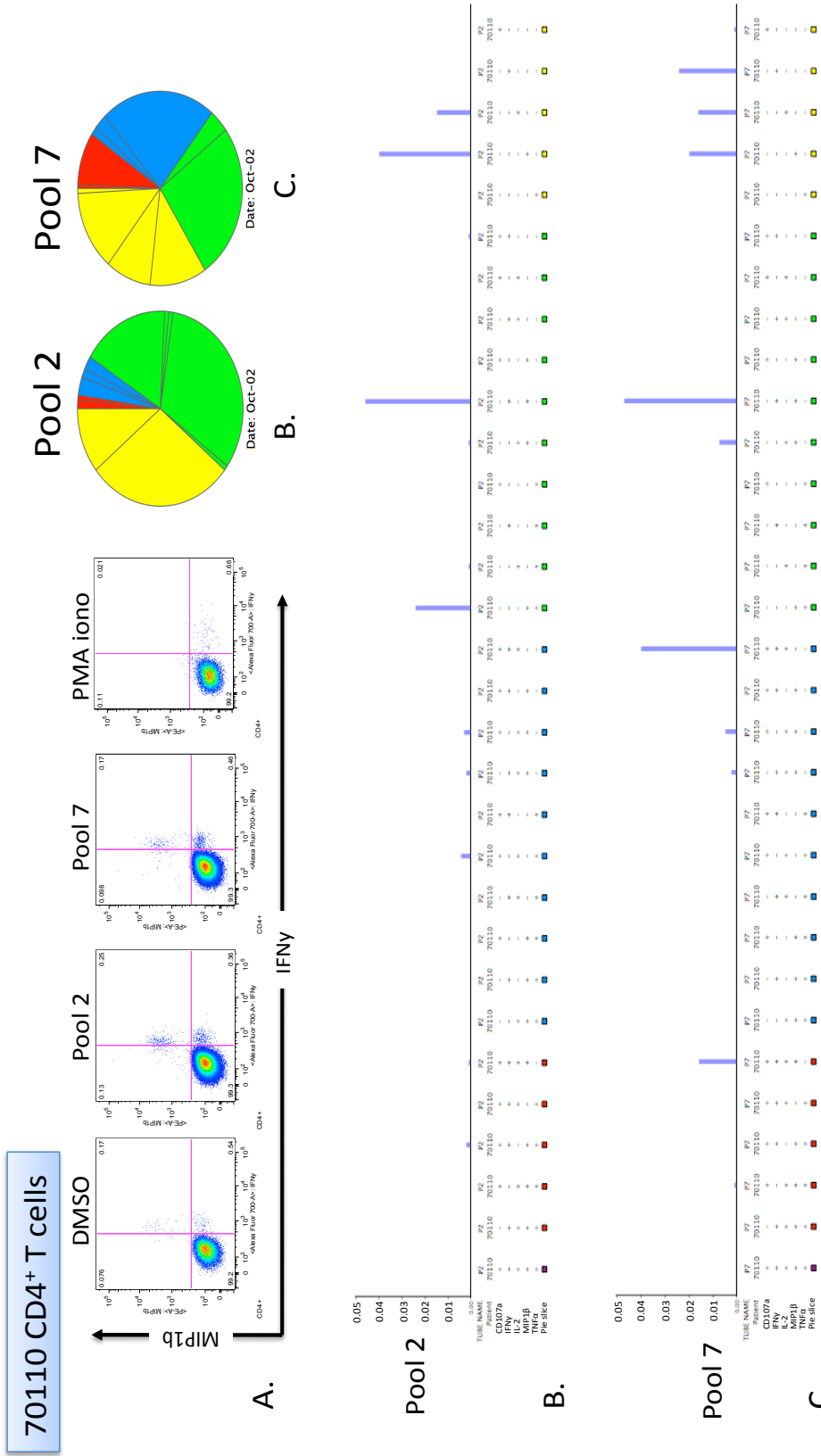


Figure 5.19 CD8⁺ T cells in patient 11067 are polyfunctional. Representative FACS plots are shown in **A.** (MIP1- β and IFN- γ) and **B.** (TNF α and IFN- γ). DMSO was used as a negative control, PMA and ionomycin ("PMA iono") were used as a positive control. FACS plots are gated on CD8⁺ T cells. SPICE pie charts and bar graphs show the polyfunctionality of the CD8⁺ T cells in response to peptide 3.6 (**C**) and epitope RMTENIVEV ("RMT", **D**). The pie chart reference of colours is also applicable to Figures 5.20 to 5.22.



Figure 5.20 The production of cytokines by CD8⁺ T cells in patient 70110. A. Representative FACS plots for the production of MIP1-β and IFN-γ. DMSO was used as a negative control, PMA and ionomycin ("PMA iono") were used as a positive control. FACS plots are gated on CD8⁺ T cells. **B, C.** SPICE pie charts and bar graphs show the polyfunctionality of the CD8⁺ T cells in response to pool 2 (**B**) and pool 7 (**C**).



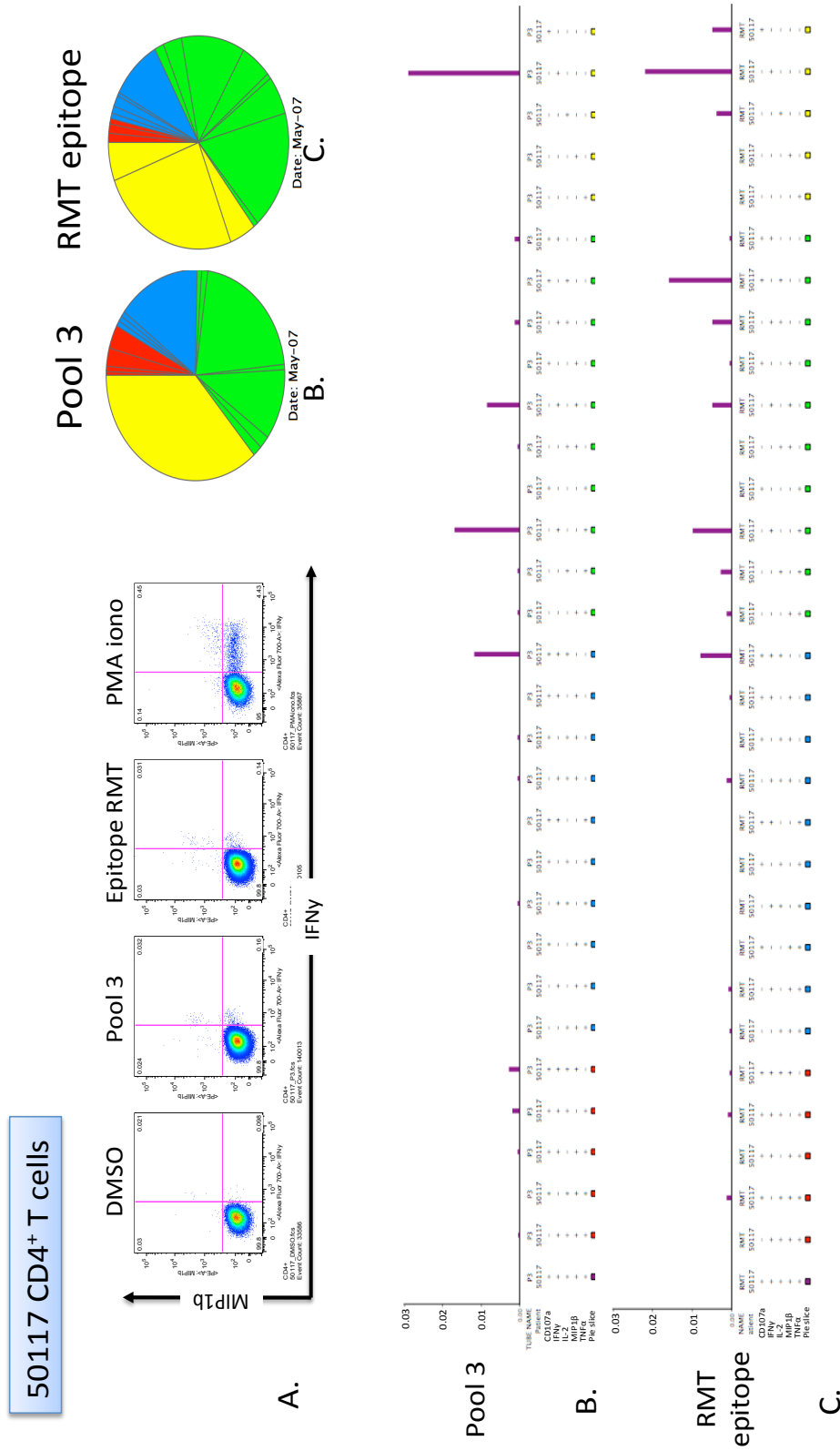


Figure 5.22 The production of cytokines by CD4⁺ T cells in patient 50117. A. Representative FACS plots for the production of MIP1-β and IFNγ. DMSO was used as a negative control, PMA and ionomycin (“PMA iono”) were used as a positive control. FACS plots are gated on CD4⁺ T cells. **B, C.** SPICE pie charts and bar graphs show the polyfunctionality of the CD4⁺ T cells in response to pool 3 (**B**) and epitope RMTENIVEV (“RMT”, **C**).

ICS summary

	CD8 Cytokines	CD4 Cytokines
11067	IFN- γ + MIP1- β + TNF α IFN- γ + MIP1- β MIP1- β	V. Low for most except 1 time point: IFN- γ MIP1- β
50114	IFN- γ + TNF α + CD107a IFN- γ + IL-2 IL-2	IL-2
50117	IFN- γ MIP1- β	IFN- γ + IL-2 + CD107a IFN- γ + IL-2 IFN- γ
70110	IFN- γ + MIP1- β MIP1- β IL-2	IFN- γ + IL-2 + CD107a IFN- γ + MIP1- β MIP1- β + TNF α MIP1- β IL-2
70184	CD107a	IFN- γ + CD107a IL-2 + CD107a

Table 5.6 Summary of the production of cytokines IFN- γ , MIP1- β , TNF α , IL-2 and the presence of degranulation marker CD107a on CD8⁺ and CD4⁺ T cells as determined by ICS assay. The “+” sign between two cytokines signifies the T cells were polyfunctional for these cytokines.

4. Discussion

4.1. PARV4 infection correlates with intravenous drug use rather than HCV co-infection

This is the first study of a large cohort of PARV4 acutely infected individuals. PARV4 has been strongly associated with intravenous drug use in many studies, including in this thesis (Chapter 4). In general, most IDUs are co-infected with HCV, which makes the study of PARV4 infection difficult to dissect from the hepatic virus. Only one study of PARV4 has previously looked at a group of 10 IDUs that were HCV-negative, and only one of these was PARV4 viremic (Lurcharchaiwong et al., 2008). The REACH cohort from Baltimore provided an ideal setting to study PARV4 infection alone.

Though these individuals were HCV- and HIV-negative, 36% of them were PARV4 seropositive. This rate is similar to other HCV-infected cohorts (see Chapters 3 and 4), strengthening the correlation between PARV4 and intravenous drug use, rather than HCV. It would seem that the individuals of this cohort have somehow escaped HCV infection to date, while maintaining a high rate of acquisition of PARV4.

4.2. High incidence of PARV4 infection

The time between the start of intravenous drug injection and PARV4 infection was calculated to be between 3.9 and 6.9 years (based intravenous drug use for 2 and 5 years respectively, prior to enrolment in the REACH cohort). Additionally,

the incidence of PARV4 was calculated to be 4.5% to 7.5% per year. This compares with the incidence of HCV that is 7.8% per year, in a different cohort of IDUs in Baltimore. The incidence of PARV4 is also substantially higher than that of HIV-1 which had decreased to 0% in the period from 2003 to 2005 in a similar population in Baltimore (Mehta et al., 2011). This suggests that PARV4 is a commonly encountered virus that could be transmitted at comparable rates to HCV. This has important consequences for the safety of blood products.

4.3. Acute PARV4 infection: viremia, IgG seroconversion and IgM

Dates of anti-PARV4 IgG seroconversion were different for every individual, thereby suggesting there was no common time of transmission (Table 5.3). No data on whether these individuals knew each other or shared injection equipment was available.

This is the first study to determine the length of viremia of PARV4. From these individuals it can be estimated that viral DNA remains in peripheral blood between 1 and 3 months. PARV4 viral loads were determined for 6/8 patients, and averaged at 5×10^3 copies/ml. These low levels are characteristic of PARV4 infection and have been observed in other studies (Schneider et al., 2008a, Tuke et al., 2009, Fryer et al., 2007d). Anti-PARV4 IgG was either detected simultaneously to viral DNA or 1 month later (being the next available sample). IgM was also detected at a similar time but with monthly samples, it was difficult to assess the exact timing of IgM with respect to IgG and viremia. Nonetheless,

acute PARV4 infection can be defined by short-lived viremia and concomitant appearance of IgM.

4.4. High antibody titres are not associated with high T cell responses

The antiviral humoral and cellular immune responses work in concert with each other, as antibodies can bind free virus and T cells can control the infection in peripheral tissues. From this data, it was determined that IgG levels appeared within the first month of viremia and increased progressively until they peaked between 4 to 7 months after seroconversion. The rate of appearance of IgG was similar to that of the HCV and HIV-1 humoral responses, estimated around 2 to 6 weeks (Tomaras et al., 2008, Netski et al., 2005). T cells responses to PARV4 NS were detected later in time and gradually increased, peaking at 5 months post seroconversion. From the T cell responses seen in patient 11067, it can be hypothesised that, as PARV4 infection persists, T cell responses become stronger and more focused on the peptides they respond to. Indeed, T cell responses were targeting peptide pools 2, 3 and 8 with very little background in other pools (Figure 5.11A). The term memory inflation has been used to define an epitope-specific T cell increase reaching high levels over time. In murine CMV infection, T cell responses in the blood to one epitope represented 6% of CD8⁺ T cells 8 days after infection. These levels began to rise 1 month later, peaking at 20% 400 days after infection (Karrer et al., 2003). This was due to repetitive CMV antigen exposure occurring through the latent phase of infection. This supports a similar mechanism of viral persistence in PARV4 infection.

T cell responses to the VP capsid protein were low and infrequent. The cellular response to parvovirus B19 capsid proteins was predominantly by CD4⁺ T cells (Kasprowicz et al., 2006). Due to limited samples, it could not be determined whether this was the case for PARV4 VP responses, but levels of response were lower than those seen to the non-structural proteins (both in B19 and PARV4).

From the plots showing antibody levels and ELISpot responses to the NS protein (Figure 5.23), it can be speculated that when antibody levels are high enough, cellular responses may not need be so high to eliminate viremia. Indeed, patients 50114, 70418 and 70429 had levels of IgG that reached the upper limits of detection of the ELISA assay, while T cell responses remained relatively low. In counterpart, 11067 and 70184 had high T cell responses but relatively low IgG levels. For the case of 11067, however, this may be because the peak of IgG seen shortly after infection was missed as no samples were taken for 3 years after the first visit (when the patient was PARV4 viremic). Both IgG and T cell responses were high in patient 11161; however, the level of T cell responses was mainly due to a combination of smaller positive responses rather than individual large responses.

Discrepancy between humoral and cellular responses was also observed in the chronically infected HCV⁺ individuals studied in Chapter 3. Differences in cellular responses may be due to variability in patient HLA types, some molecules able to bind PARV4 peptides better than others. No HLA information was available for this current group; additionally, a very large cohort would be needed to show any effect of host genetics. It is also possible that, if PARV4 replicates in actively dividing cells like its family members, exposure of the virus to extracellular antibodies may be limited.

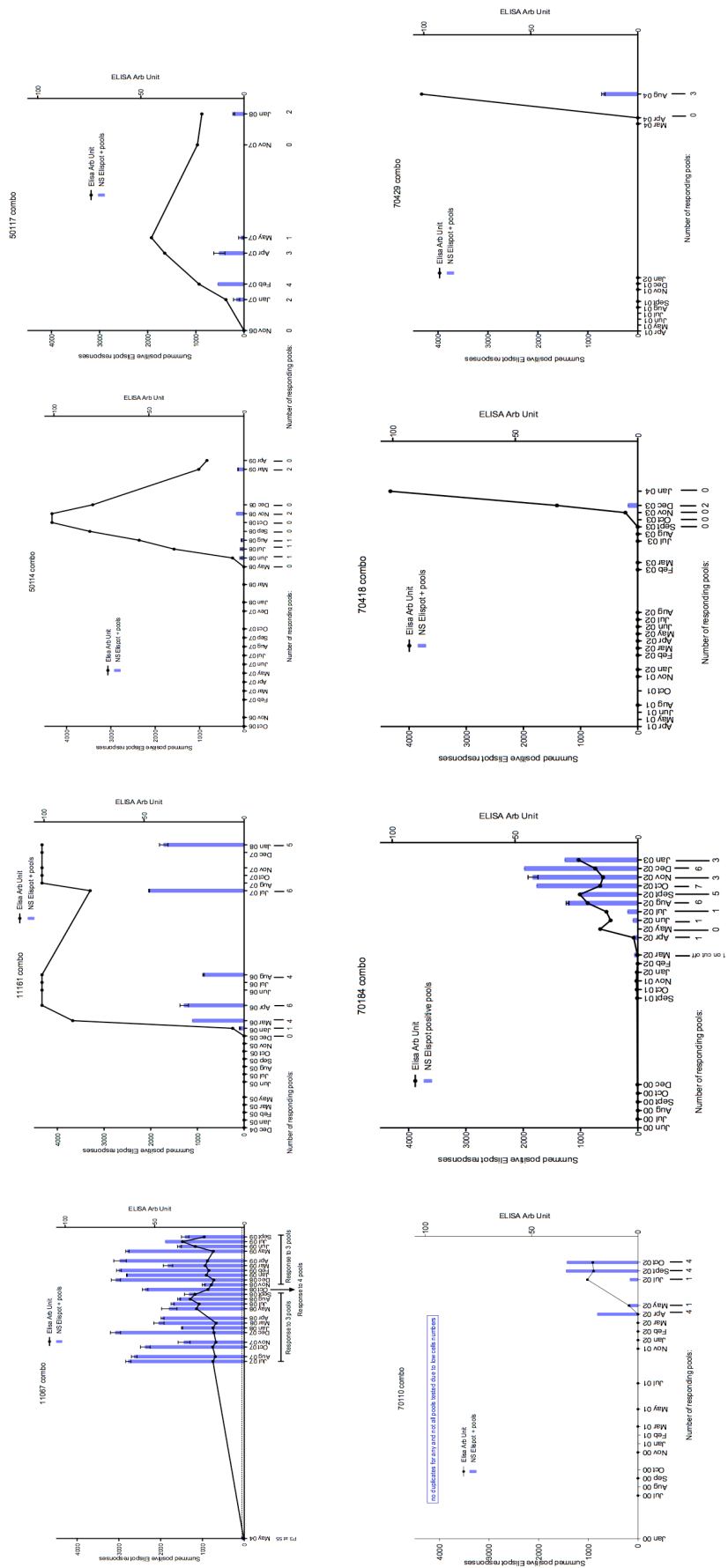


Figure 5.23 Summary of T cell responses and anti-PARV4 IgG levels in 8 IDUs who seroconverted for PARV4 during their attendance to clinic. Summed T cell responses are shown in columns (left axes), anti-PARV4 IgG levels are expressed in arbitrary units (right axes).

Alternatively, T cells may be serving as a second line of defence when the humoral response fails to clear PARV4. This would resemble LCMV infection, where a cellular response is critical to clear an established infection when the antibodies fail to clear the initial attack (Thomsen and Marker, 1988). It would be interesting to know whether individuals with high PARV4 humoral responses do indeed go on to clear infection totally. This may be suggested by the relatively low T cell responses that would have no ongoing stimulation if antigen were absent. On the other hand, it may be that PARV4 persists at much lower levels in individuals who have a strong humoral response.

This discordance in intensity of immune responses has been suggested to be behind the clinical differences seen between individuals with CMV, HIV-1 and HCV infections. Cellular immunity has been suggested to control disease in HIV-1 infection and in CMV-induced inflammation that leads to coronary artery disease, even in the absence of a humoral response (Zhu et al., 1999, Zhu et al., 2001, Rowland-Jones et al., 1995, Clerici et al., 1992, Rowland-Jones et al., 1993). T cells have also been suggested to protect against HCV infection of family members of chronic HCV-infected individuals, in the absence of anti-HCV antibodies (Scognamiglio et al., 1999).

4.5. PARV4-specific T cells are frequent and display a mature phenotype

RMTENIVEV epitope-specific T cells represented between 0.1% to 1.5% of CD8⁺ T cells. These levels were lower than those seen for EBV-specific T cells as these can range from 10% to 45% of CD8⁺ T cells to a single epitope in acute infectious

mononucleosis (Callan et al., 1998, Callan et al., 2000). However, these RMT-specific responses were similar to levels seen for CMV, HCV, HIV-1 or HBV-specific T cells (Gillespie et al., 2000, Klenerman et al., 2002, Wilson et al., 2000, Maini et al., 1999, Lechner et al., 2000, Jin et al., 2000).

These data show that the number of antigen-specific cells was higher than the number of cells producing IFN- γ (as calculated for Figure 5.13C). This may be due to different assays having different sensitivities, but it is also common that not all antigen-specific T cells are able to produce this cytokine, such as is seen in HCV- and HIV-specific T cells (Gruener et al., 2001, Goepfert et al., 2000).

The HLA type of the subjects was unknown however, as subject 11067 responded to an HLA-A*02-restricted epitope RMT, it can be inferred that they carry this HLA allele. Going on this assumption and the high responses to peptides 2.3 and 3.6, epitope prediction programmes Syfpeithi, Bimas and NetMHC pan suggest two further epitopes within these peptides (Table 5.7). Due to these peptides being targeted by only one individual in the cohort, further investigation into these epitopes was not pursued.

Through the use of MHC class I tetramers, antigen-specific T cells can also be classified into memory subsets according to cell-surface markers such as CD45RA, CCR7, CD127 and CD57. Antigen-specific CD8⁺ T cells express different combinations of these markers depending on the nature of the viral infection. As discussed in Chapter 1, influenza elicits an “early memory” phenotype where CD8⁺ T cells are CCR7⁺ CD27⁺ CD28⁺ CD45RA⁻ whereas CMV elicits a “late memory” phenotype with CCR7⁻ CD27⁻ CD28⁻ CD45RA⁺ T cells (Appay and Rowland-Jones, 2004; van Lier et al., 2003). CD8⁺ T cells for persistent viruses are also characteristically CD127⁻ and CD57⁺ as is seen in CMV and HIV (Le Priol

Restriction	Peptide	Peptide sequence	Epitope predicted	Length of epitope	Syfeithi score	BIMAS score	NetMHCpan 2.4 score*
HLA-A2	2.3	CILQQLFASHAVTLP	QLFASHAVTL	10mer	25	79.041	WB
HLA-A2	3.6	YLAPVWTLNIRE	YLAPVWTL	9mer	29	1695.106	SB

Table 5.7 Peptides 2.3 and 3.6 identified through ELISpot assays of patient 11067 contain predicted epitopes. *SB = strong binder, WB = weak binder as defined by NetMHCpan server. For epitope prediction details see Chapter 2.

et al., 2006, van Leeuwen et al., 2005). The expression of these markers was tested in PARV4-specific CD8⁺ T cells and is detailed below.

This study found that the PARV4 T cell response from one individual (50117) had an effector memory phenotype (CCR7⁻CD45RA⁻, also known as T_{EM} cells), whereas another individual (11067, non-acute) had a more differentiated effector memory CD45RA⁺ phenotype (CCR7⁻ CD45RA⁺, T_{EMRA} cells). T_{EMRA} cells are also known as “revertants” as they re-acquire CD45RA, as in the naïve state. It has been suggested this may be as a result of antigen exposure. It is hypothesised that low or absent antigen, as is observed in latent CMV infection, may allow for the differentiation to a T_{EMRA} phenotype, whereas high antigen load, as in HIV-1 infection, may lead to an early abrogation of differentiation, explaining why most HIV-1-specific T cells are T_{EM} (Waller et al., 2008, Champagne et al., 2001).

By losing CCR7 expression, these cells lose their capacity to home to lymph nodes, and are thereby able to patrol peripheral tissues for a possible secondary infection. It could be hypothesised that the samples studied in this cohort represent a time course for the development of immune memory cells, as 50117 is acutely infected, whereas the samples from 11067 were taken 3 years after PARV4 infection. Thus, initial responses would have an effector memory phenotype (CCR7⁻ CD45RA⁻, Figure 5.14B, April 2007 sample) which becomes increasingly CD45RA⁺, as seen in January 2008 (Figure 5.14B), and in subsequent 11067 samples (Figure 5.14A). In another persistent viral setting, although B19-specific CD8⁺ T cells were CD45RO⁺ in acute infection (2 months post symptom development), they were then also shown to revert to CD45RA⁺ 12 months after the onset of symptoms (Isa et al., 2005, Kasprovicz, 2005). This

was also observed in CMV-specific CD8⁺ T cells that differentiate to a T_{EMRA} phenotype, whereas HIV-1-specific CD8⁺ T cells have a T_{EM} phenotype (Champagne et al., 2001).

80% of PARV4-specific T cells were also CD57⁺. CD57 has been described as a marker of terminal differentiation that is often co-expressed with CD45RA, and frequently found in chronic immune activation (Focosi et al., 2010). The expression of CD57 and CD45RA increased over time in B19 antigen-specific T cells (Isa et al., 2005). Similarly, HIV- and CMV-specific T cells expressed increased levels of CD57. Unlike, however, yellow fever virus- and vaccinia virus-specific T cells that are also CCR7⁻CD45RA⁺ but CD57⁻, potentially reflecting the acute nature of these latter viruses (Moss and Khan, 2004; Brenchley et al., 2003; Ahmed and Akondy, 2011).

CD8⁺ CD57⁺ T cells have been characterised by high cytotoxic potential; however, CD57 is known as a marker of cellular senescence and clonal exhaustion due to their sensitivity to apoptosis, their short telomeres, reflecting an extended replicative history and their poor ability to proliferate (Brenchley et al., 2003; reviewed by Strioga et al., 2011; Focosi et al., 2010). However, this concept has been challenged as many of these T cells rely on their proliferation potential to fight against secondary infection (Moss and Khan, 2004, Focosi et al., 2010). The CD8⁺ CD57⁺ T cell subset, also normally CD28⁻, has been shown to expand during chronic activation in several viral infections and is thought to be a result of persistent antigenic stimulation (Brenchley et al., 2003; Mojumdar et al., 2011). In their review, Strioga et al. (2011) define this subset as “highly antigen experienced CD8⁺ T cells”. Indeed, the increase in CD57 expression has been shown to positively correlate with plasma HIV-1 viral load (Mojumdar et al.,

2011). Thus, the expression of CD57 on PARV4-specific CD8⁺ T cells suggests that antigen may be persisting.

Le Priol *et al.* show that CD8⁺ CD57⁺ T cells express more adhesion molecules and fewer chemokine receptors than CD8⁺ CD57⁻ cells, enabling them to remain in peripheral tissues (Le Priol *et al.*, 2006). This is consistent with the data generated in this study, as CCR2 and CCR6 were not found on PARV4-specific T cells. The majority of PARV4-specific cells were also CD161⁻, an NK cell marker found on HCV- and HBV-specific T cells (Northfield *et al.*, 2008). CD161⁺ cells are often CCR6⁺, CCR2⁺, so the absence of all these markers on PARV4-specific cells is consistent with other studies (Billerbeck *et al.*, 2010).

PARV4-specific T cells did express CX3CR1. Le Priol *et al.* showed that CD8⁺ CD57⁺ T cells preferentially expressed CX3CR1, thereby homing these cells to tissues rather than lymphoid organs. CX3CR1 is also present in areas of necrosis and inflammation in acute HCV liver injury (Wasmuth *et al.*, 2008). In both HCV and HIV-1 infections, the serum levels of fractalkine, the ligand of CX3CR1, increased. They correlated with the level of HIV-1 viremia and returned to normal levels when patients were treated with anti-retroviral therapy (Wasmuth *et al.*, 2008, Foussat *et al.*, 2001). In PARV4 infection, CX3CR1 may be directing T cells to the sites of antigen persistence, as fractalkine is expressed by stressed endothelial cells. These are one of the targets of CMV, and CX3CR1 is expressed abundantly by CMV-specific effector T cells (Hertoghs *et al.*, 2010).

Harari *et al.* suggested that CD127, in combination with CD45RA, allowed a clearer definition of differentiation subsets, as this marker distinguished between influenza virus-specific T cells that efficiently cleared infection, and

HIV-1 specific T cells that were unable to control viral replication and allowed persistence (Harari et al., 2009).

CD127 is the receptor for IL-7 (IL-7R α), which is required for T cell survival in the absence of antigen (Kaech et al., 2003). CD127^{hi} T cells represent 5-10% of the T cell pool, and they are the subset that differentiate into memory CD8⁺ T cells which is maintained by IL-7 signalling during memory homeostasis (Wherry and Ahmed, 2004; Schluns et al., 2000). Several studies have shown that CD8⁺ T cells specific for viruses that are successfully cleared, such as influenza, respiratory syncytial virus, or acute HBV that was spontaneously cleared, expressed CD127 (van Leeuwen et al., 2005; Boettler et al., 2006). In contrast, CD8⁺ T cells specific for persistent viruses such as CMV and HIV, were CD127⁻. Moreover, in the acute phase of HIV and CMV infections, the decrease in CD127 expression correlated with plasma viremia, and the emergence of CD127⁺ T cells was observed over the course of HBV infection as antigen was contained (Mojumdar et al., 2011; van Leeuwen et al., 2005; Boettler et al., 2005). This has also been shown in mice infected with LCMV, where persistent antigen suppressed CD127 expression, whereas short-lived antigen only led to a temporary decrease of CD127 expression (Lang et al., 2005). These studies provide evidence that CD8⁺ T cells express CD127 in the absence of antigen.

It has been established that patient 11067 was not acutely infected with PARV4, and that infection most likely occurred three years prior to most of the samples tested, as this was when they were PARV4 viremic. RMT-specific cells from 11067 were CD127^{lo} for the duration of the study, spanning 3 to 5 years, which suggests that antigen may still be present 5 years post infection, otherwise it would be expected that CD127 expression would be upregulated. This is similar

to the study by Harari and colleagues who found that 80% of HIV-1 specific CD8⁺ T cells were CD127⁻ (Harari et al., 2009).

As patient 11067 is an active IDU, it is also possible that continuous exposure to PARV4 could result in a constant renewal of antigen, maintaining low CD127 levels. However, one sample was tested three years after primary infection and no viral DNA was detected. This supports the assertion that PARV4 persists at levels (below detection) leading to low CD127 expression.

Thus, PARV4-specific T cells are frequent and display a mature effector phenotype. However, high T cell frequencies do not mean they will be efficient at clearing the infection. This is covered in the next section.

4.6. PARV4-specific T cells in peripheral blood are activated following seroconversion and ready to kill

PARV4-specific T cells had an activated phenotype, evident by the presence of CD38. The frequency of expression of CD38 has been shown to decrease in parallel with the HIV-1 viral load – as HIV-1 replication is limited, cells become less activated (Tilling et al., 2002). With just over 50% of PARV4-specific T cells being CD38⁺, this may reflect an intermediate stage of activation due to low levels of persisting PARV4. CD107a is a lysosome-associated membrane protein (LAMP) which is brought to the cell surface as these lysosomes release their cytotoxic granules (Betts et al., 2003). Its presence on stimulated PARV4-specific T cells (by ICS) suggests these cells were degranulating in response to PARV4 peptides, which is an essential step in the release of cytotoxic molecules such as granzymes and perforin.

With 80% RMT-specific cells expressing PD-1, it was possible these cells were exhausted from over-exposure to antigen. PD-1 has been characterised as a marker for T cell exhaustion on cells unable to produce cytokines such as IL-2. The surface expression of PD-1 have been observed in HIV and HCV infections, where T cell exhaustion is thought to be a possible mechanism of viral evasion. Blocking the PD-1/PD-L1 pathway has been shown to restore T cell function (Day et al., 2006, Urbani et al., 2006, Barber et al., 2006). However, PD-1 is also expressed on 60% of memory CD8⁺ T cells in healthy individuals, and on yellow fever virus- and vaccinia virus-specific CD8⁺ T cells (Duraishwamy et al., 2011, Ahmed et al., 2011). These studies emphasise that the presence of PD-1 is not always indicative of exhaustion.

My data would suggest PD-1 is simply a marker of prolonged antigen exposure, as shown by the polyfunctional CD8⁺ T cells observed by ICS. Wherry *et al.* define several phases of exhaustion by the cytokines the cells are able to produce (Wherry and Ahmed, 2004). Table 5.6 illustrates that PARV4-specific CD8⁺ and CD4⁺ T cells produce a range of cytokines. Additionally, the ELISpot data detects the production of IFN- γ , and with levels exceeding 1,000 SFU/10⁶ PBMCs, these cells are still functional.

Many studies have shown that the quality of the T cell response correlates with immune protection (Betts et al., 2006, Ciuffreda et al., 2008). Polyfunctional CD8⁺ and CD4⁺ T cells, and especially their ability to produce IL-2, have been correlated with increased viral control in several HIV-1 studies, either in virus suppression assays or as the predominant T cell subset in non-progressors compared to progressors (Betts et al., 2006, Akinsiku et al., 2011, Kannanganat et al., 2007, Julg et al., 2010, Zimmerli et al., 2005). Although IFN- γ and TNF- α

are important in mediating cytotoxicity, IL-2 is crucial for CD4⁺ and CD8⁺ T cell proliferation, thereby providing the tools for a more efficient effector response (Iyasere et al., 2003, Darrah et al., 2007, Zimmerli et al., 2005). This has also been observed in HCV infection, where the presence of polyfunctional effector memory cells (CCR7-CD45RA⁻) in acute infection correlated with spontaneous resolution (Badr et al., 2008). Ciuffreda and colleagues (2008) also showed that HCV-monoinfected individuals had more polyfunctional and proliferating HCV-specific CD4⁺ and CD8⁺ T cells, whereas this was impaired in HCV/HIV co-infected individuals with more severe liver fibrosis. Moreover, Harari and colleagues (2005) found that CD8⁺ T cells specific for HLA-B restricted HIV epitopes were more polyfunctional and had a greater proliferation potential, suggesting polyfunction may explain the protection seen in HLA-B genotypes (Harari et al., 2005). Nonetheless, it is likely that a combination of high magnitude, broad and polyfunctional T cell responses are important for optimal protection (Julg et al., 2010, Iyasere et al., 2003).

These cells are evidently prepared for cytotoxic activity when they encounter antigen with the frequent expression of granzymes A and B, and perforin detected in CD8⁺ T cells of patient 11067. This profile, along with their CD57⁺ CD127⁻CD45RA⁺ expression, matches that of CMV-specific T cells where strong cytotoxic profiles were associated with antigen re-exposure (Harari et al., 2009). Interestingly, CD8⁺ T cells in patient 50117 expressed granzyme A, but very little granzyme B or perforin. This decrease is surprising as 50117 was acutely infected with PARV4, where a more activated phenotype would be expected. Granzyme A is more ubiquitously expressed than granzyme B, and higher concentrations are required to induce apoptosis (Anthony et al., 2010).

Additionally, Harari *et al.* found that the expression of granzyme A did not correlate with cytotoxicity in influenza virus, CMV, EBV or HIV-1 infections (Harari *et al.*, 2009). *GZMB* (the gene for granzyme B) was upregulated specifically in EBV and CMV effector memory T cells, whereas *GZMA* expression was high regardless of differentiation status (Hertoghs *et al.*, 2010). Hence it is possible that the ability of 50117 CD8⁺ T cells to fight PARV4 infection may be reduced compared to 11067, possibly due to differences in host genetics.

Nonetheless, granzyme A is thought to be important in inflammation, as it induces the production of pro-inflammatory cytokines and is present in the inflamed joints of rheumatoid arthritis patients (Metkar *et al.*, 2008, Tak *et al.*, 1999). Granzyme A can also interfere directly with viral replication (Simon *et al.*, 1987). Thus, it is possible that granzyme A has an antiviral capacity against PARV4 that is independent from perforin.

Thus, PARV4-specific T cells are activated and able to release cytotoxic granule molecules. This is reminiscent of B19- and HCV-specific T cells that remain activated long after viral DNA or RNA is no longer detected in peripheral blood, thereby suggesting viral persistence that is below the threshold of detection (Pham *et al.*, 2004, Kasprovicz, 2005, Isa *et al.*, 2005).

These data clearly show that PARV4-specific cells are mature and activated, which is very similar to B19- and CMV-specific CD8⁺ T cells (Isa *et al.*, 2005, Moss and Khan, 2004). The presence of CD38, CD57 and absence of CD127 all suggest that, similar to B19 and CMV, PARV4 is a persistent virus. This is unlike virus-specific T cells in other persistent infections such as HIV and HCV, potentially reflecting their inability to control viral replication contrary to

immunocompetent individuals infected with B19 and CMV (van Lier et al, 2003). This may be due to T cell exhaustion and viral variation in HCV and HIV, which does not occur in PARV4. The combination of markers and cytokines produced all suggest that the T cells are encountering antigen months and years after primary infection, which is consistent with PARV4 being a persistent virus.

5. Summary of findings

- The transmission of PARV4 is associated with intravenous drug use, rather than directly with HCV infection
- The incidence of PARV4 was estimated to be between 4.5% and 7.5% per year in this cohort of IDUs
- Anti-PARV4 IgG levels are detectable many years after primary infection
- High IgG levels do not correlate with high T cell responses
- Different individuals have varying levels of IgG and T cell responses over the years, which may in part explain the discrepancy seen within individuals in Chapter 3
- 0.5-1.5% of CD8⁺ T cells are specific for a single epitope (RMTENIVEV) in acute infection
- PARV4-specific T cells have a mature and activated effector memory phenotype (CD45RA⁺ or CD45RA⁻)
- These cells are polyfunctional and produce granzymes and perforin
- My data provide further immunologic evidence for PARV4 antigen persistence years after infection

Chapter 6

Conclusion

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Jones *et al.* wrote at the time of PARV4 discovery in 2005 that “the detection of a parvovirus highly distinct from the B19 group or AAV group suggests that the number of parvoviruses able to replicate in humans may be larger than currently appreciated” (Jones et al., 2005). In the past 6 years this has been confirmed with the discovery of human bocavirus (HBoV) and additional genotypes for previously known human parvoviruses (Allander et al., 2005). The immune response to PARV4 had never been characterised prior to this study. Here I summarise the results from the chapters of this thesis, projecting how they contribute to what was previously known about PARV4 and the antiviral immune response.

1. New approaches to studying PARV4

In the studies presented in this thesis, several assays were used to detect past and present PARV4 infection. PARV4 viral DNA was detected in plasma samples using DNA extraction followed by PCR. This was only possible in acute samples as discussed in Chapter 5; however, due to low viral loads this is not a reliable technique to detect PARV4 infection beyond 1 month after transmission. Other studies have also found that PARV4 DNA is difficult to detect in peripheral blood (Manning et al., 2007, Lau et al., 2008, Sharp et al., 2009, Schneider et al., 2008b). Hence, different methods have been developed to assess the prevalence of PARV4. Sharp and colleagues developed an ELISA to detect anti-PARV4 IgG, an assay extensively used in my studies, while Yang *et al.* developed an immunoblot assay to detect these antibodies (Yang et al., 2011, Sharp et al., 2009). These assays facilitated the assessment of the seroprevalence of PARV4, and the study

of the humoral immune response over the course of infection. T cells retain an imprint of previous and present infections, and provide information on the cellular immune response profile to a particular virus. My study is the first to look at the cellular response to PARV4 and to use a combination of ELISpot, ICS and tetramer-stain assays to study PARV4 infection.

Thus, many tools are now available to study this novel virus, and combined they provide the most complete picture of PARV4 infection. For example, Chapter 5 showed it was important to use both tetramer-stains and ELISpot assays to study antigen-specific cells, as they do not all produce cytokines upon stimulation *ex vivo*. Discrepancy in the detection of anti-PARV4 IgG and T cell responses in several subjects also suggests that using one method alone does not allow a complete assessment of the prevalence of this virus.

Through these techniques I have determined that PARV4 infection is nearly universal in a HIV⁺ HCV⁺ IDU cohort (REACH cohort), present in around 30% of HCV⁺ cohorts, 10% of HIV⁺ MSM cohorts, whilst remaining absent from healthy subjects in Western countries. This reaffirms the likelihood of parenteral transmission, where the added co-infection with HIV-1 may be a marker of more frequent exposure. There remains the possibility of sexual transmission, as 11% of MSMs from the Swiss HIV cohort were PARV4 seropositive, which echoed the detection of PARV4 in other individuals whose risk factor was heterosexual exposure (Simmonds et al., 2008).

A study that found PARV4 in infants in several neighbouring villages in Ghana suggests that PARV4 was common and ubiquitous, and possibly spread through contact, although these studies require replication. Sharp *et al.* (2010a) found PARV4 homologues in 63% of chimpanzees tested in Cameroon, suggesting a

possible environmental source that would primarily affect non-human primates. The authors commented that “a possible source in sub-Saharan Africa for PARV4 would contain many potential parallels with the emergence and global spread of HIV-1 and HCV in the 20th century”. Thus, PARV4 could also pose a threat for blood product recipients. Feline panleukopenia virus (FPV) spread to dogs in the 1970s through the mutation of two structural regions in the viral capsid protein (Parrish, 1999). It is possible that a similar event occurred for PARV4 from chimpanzees to humans, as the crossreactivity of antibodies to the VP capsid proteins suggests the homologues are very similar (Sharp et al., 2010a). This also seems plausible as the transmission of simian erythroviruses (similar to B19) to animal handlers has been reported (Brown et al., 2004).

2. The cellular response to PARV4

In this thesis I have demonstrated that PARV4 elicits strong T cell responses in HCV⁺ and HIV-1⁺ individuals, whether acutely or chronically infected. These reached over 2000 SFU/million PBMC detected by IFN- γ ELISpot, or 1.5% CD8⁺ T cells as detected by tetramer stain. T cell response levels were similar in PARV4 acute and chronic individuals, although possibly focussed on fewer pools in persistent infection. For a virus with such a small genome, these levels are extremely high. These responses were also frequent, broad and had a mature and activated effector memory phenotype, providing an ideal immune response to eliminate the virus, such as is seen in HCV (Lechner et al., 2000). However, T cell responses were also long-lasting (over 5 years post infection) suggesting that persisting antigen provided a source of stimulation. This is confirmed by the

general absence of CD127 on PARV4-specific T cells (Chapter 5). If PARV4 induces a robust immune response, why is the virus not eliminated? We can speculate this could be for several reasons. First, it may be that the immune system is failing to completely eliminate the virus, whilst keeping viral levels under control, as is seen in HCV, CMV and EBV infection (Klenerman and Kim, 2007, Khanna and Burrows, 2000, Ho, 1980). Viruses can evolve rapidly and thereby exploit defects in the immune system. Additionally, if the virus were to integrate into the genome, as discussed below, eliminating the virus would be very difficult. Finally, as I have found no clear association of PARV4 with pathology it may be that the immune system co-evolves with the virus only as it is necessary; if the virus is not a danger to the host, the immune system has no need to eliminate it completely.

I have also identified the first PARV4 epitope (HLA-A*02-restricted RMTENIVEV) together with other possible epitopes in Chapter 5 that require experimental confirmation. Additional epitopes would allow further dissection of the immune response to PARV4 in the general population. Peptide-specific $\gamma\delta^+$ T cells were also stimulated by PARV4 NS peptides, suggesting a potential new role for these cells in the antiviral response. The results presented in Chapter 3 are preliminary and require further investigation into the mode of action of these cells.

3. Models of PARV4 infection

Several subjects presented in this study showed a discrepancy between the presence of PARV4-specific T cells and antibodies (Chapter 3). The study of both

these branches of the immune system during acute PARV4 infection (Chapter 5) showed that this was not an experimental artefact. Indeed, individuals presented varying levels of antibodies and T cell responses to PARV4 antigens, potentially reflecting differences in the ability of the immune system to fight infection. Similar scenarios have been observed in LCMV, CMV, HIV-1 and HCV, discussed in detail in Chapter 5 (Thomsen and Marker, 1988, Zhu et al., 1999, Zhu et al., 2001, Scognamiglio et al., 1999, Rowland-Jones et al., 1995, Clerici et al., 1992).

These characteristics place PARV4 between the conventional classification of lytic and persistent viruses. Antibodies are critical for adaptive immune defence; if these are not sufficient to eliminate circulating virus, T cells play an important role in killing infected cells. These T cells are crucial against non-lytic persistent viruses as secreted antibodies do not have access to internal antigen. It is unknown whether PARV4 is cytopathic, nonetheless strong T cell responses are mounted against this antigen to maintain it under control.

From my work, a model of PARV4 infection and the elicited immune response can be drawn. This is shown in Figure 6.1. Similar to parvovirus B19, PARV4 is an ideal candidate for studying the role of the immune response during acute and chronic infection. This thesis has focused on the presence of IgM, levels of IgG and PARV4-specific CD8⁺ and CD4⁺ T cells. However, it is clear that $\gamma\delta$ ⁺ T cells are also strongly stimulated by PARV4 peptides. Further characterisation of these and other immune cells, as well as the targets of the humoral response would provide a more complete picture of the immune response to a persistent virus.

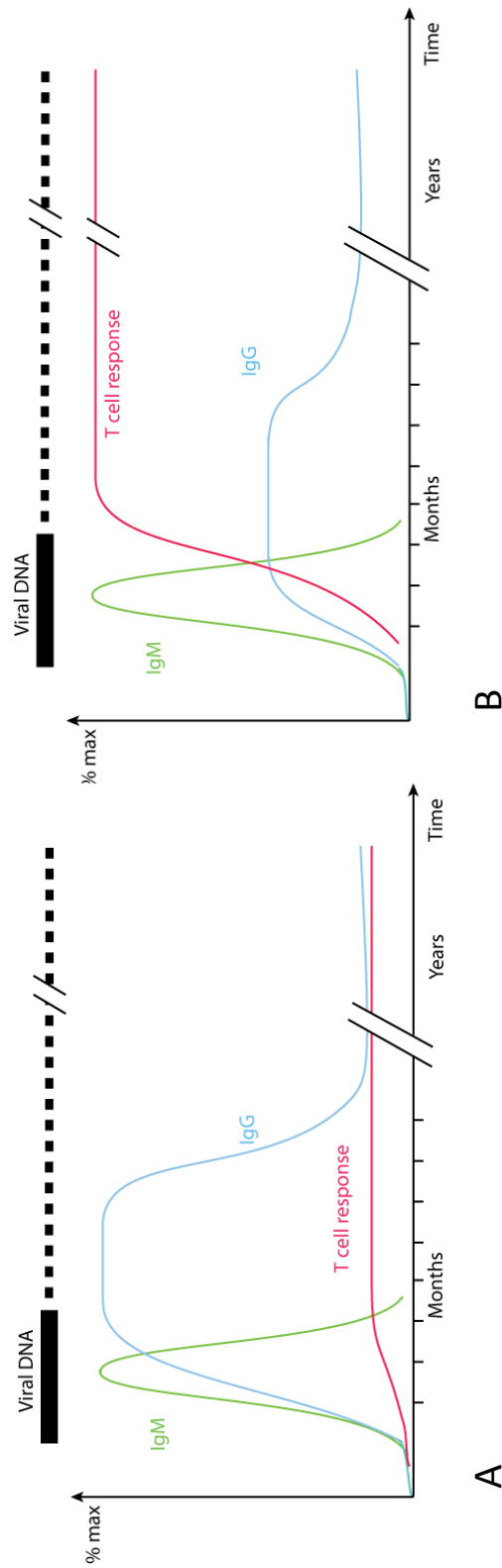


Figure 6.1 Simplified course of PARV4 infection. Viral DNA is detected in peripheral blood in the acute phase, between 1 and 3 months, levels then drop below the threshold of detection but it is hypothesised these persist even at low levels (indicated by the dashed line). IgM is detected simultaneously to viral DNA and disappears within 1-2 months. Two scenarios were observed for IgG and the cellular response in Chapter 5. **A.** IgG levels were high and persisted for several months, while the T cell response remained low or undetectable. **B.** IgG levels remained comparatively low while T cell responses reached high levels.

4. PARV4 persistence

All three data chapters of this thesis point towards a persisting virus. The high levels of T cell responses years after infection (Chapter 3 and individual 11067 in Chapter 5) suggest they are maintained by continuous exposure to antigen. This is confirmed by the absence of CD127 on the surface of PARV4-specific T cells. These cells are also mature and differentiated (CD57⁺) as is seen in many other chronic infections (Strioga et al., 2011). Additionally, parvovirus B19, which is the closest related virus to PARV4 and also elicits a strong T cell response, is a persistent virus that can be detected over two years after infection, but may remain at very low levels for much longer (Lindblom et al., 2005, Young and Brown, 2004).

There are several possibilities as to what form PARV4 may persist as. Firstly, PARV4 could be actively replicating at a very low level (Figure 6.2A). The cellular tropism of PARV4 is unknown, even though viral DNA has been found in many tissues. Hence it is possible that PARV4 is replicating in a peripheral site. As PARV4 most likely requires actively dividing cells to replicate and if, like parvovirus B19, it is cytopathic, one would expect to see substantial pathology. B19, although unable to replicate in non-permissive cells, is still able to express NS1 in these cells, and therefore present NS1 peptides on their surface (Poole et al., 2004). If applied to PARV4, this would allow the presentation of antigen to T cells long after acute infection (Figure 6.2B).

Alternatively, PARV4 could remain latent within a cell (Figure 6.2C-D). This could occur by integrating the host genome, as human T-lymphotropic virus (HTLV) does, or in an episomal form, such as Herpes Simplex virus 1 (HSV-1) or

EBV. Although integrating the genome would be a challenge for a virus with so few proteins and without an apparent nuclear localisation signal (NLS), several recent studies have found endogenous parvovirus sequences integrated into several animal and human genomes with evidence of established germline infection (Kapoor et al., 2010b, Katzourakis and Gifford, 2010, Liu et al., 2011, Belyi et al., 2010). Moreover, AAV is able to integrate the host genome through shared sequences (Kotin et al., 1990). Kerr *et al.* identified regions of sequence identity between several human and rodent parvoviruses with their host genomes, suggesting integration through recombination would be possible (Kerr and Boschetti, 2006).

Additionally, AAV is also able to persist in an episomal form (Duan et al., 1998). PARV4 contains a rolling circle replication motif (Ding et al., 2002, Fryer et al., 2007a), and recently two groups found human bocavirus (HBoV) in an episomal form in clinical samples (Kapoor et al., 2011, Lusebrink et al., 2011). HIV-1 DNA has been found in an episomal form, and several studies have shown evidence of protein expression from these episomes, including the construction of an integrase-deficient virus able to produce functional virions (Cara et al., 1996, Brussel and Sonigo, 2004, Sloan and Wainberg, 2011, Engelman et al., 1995). If PARV4 DNA were maintained as episomes and transcribed, this would provide a way by which antigen could persist. These are summarised in Figure 6.2.

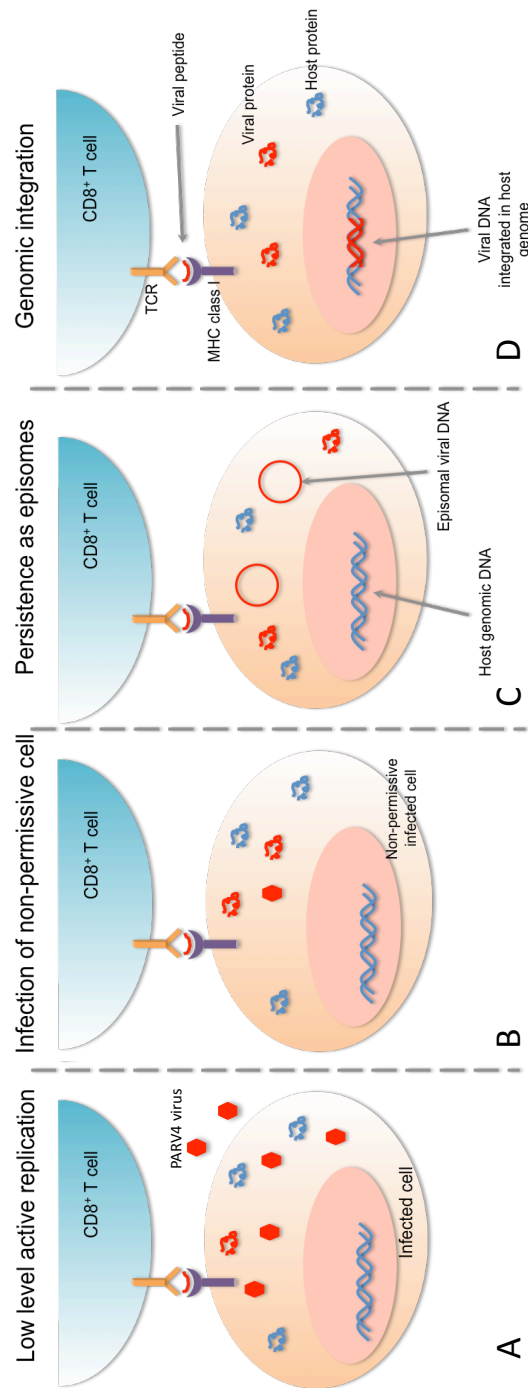


Figure 6.2 Models of PARV4 persistence. There is strong evidence for the persistence of PARV4, however it is unknown which form this takes. Each of the models presented here would allow for antigen presentation to T cells. **A.** PARV4 may be actively replicating at low levels and producing new virus. **B.** Non-permissive cells may also be infected, and whilst PARV4 cannot replicate within these cells, the NS protein may still be expressed, as occurs for parvovirus B19. **C.** PARV4 viral DNA may persist as episomes and produce some viral proteins. **D.** Viral DNA may integrate the genome, thereby allowing the transcription of proteins through host machinery.

5. PARV4 – a risk for humans?

Chapter 4 demonstrated that PARV4 appears to have no effect on HCV disease progression or treatment outcome, and that the earlier occurrence of HIV CDC-B symptoms in PARV4 seropositive subjects, was intrinsically linked to their HCV co-infection and IDU status. Parvovirus B19 may be non-pathogenic in a healthy individual, but can be very dangerous to a foetus or immuno-compromised person, much as other parvoviruses can be lethal to animals (Young and Brown, 2004; Patel and Heldens, 2009). The near universal PARV4 infection in HIV⁺ HCV⁺ IDU cohorts warrants further investigation into the clinical significance of this blood-borne agent. As discussed in Chapter 1, the safety of blood products administered to vulnerable populations is of particular concern as there is currently no requirement for PARV4 screening of blood products. With no known tissue tropism and little known about PARV4 protein function, it is difficult to investigate PARV4 pathology. The recent link of PARV4 with encephalitis warrants further investigation into cohorts of patients with encephalitis of unknown aetiology (Glaser et al., 2003, Benjamin et al., 2011). Interestingly, HCV infection has also been associated with neuropathy and it would be interesting to investigate the prevalence of PARV4 in these subjects (Nemni et al., 2003).

6. PARV4 as a potential vaccine vector

A recent phase I/II clinical trial for a B19 vaccine has recently been completed (Clinical Trials.gov, 2011). While currently there is no need for a vaccine for

PARV4, with such a strong humoral and cellular immune response, PARV4 may be useful as a vector in vaccine design, as has been parvovirus minute virus of mice (MVM) (Palmer et al., 2004). It would be necessary first to determine the tissue tropism of this virus, although it has been found in many tissues that would be useful vaccine targets. It would then be necessary to investigate whether the NS protein has a significant role in infection or host-virus interactions, or whether any DNA packaged into the viral capsid under the right conditions could elicit similar T cell and antibody responses. It has also been difficult to culture B19 and PARV4. Attempts to culture PARV4 in bone marrow, hematopoietic and adherent cells were unsuccessful (Fryer et al., 2007b). However, there have been a few reported successes of B19 culture, providing hope for PARV4 expansion *in vitro* (reviewed in Pillet et al., 2008). Thus, though there are currently many obstacles to using PARV4 as a vaccine vector, further investigations will shed light on whether this new virus could be an appropriate tool.

7. Final remarks

PARV4 remains an intriguing virus that, despite a marked host adaptive response, is not associated with clear symptoms. Further research on the pathogenesis of PARV4 infection is warranted given the extremely high prevalence of infection in IDUs and the profound and long-lived T cell responses elicited. Future studies to define its molecular, cellular and tissue targets may shed further light on its pathogenesis both as a single agent and in the context of co-infection.

Norja *et al.* suggested that persistent viruses such as parvovirus B19 constitute a “biportfolio” defined as a “registry of one’s infectious encounters” (Norja et al., 2006). Additionally, the recent discovery of endogenous parvovirus sequences within the human genome provide a “fossil record” of previous infections, offering clues to the co-evolution of parvoviruses and their hosts (Liu et al., 2011). These bioinformatic studies combined with experiments such as those described in this thesis, will clarify the role and impact of PARV4, as well as advance our knowledge of the immune response in the context of viral infection.

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Table AT1. PARV4 NS, ARF and VP peptides used in this project.**PARV4 NS peptides**

1. 1	MDAPAWIAVLQIPTG	5. 8	VNKLSYRSFLGSSGG
1. 2	WIAVLQIPTGFSLNP	5. 9	YRSFLGSSGGVLQAT
1. 3	QIPTGFSLNPANWRD	5. 10	GSSGGVLQATNALQI
1. 4	FLSNPANWRDWDGLQ	6. 1	VLQATNALQIAKREM
1. 5	ANWRDWDGLQRPRNL	6. 2	NALQIAKREMLAHP
1. 6	WDGLQRPRNLLADDW	6. 3	AKREMLAHPLLSYL
1. 7	RPRNLLADDWPIQEL	6. 4	VLAHPLLSYLTKNAS
1. 8	LADDWPIQELRESVP	6. 5	LLSYLTKNASAFEES
1. 9	PIQELRESVPFFDHA	6. 6	TKNASAFEESNKVAQ
1. 10	RESVPFFDHAVNLGY	6. 7	AFEESNKVAQLFSLN
2. 1	FFDHAVNLGYCILQQ	6. 8	NKVAQLFSLNGYNPV
2. 2	VNLGYCILQQLFASH	6. 9	LFSLNGYNPVDAAWY
2. 3	CILQQLFASHAVTLP	6. 10	GYNPVDAAWYFAAWA
2. 4	LFASHAVTLPCRVPK	7. 1	DAAWYFAAWARGVWP
2. 5	AVTLPCRVPKPSMFLQ	7. 2	FAAWARGVWPKRRAI
2. 6	CRVKPSMFLQLEPSS	7. 3	RGVWPKRRAIWLWGP
2. 7	SMFLQLEPSSGEENE	7. 4	KRRAIWLWGPPASTGK
2. 8	LEPSSGEENEMHYHL	7. 5	WLWGPPASTGKTLAA
2. 9	GEENEMHYHLVVNQA	7. 6	ASTGKTLAAAIALNL
2. 10	MHYHLVVNQADMVGR	7. 7	TLLAAAIALNLSPSYG
3. 1	VVNQADMVGRECSNW	7. 8	AIANLSPSYGCVNWT
3. 2	DMVGRECSNWLRTWK	7. 9	SPSYGCVNWTNQNF
3. 3	ECSNWLRTWKVFMAG	7. 10	CVNWTNQNFNFNDCH
3. 4	LRTWKVFMAGYLVAP	8. 1	NQNFNFNDCHCQSLV
3. 5	VFMAGYLVAPVWTL	8. 2	FNDCHCQSLVWWEWG
3. 6	YLVAPVWTLWNIRE	8. 3	CQSLVWWEWGRMTEN
3. 7	VWTLWNIRETRQGR	8. 4	WWEWGRMTENIVEVA
3. 8	WNIRETRQGRLYQAD	8. 5	RMTENIVEVAKAVLG
3. 9	TRQGRLYQADMSFVK	8. 6	IVEVAKAVLGGAPVR
3. 10	LYQADMSFVKNYLLP	8. 7	KAVLGGAPVRLDVKN
4. 1	MSFVKNYLLPKLPLN	8. 8	GAPVRLDVKNKGSED
4. 1b	NYLLPKLPLNDCYYA	8. 9	LDVKNKGSEDIPTC
4. 1c	KLPLNDCYYAWTNID	8. 10	KGSEDIPTCVIITS
4. 2	DCYYAWTNIDRFEAA	9. 1	YIPTCVIITSNGDLT
4. 3	WTNIDRFEAAVLSVR	9. 2	VIITSNGDLTVDG
4. 4	RFEAAVLSVRNRQLS	9. 3	NGDLTVDGPPVST
4. 5	VLSVRNRQLSGPQGA	9. 4	VDGPPVSTQHQEA
4. 6	NRQLSGPQGAIALPF	9. 5	PVSTQHQEALQTRI
4. 7	GPQGAIALPFTDAPP	9. 6	QHQEALQTRITMFQF
4. 8	IAPFTDAPPRTPA	9. 7	LQTRITMFQFQRMVP
4. 9	TDAPPRTPAAGVPP	9. 8	TMFQFQRMVPDGLAP
4. 10	RTPAAGVPPTMAGK	9. 9	QRMVPDGLAPLPEEE
5. 1	EGVPPTMAGKGTQRF	9. 10	DGLAPLPEEEVRSFF
5. 2	TMAGKGTQRFMDLID	10. 1	LPEEEVRSFFKLGEQ
5. 3	GTQRFMDLIDWLVEN	10. 2	VRSFFKLGEQELNMK
5. 4	MDLIDWLVENGIAE	10. 3	KLGEQELNMKGTPPE
5. 5	WLVENGIAEKRWLS	10. 4	ELNMKGTPPEEFRVP
5. 6	GIATEKRWLSVNKLS	10. 5	GTPPEEFRVPRNFDK
5. 7	KRWLSVNKLSYRSFL	10. 6	EFRVPRNFDKQPMAS

PARV4 NS peptides - continued

10. 7	RNFDKQPMASTSNLP
10. 8	QPMASTSNLPKALCA
10. 9	TSNLPKALCAPMEDN
10. 10	KALCAPMEDNQVQWD
11. 1	PMEDNQVQWDEDDW
11. 2	QVQWDEDDWFPPPT
11. 3	SEDDWFPPPTQKKRR
11. 4	FPPPTQKKRRELQET
11. 5	QKKRRELQETPPTTP
11. 6	ELQETPPTTPSEVIE
11. 7	SEVIELSSPSPLADA
11. 8	LSSPSPLADAPPRTTP
11. 9	PLADAPPRTPD SLGE
11. 10	PPRTPD SLGELSLTP
12. 1	DSL GELSLTPTS VSQ
12. 2	LSLTPTS VSQIVSAP
12. 3	TSVSQIVSAPFPDET
12. 4	IVSAPFPDETAERYG
12. 5	FPDETAERYGAGDIE
12. 6	AERYGAGDIESFWSE
12. 7	AGDIESFWSEHVFDA
12. 8	SFWSEHVFADWATR
12. 9	HVFADWATRLHICP
12. 10	DWATRLHICPPGGPK
13. 1	LHICPPGGPKPYGLF
13. 2	PGGPKPYGLFWTYLW
13. 3	PYGLFWTYLWSREFW
13. 4	WTYLWSREFWRFKQS
13. 5	SREFWRFKQSLSRSE
13. 6	RFKQSLSRSEAHLIN
13. 7	LSRSEAHLINRRFIW
13. 8	AHLINRRFIWAWW

PARV4 ARF1 peptides

1. 1	MSESTLLVTAAMKEL
1. 2	LLVTAAMKELIPLLN
1. 3	AMKELIPLLNQTL SG
1. 4	IPLLNQTL SGLPEAG
1. 5	QTL SGLPEAGLLSPG
1. 6	LPEAGLLSPGIIMLV
1. 7	LLSPGIIMLVLVILW
1. 8	IIMLVLVILWIVVPL
1. 9	LVILWIVVPLRDQWM
1. 10	IVVPLRDQWMRQQNI
1. 11	RDQWMRQQNIMMNGTQR

PARV4 ARF2 peptides

2. 1	MLLEVYGR LRKLLVI
2. 2	YGR LRKLLVILLMFN
2. 3	KLLVILLMFN FLRSY
2. 4	LLMFN FLRSYRPLLL
2. 5	FLRSYRPLLLRTNN
2. 6	RPLLLRTNNRLIPQ
2. 7	LRTNNRLIPQESPQP
2. 8	RLIPQESPQPRRRGL
2. 9	ESPQPRRRGLVPLTS
2. 10	RRRGLVPLTSLTRPC
2. 11	VPLTSLTRPCFCSLI
2. 12	LTRPCFCSLIPIQCP
2. 13	FCSLIPIQCPWNQLE
2. 14	PIQCPWNQLEEEVEL
2. 15	WNQLEEEVELKLKLSG

PARV4 VP peptides

A. 1	MSAADAYRPGGKLPL
A. 2	AYRPGGKLPLDELMQ
A. 3	GKLPLDELMQRMNRA
A. 4	DELMQRMNRAIPVGP
A. 5	RMNRAIPVGPEPSSQ
A. 6	IPVGPEPSSQANRGG
A. 7	EPSSQANRGGGPYQT
A. 8	ANRGGGPYQTHFAIG
A. 9	GPYQTHFAIGIMYSK
A. 10	HFAIGIMYSKAFQGL
A. 11	IMYSKAFQGLLRFAN
A. 12	AFQGLLRFANALPPE
A. 13	LRFANALPPELSPVK
A. 14	ALPPELSPVKQLVNQ
A. 15	LSPVKQLVNQLENYR
A. 16	QLVNQLENYRRKTS
A. 17	LENYRRKTS DTRVWY
A. 18	RKTS DTRVWYRVYLD
B. 1	TRVWYRVYLD MTRLL
B. 2	RVYLD MTRLLISVAP
B. 3	MTRLLISVAPPGAAN
B. 4	ISVAPPGAANKLRQA
B. 5	PGAANKLRQAAAGIT
B. 6	KLRQAAAGITHSKAP
B. 7	AAGITHSKAPNAESL
B. 8	HSKAPNAESLRGIVR
B. 9	NAESLRGIVRFAAAA
B. 10	RGIVRFAAAAFVPTV
B. 11	FAAAAFVPTVENIDR
B. 12	FVPTVENIDRFFEDS
B. 13	ENIDRFFEDSLTNFA
B. 14	FFEDSLTNFAKEDLD
B. 15	LTNFAKEDLDTWQQL
B. 16	KEDLDTWQQLHEQFI
B. 17	TWQQLHEQFIKLFHP
B. 18	HEQFIKLFHPPDVG
C. 1	KLFHPPDVG VHLVSD
C. 2	PDVG VHLVSDSRDEG
C. 3	HLVSDSRDEGADSLV
C. 4	SRDEGADSLVEPDLE
C. 5	ADSLVEPDLERPAGG
C. 6	EPDLERPAGGGLTLP
C. 7	RPAGGGLTLP GYNYV
C. 8	GLTLP GYNYV GPGNP
C. 9	GYNYV GPGNP LDSGP
C. 10	GPGNP LDSGP PPGPV
C. 11	LDSGP PPGPV DEAAK
C. 12	PQGPV DEAAK HHD
C. 13	DEAAK HHD ERYAEMI
C. 14	HHD ERYAEMI EHGD
C. 15	YAEMI EHGDIPYLHG

C. 16	EHGDIPYLHGHGADR
C. 17	PYLHGHGADR LMNKE
C. 18	HGADR LMNKE EEEKE
D. 1	LMNKE EEEKERRGDI
D. 2	LEEKERRGDITHLAD
D. 3	RRGDITHLADV VVGN
D. 4	THLADV VVGN AIRGL
D. 5	VVGN AIRGLWQAKE
D. 6	AIRGLWQAKETV GDI
D. 7	WQAKETV GDIADVQL
D. 8	TVGDIADVQLS QVLP
D. 9	ADVQLS QVLP PAPP
D. 10	SQVLP PAPPSSDQQP
D. 11	PAPPSSDQQPAYSAG
D. 12	SDQQPAYSAG EPSAK
D. 13	AYSAG EPSAK KARIG
D. 14	EPSAK KARIGTPDES
D. 15	KARIGTPDES DPALL
D. 16	TPDES DPALL LQPH
D. 17	DPALL LQPH TMTSV
D. 18	LQPH TMTSV EPAGG
E. 1	NTMSV EPAGG GGGVK
E. 2	EPAGG GGGVKVKAQW
E. 3	GGGVKVKAQWIGGTS
E. 4	VKAQWIGGTSFSDSV
E. 5	IGGTSFSDSVVITSH
E. 6	FSDSVVITSH TRTSM
E. 7	VITSH TRTSM LADR
E. 8	TRTSM LADRGGYVPV
E. 9	LADRGGYVPVYKQGS
E. 10	GYVPVYKQGSHVDSS
E. 11	YKQGSHVDSSQPVMG
E. 12	HVDSSQPVMGMKTPY
E. 13	QPVGMGMKTPYSYIDV
E. 14	MKTPYSYIDV NALSA
E. 15	SYIDV NALSAHFTPR
E. 16	NALSAHFTPRDFQQL
E. 17	HFTPRDFQQLLDEYD
E. 18	DFQQLLDEYDEIKPK
F. 1	LDEYDEIKPKSLTIA
F. 2	EIKPKSLTIAISAIV
F. 3	SLTIAISAIVIKDVA
F. 4	ISAIVIKDVATNQTG
F. 5	IKDVATNQGTGTTVSD
F. 6	TNQGTGTTVSDSASGG
F. 7	TTVSDSASGGITVF
F. 8	SASGGITVFADDSYD
F. 9	ITVFADDSYDYPYVL
F. 10	DDSYDYPYVLGHNQD
F. 11	YPYVLGHNQDTLP
F. 12	GHNQDTLPGLPGEN

PARV4 VP peptides - continued

F. 13	TLPGHLPGENYVLPQ
F. 14	LPGENYVLPQYGYIT
F. 15	YVLPQYGYITRGREI
F. 16	YGYITRGREIDQQNS
F. 17	RGREIDQQNSIVAIS
F. 18	DQQNSIVAISDHKTE
G. 1	IVAISDHKTELFFLE
G. 2	DHKTELFFLEHHDAE
G. 3	LFFLEHHDAECLGTG
G. 4	HHDAECLGTGDHWSH
G. 5	CLGTGDHWSHHYEF
G. 6	DHWSHHYEFDDLPW
G. 7	HYEFDDLPWRKLST
G. 8	DDLPWRKLSTPNQTL
G. 9	RKLSTPNQTLYARHN
G. 10	PNQTLYARHNPISS
G. 11	YARHNPISSRLAIM
G. 12	PISSRLAIMTGVDN
G. 13	RLAIMTGVDNDGTAI
G. 14	TGVDNDGTAIWKRPE
G. 15	DGTAIWKRPEGMDVG
G. 16	WKRPEGMDVGRPLN
G. 17	GMDVGRPLNYVPGP
G. 18	RLPLNYVPGPALMMP
H. 1	YVPGPALMMPDQI
H. 2	ALMMPDQIIRNTTF
H. 3	TDTQIRNTTFRDPVA
H. 4	RNTTFRDPVAIGNPA
H. 5	RDPVAIGNPATSDRY
H. 6	IGNPATSDRYSVAPL
H. 7	TSDRYSVAPLVHQPW
H. 8	SVAPLVHQPWSVRTE
H. 9	VHQPWSVRTEEWLAN
H. 10	SVRTEEWLANKTDYA
H. 11	EWLANKTDYAVHNYL
H. 12	KTDYAVHNYLGGVAY
H. 13	VHNYLGGVAYTRRKH
H. 14	GGVAYTRRKHEESYD
H. 15	TRRKHEESYDKHEED
H. 16	EESYDKHEEDRDGRV
H. 17	KHEEDRDGRVTNPSR
H. 18	RDGRVTNPSRVVQID
J. 1	TNPSRVVQIDGDLAA
J. 2	VVQIDGDLAAPHVGH
J. 3	GDLAAPHVGHTFFVP
J. 4	PHVGHTFFVPGHTRV
J. 5	TFFVPGHTRVTSGGT
J. 6	GHTRVTSGGTDTVYS
J. 7	TSGGTDTVYSPKLYQ
J. 8	DTVYSPKLYQEPVFP
J. 9	PKLYQEPVFPLFPGA

J. 10	EPVFPLFPGA/VWNP
J. 11	LFGA/VWNP/PLSYD
J. 12	VWNP/PLSYDCQIWT
J. 13	PLSYDCQIWT/KIPNT
J. 14	CQIWT/KIPNTECHFF
J. 15	KIPNTECHFFAQYPL
J. 16	ECHFFAQYPLLGGWG
J. 17	AQYPLLGGWGVLTTP
J. 18	LGGWGVLTTPPMIFV
K. 1	VLTPPMIFVKLRSQ
K. 2	PMIFVKLRSQGPSPS
K. 3	KLRSQGPSPSPAHT
K. 4	PGPSPAHTVPQSN
K. 5	PGAHTVPQSNLNQYA
K. 6	VPQSNLNQYAFHLH
K. 7	LNQYAFHLHYSMQF
K. 8	IFHLHYSMQFLVKRR
K. 9	YSMQFLVKRRKRSRR
K. 10	LVKRRKRSRRHNPEK
K. 11	KRSRRHNPEKPAPFP
K. 12	HNPEKPAPFPTDSDG
K. 13	PAPFPTDSDGRMPFT
K. 14	TTDSDGRMPFTLANSL
K. 15	RMPFTLANSLKDPNT
K. 16	LANSLKDPNTPVYEV
K. 17	KDPNTPVYEVPSDQW
K. 18	PVYEVPSDQWIARNY
K. 19	PSDQWIARNYSHLL

Table AT2. Fluorochrome-conjugated antibodies used in this project.

Antibody to	Fluorochrome	Brand
CCR2 (CD192)	PerCP Cy5.5	Biolegend
CCR6 (CD196)	PECy7	eBioscience
CCR7 (CD197)	PECy7	BDPharmingen
CD107a	PECy5	BD Pharmingen
CD127	APC	MACS Miltenyi Biotech
CD14	Pacific Blue	eBioscience
CD14	Pacific Blue	Invitrogen
CD16	Pacific Blue	eBioscience
CD161	PerCP	BioLegends
CD161	FITC	Macs
CD161	APC	Macs
CD19	Pacific Blue	Invitrogen
CD19	Pacific Blue	eBioscience
CD3	Pacific Orange	Invitrogen
CD38	PerCP Cy5.5	Biolegend
CD4	Qdot 605	Invitrogen
CD45RA	APC	Abcam
CD45RA	FITC	BD Pharmingen
CD57	Pacific Blue	Biolegend
CD8	APC	BDPharmingen
CD8	PECy7	BDPharmingen
CD8	Pacific Blue	BD Pharmingen
CD8	APC	MACS Miltenyi Biotech
CX3CR1	FITC	Biolegend
CXCR4 (CD184)	PECy7	BD Pharmingen
CXCR6 (CD186)	Alexa 647	Biolegend
Gamma-delta TCR	PE	BDPharmingen
Granzyme A	PerCP Cy5.5	Biolegend
Granzyme B	Alexa 700	BD Pharmingen
HLA-A*0201-RMT tetramer	PE	made in house
HLA-DR	Alexa 700	BD Pharmingen
IFN- γ	Alexa 700	BDPharmingen
IFN- γ	FITC	BD Biosciences
IL-2	APC	BD
Live/dead marker L10119	Near IR	Invitrogen
Live/dead marker L34955		Invitrogen
MIP1b	PE	BD Pharmingen
PD-1	PECy7	BD Pharmingen
Perforin	FITC	BD Pharmingen
TNF α	PECy7	BD Pharmingen

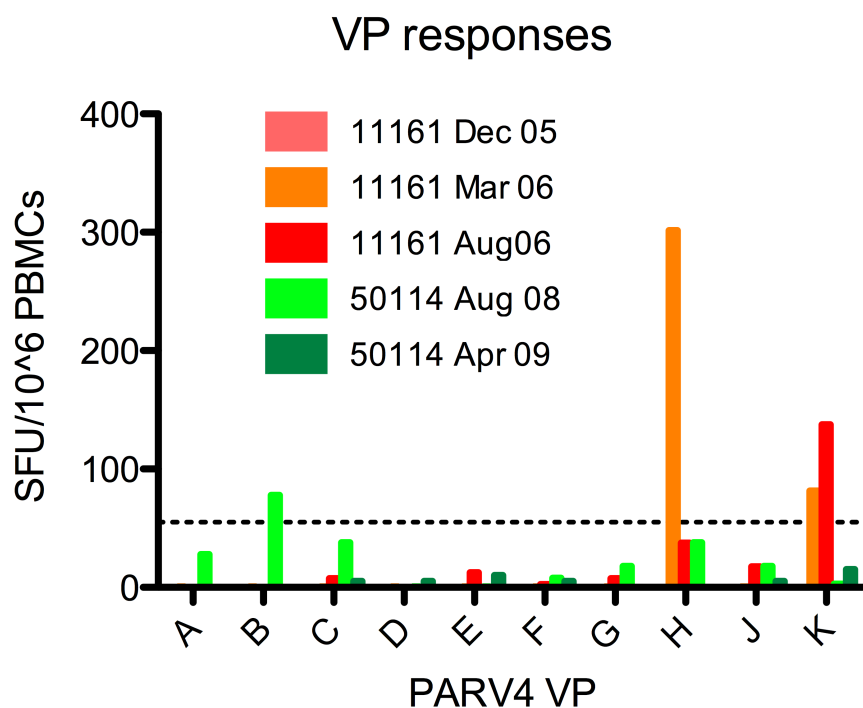


Figure A1. T cell responses to PARV4 VP peptides. Due to limited sample availability, only 4/8 patients were tested for PARV4 VP responses. 2/4 patients tested had T cell responses below the cut off. The other two, 11161 and 50114, are presented here.

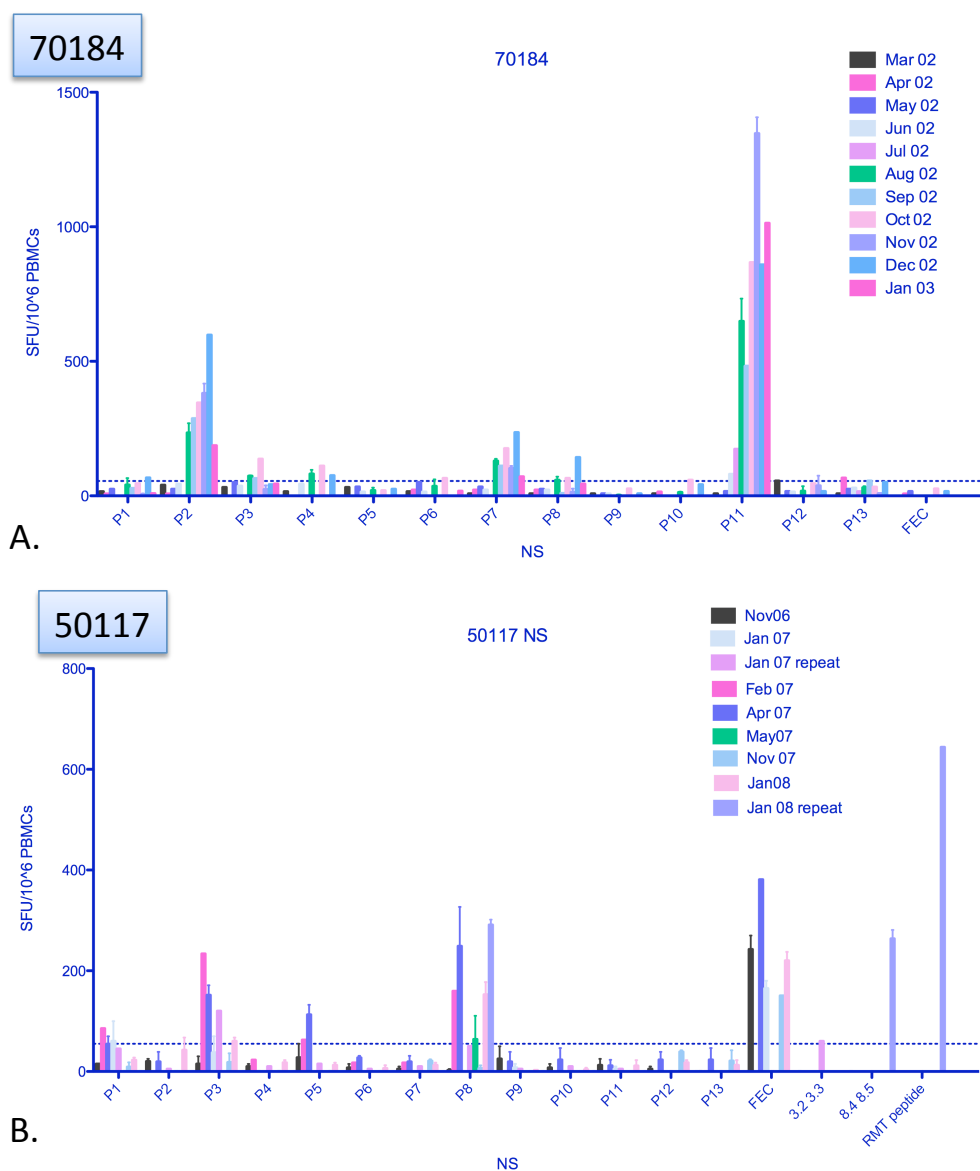


Figure A2. T cell responses over time for patients 70184 and 50117. A. ELISpot results over 11 time points (Mar 02 is the last seronegative sample). Due to limited samples, not all time points were tested in duplicate. **B.** ELISpot results over 7 time points (Nov 06 is the last seronegative sample). “RMT” is the epitope defined in Chapter 3.

70110

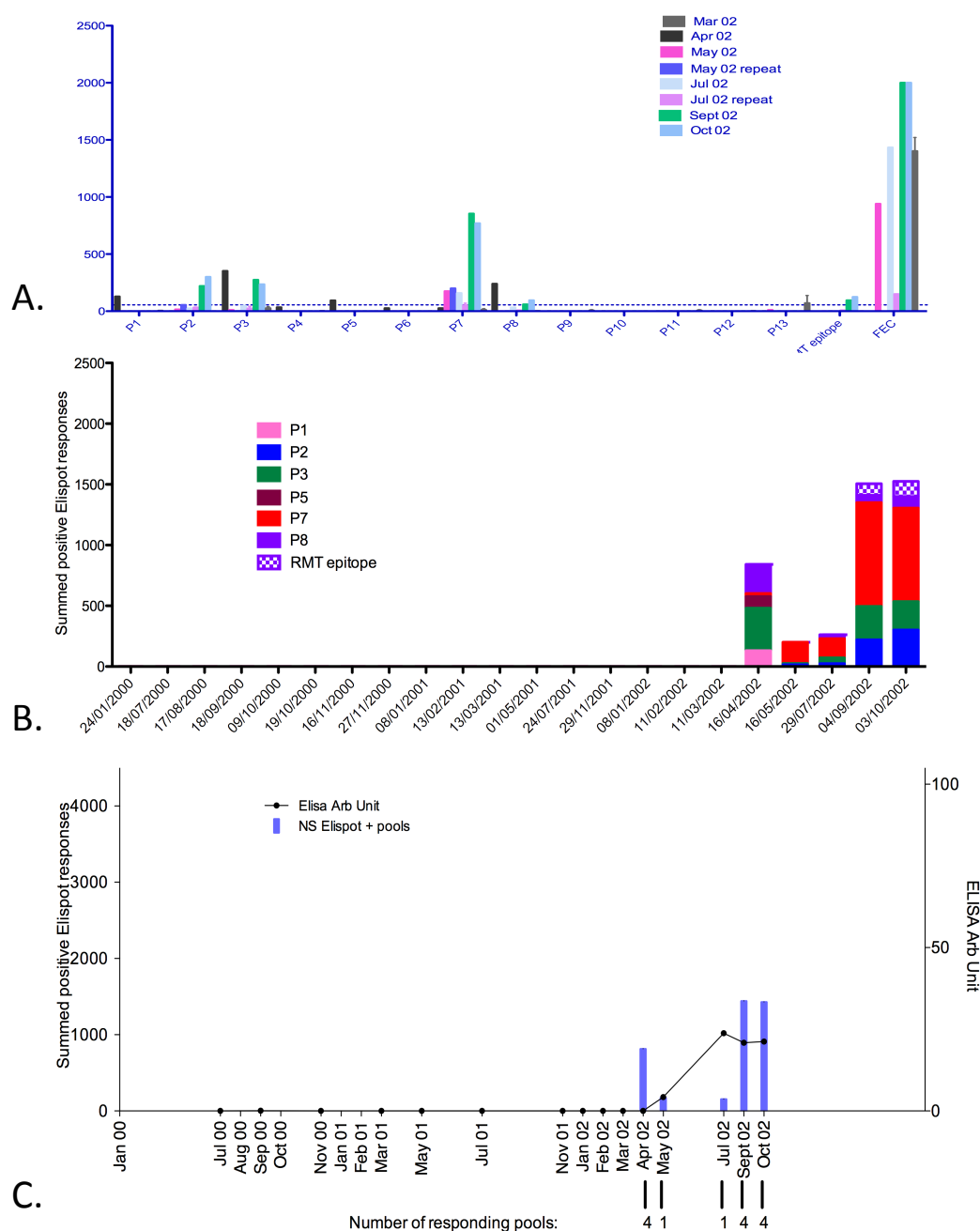


Figure A3. Cellular and humoral responses to PARV4 in patient 70110. A. ELISpot results over 6 time points (Apr 02 is the last seronegative sample). FEC is the positive control for this assay. “RMT” is the epitope defined in Chapter 3. **B.** Changes in responses to PARV4 NS peptide pools over time. **C.** T cell responses (left axis) and IgG levels (right axis) over time. Due to limited samples, none of these tests were carried out in duplicate and not all pools could be tested at every time point.

11161

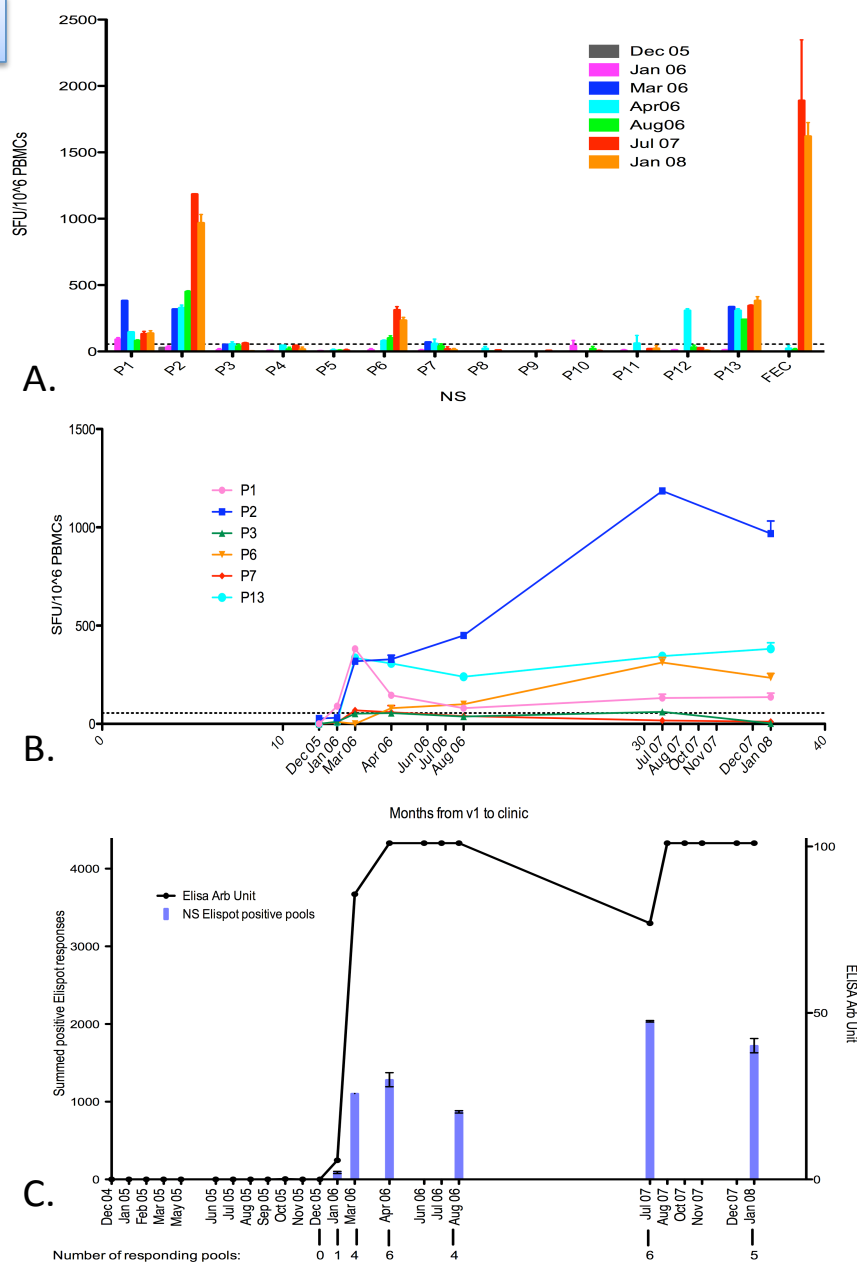


Figure A4. Cellular and humoral responses to PARV4 in patient 11161. **A.** ELISpot results over 7 time points (Dec 05 is the last seronegative sample). FEC is the positive control for this assay. **B.** Changes in responses to PARV4 NS peptide pools over time. **C.** T cell responses (left axis) and IgG levels (right axis) over time.

70429

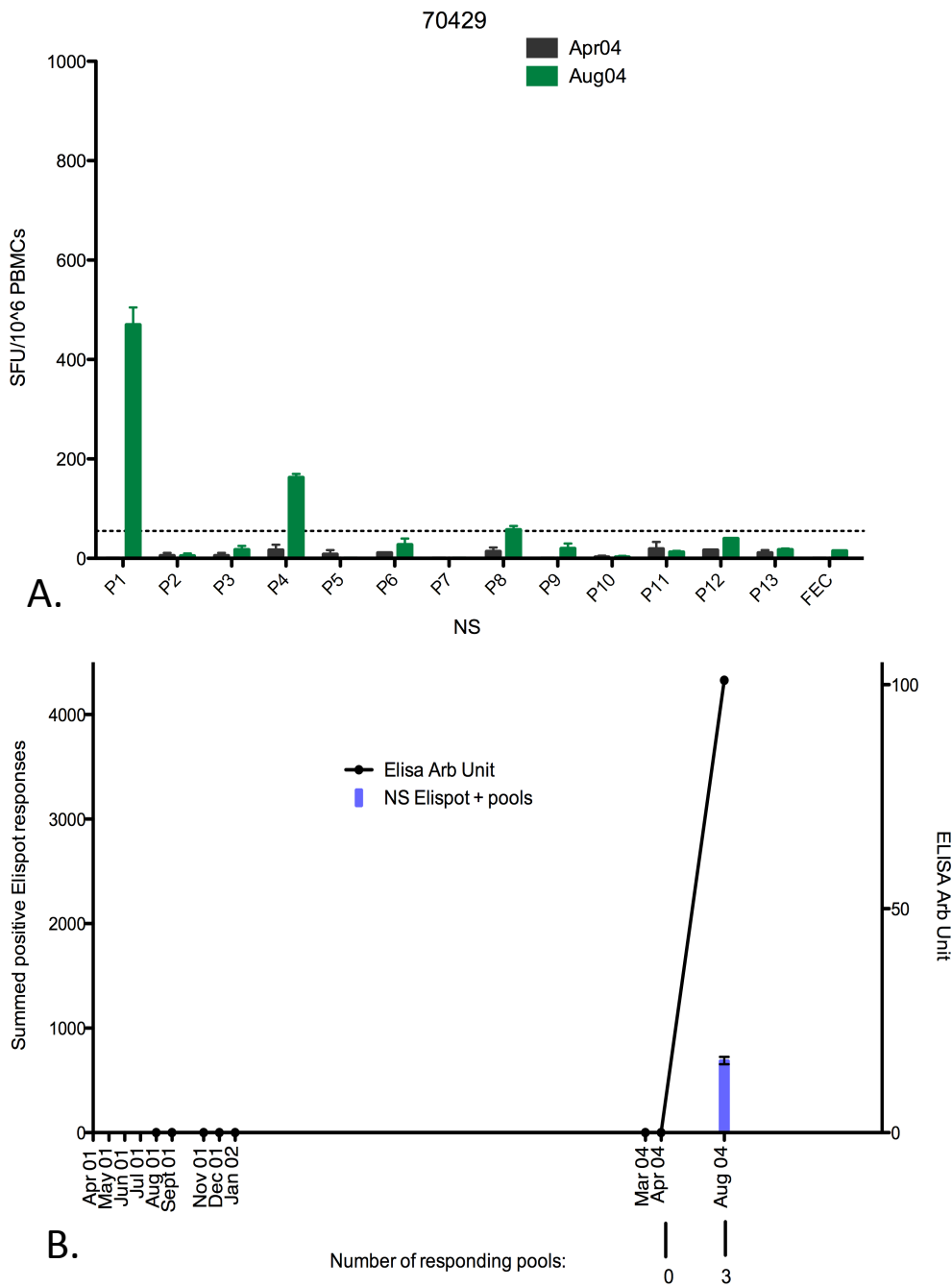


Figure A5. Cellular and humoral responses to PARV4 in patient 70429. A. ELISpot results over 2 time points (Apr 04 is the last seronegative sample). **B.** Comparison of T cell responses (left axis) and IgG levels (right axis) over time.

70418

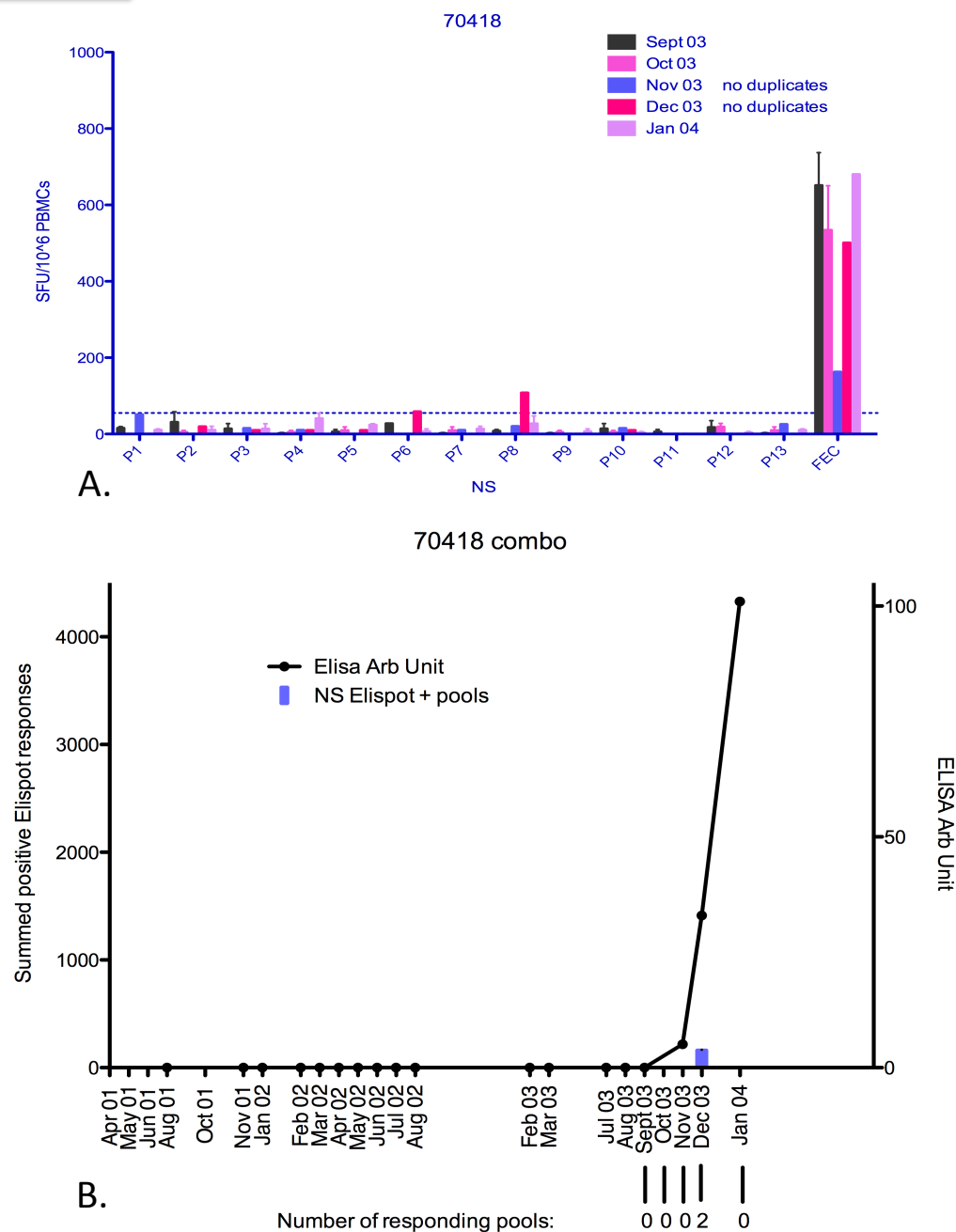


Figure A6. Cellular and humoral responses to PARV4 in patient 70418. **A.** ELISpot results over 5 time points (Sept 03 is the last seronegative sample). Due to limited sample, two time points were not tested in duplicate, as indicated. **B.** Comparison of T cell responses (left axis) and IgG levels (right axis) over time.

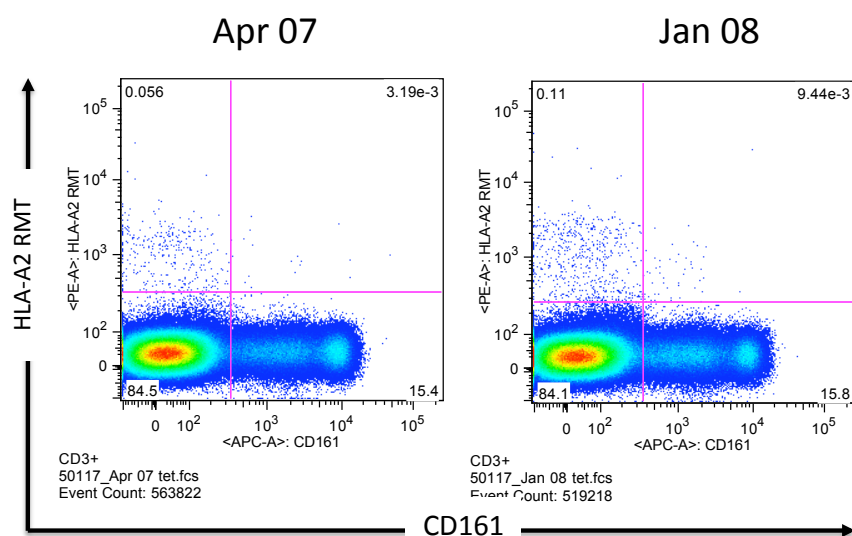


Figure A7. PARV4-specific T cells from patient 50117 are also predominantly CD161⁻. These cells are gated on CD3⁺. No chemokine receptors staining was carried out due to limited samples.

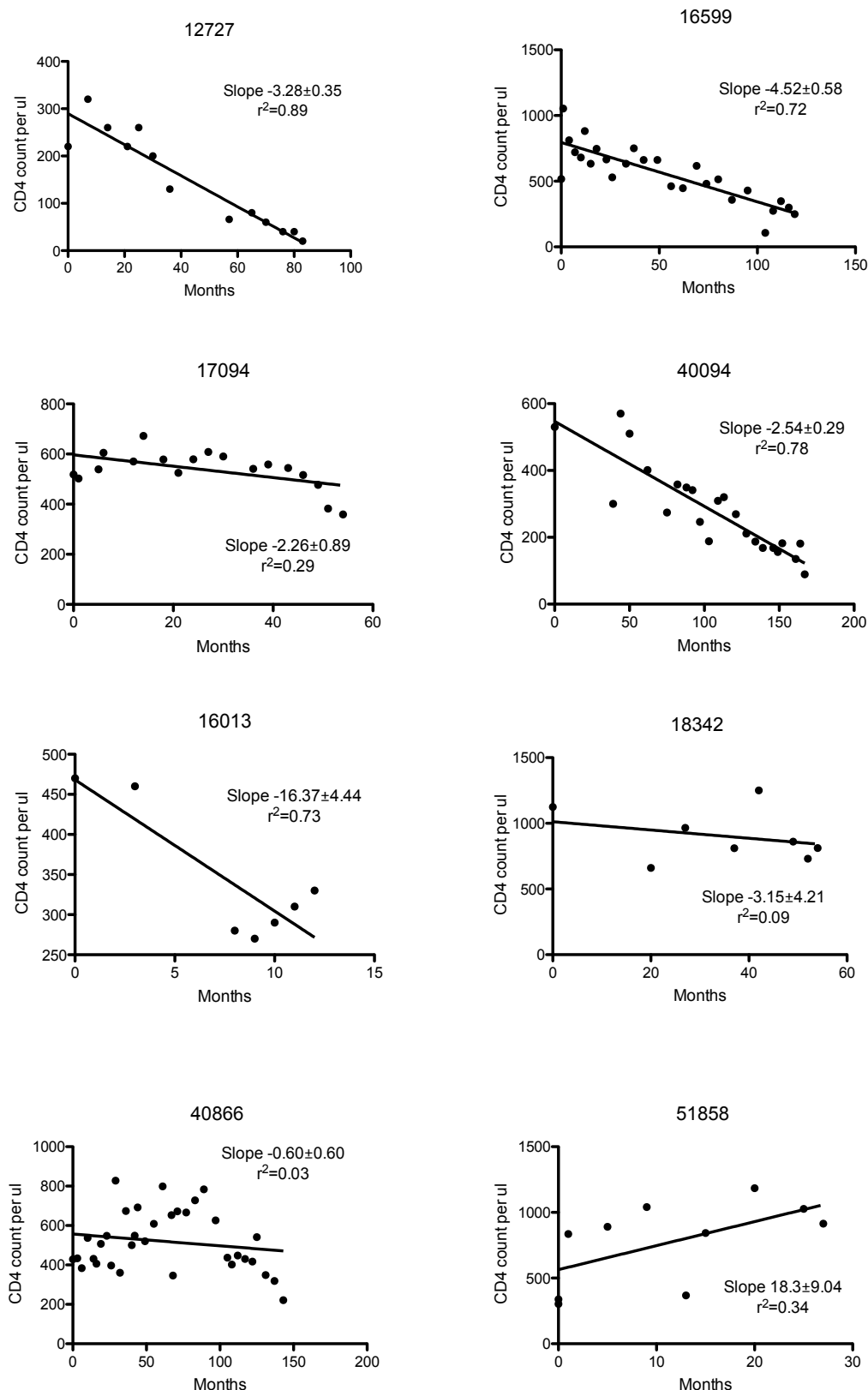


Figure A8. Calculating CD4 slopes from linear regression analysis of CD4 counts. Slopes, standard error and a measure of goodness of fit, r^2 , are shown for 8 individuals. The number of CD4 counts and the time they cover is varied across the individuals from the Swiss HIV cohort. This must be taken into consideration whilst interpreting the data.

Table AT3. Summary table of literature review presented in the introduction (Chapter 1).

Study	PARV4 DNA, IgG/IgM, PBMC	Prevalence of PARV4 discovery in 1 individual	Percentage	PARV4 Genotype	Sample population	Tissue	Origin of samples	Characteristic of positive samples	PARV4 DNA levels
Jones et al, 2005	DNA	discovery in 1 individual		Geno 1	Patients with acute infection syndrome negative for HIV	Plasma	San Francisco, USA	HBV+ IDU with acute infection syndrome	1x 10 ⁶ copies/ml (ref Tube 2009 personal comm)
Fryer et al, 2006	DNA	7/137	5%	Geno 1 + 2	Healthy, asymptomatic individuals	Plasma pools for fractionation	Europe and North America	Healthy from North America (no European positives)	max: 3.9x10 ⁶ copies/ml, several pools <500 copies/ml
Manning et al., 2006	DNA	0/574	0%		Patients referred to the Specialist Virology Laboratory	Respiratory samples	Edinburgh, UK		
Schneider et al, 2008	DNA	13/87	15%	Geno 1 + 2	Biopsies in the context of transplant, HIV- except 1	Liver	Germany?	7 were HIV+, all 100%+ 12 were non-HIV+ removed during transplant, 1 from HIV+	
Manning et al, 2007	DNA	17/24	71%	Geno 1 + 2	Autopsies of HIV infected subjects	Bone marrow, lymphoid tissue, brain, plasma	Edinburgh, UK	HIV+ (both pre-AIDS and AIDS)	>2000 copies/10 ⁶ cells in bone marrow
		0/8	0%		Autopsies of HIV uninfected subjects	Bone marrow, lymphoid tissue, brain, plasma		HIV-	
Fryer et al, 2007, EID	DNA	3/26	12%	Geno 1 + 2	HCV infected RNA+ or RNA-, IVDUs and non-IVDUs	Blood samples from cadavers	London, UK	3 out of 10 of the RNA+ IVDUs were positive, ie 30%	<700 copies/ml of plasma
		14/351	4%		Plasma pools from 10 manufacturers	Recent plasma samples			
		24/115	21%		Plasma pools from 3 manufacturers	Archived plasma samples	Europe and North America		max: 6.58log10 copies/ml plasma
Fryer et al, 2007, Transfusion	DNA	29/4800	1%		Healthy blood donors	Test pools			
		4/200	2%		Healthy blood donors	Individual plasma samples			
		13/216	6%		Febrile MSM or IVDU patients but HIV-	Plasma			
Longhi et al, 2007	DNA	16/35	46%	Geno 1	AIDS patients	Bone marrow aspirates	Italy	11/16 were HCV+ IDUs, 2/13 HCV- non IDU (MSM or unprotected heterosexual)	<3log10 copies/150 000 cells, except 1 case
		11/13	85%		HIV+ AIDS IDU	autopsy bone marrow or lymphoid tissue			
		6/11	55%		HIV+ pre-AIDS IDU	autopsy bone marrow or lymphoid tissue			
		0/13	0%		HIV+ HCV- AIDS MSM non-IDU	autopsy bone marrow or lymphoid tissue	Edinburgh, UK		
Simmonds et al, 2007	DNA	1/12	8%		HIV- HCV+ IDU	autopsy bone marrow or lymphoid tissue			
		1/2	50%		Haemophiliacs HIV+ HCV+	autopsy bone marrow or lymphoid tissue			
		0/8	0%		low risk controls	autopsy bone marrow or lymphoid tissue			
Fryer et al, 2007, Vox Sanguinis	DNA	28/175	16%	Geno 1 + 2	products from various manufacturers	Factor VII concentrate products	NIBSC, some USA	27 of them were from old samples pre 1989	max: 5log10 copies/ml
Lau et al, 2008	DNA	7/362	2%	Geno 1	Serum sent in for HIV/HCV testing	Serum	Hong Kong, China	5/7 were HCV Ab+	
Lau et al, 2008	DNA	other previous serosurvey parvos of same family found							
Simmonds et al, 2008	DNA	2/13	15%	Geno 3	Subjects with AIDS from sub-saharan Africa	bone marrow (6) or lymph node (7)	Sub-Saharan Africa	heterosexual, HCV- HBV-	7.3 and 620 DNA copies per 10 ⁶ cells
		7/21	33%		Lots not virally inactivated, admin until early 1980s, 8 diff products	Coagulation factor concentrates, including Factors VII, VIII, IX			100 to 6x10 ⁶ copies/ml

non-human studies
Healthy individuals
commercial products

Study	PARV4 DNA IgG/IgM/PBMC	Prevalence of PARV4	Percentage	PARV4 Genotype	Sample population	Tissue	Origin of samples	Characteristic of positive samples	PARV4 DNA levels
Schneider et al, 2008, Haemophilia	DNA	13/146	9%	Geno 1 + 2	Lots past expiry date, still admin now, representing 13 diff. products	Coagulation factor concentrates	Commercial products (German lab)		
		1/2	50%		Lots used in 1997, past expiry date, still admin now	Coagulation factor concentrates			
Lurchachaiwong et al, 2008	DNA	7/88	8%	Geno 2	IVDU subjects (78 HCV+, 5 HBV+, 35 HIV+)	Serum	Thailand	5 were PARV4+ HCV+, 1 PARV4+ HCV+ and possibly HIV+, 1 only PARV4+	
		7/176	4%		Blood donors	Serum			
Vallerini et al, 2008	DNA	1/100	1%	Geno 1 + 2	Blood donors, HIV-	Serum	Italy		
		1/84	1%		Patients with suspected viral disease, HIV-	Serum			
		0/107	0%		BV/peripheral blood stem cell transplant patients, HIV-	Serum		Patient had Wegener granulomatosis, died	
		2/126	2%		Kidney transplant recipients, HIV-	Serum			
Servant-Delmas, 2009	DNA	0/78	0%		Non-immunosuppressed patients multiply transfused with RBCs for Blood donors (280 healthy, 105 + for HBsAg, 93 + for HCV Ab)	Serum/plasma	France		
		0/478	0%			Serum/plasma			
Biagini et al, 2008	DNA	0/214	0%	Geno 1	Hemodialysis patients	Plasma	France		
		5/164	3%		Kidney transplant recipients	Plasma		1 of the 5 was DNA neg before transplant, others unknown	
		0/192	0%		Healthy blood donors	Plasma			
Sharp et al, 2009	IgG	8/12	67%		HCV+ HIV+ IDU	Plasma	UK		
		7/21	33%		HCV+ HIV- IDU	Plasma			
		4/17	24%		HCV- HIV+ MSM	Plasma			
		0/14	0%		HCV- HIV+ heterosexual	Plasma			
		11/20	55%		HCV+ HIV+ haemophiliacs	Plasma			
		4/15	27%		HCV+ HIV- haemophiliacs	Plasma			
		1/35	3%		Haemophilic control siblings	Plasma			
		0/50	0%		Low risk controls	Plasma			
Tuke et al 2009	DNA	3/94	3%	Geno 1	HCV Ab - RNA +	Plasma, sera, whole blood	USA	of these 3 had the highest PARV4 viral tit	
		2/88	2%		Samples for routine HCV RNA test	Plasma, sera, whole blood			4 were HCV+, 1 was HBV+, 1 was HIV+, 1 was HCV+HBV+, 1 was HCV+HIV+, 1 was HCV+HBV+HIV+ Max: 5x10 ⁸ DNA copies/ml
		2/140	1%		Samples for routine HBV/DNA test	Plasma, sera, whole blood	UK?		
		0/50	0%		HIV-1 pro-viral DNA + IDUs	Plasma, sera, whole blood			
		1/88	1%		Samples for routine HIV-1 RNA viral load test	Plasma, sera, whole blood	UK		
		0/96	0%		UK blood donors	Plasma, sera, whole blood			
Tounissi et al 2010	DNA	71/304	23%	Geno 1?	Blood donors	Plasma	France	2 different sets of primers, more found for 2nd set	V. low <500 copies/ml
		2/53	4%		Healthy blood donors	Blood			

Study	PARV4 DNA/ IgG/IgM/ PBMC	Prevalence of PARV4	Percentage	PARV4 Genotype	Sample population	Tissue	Origin of samples	Characteristic of positive samples	PARV4 DNA levels
Botto et al., 2009	DNA	3/37	8%	Geno 1 + 2	Healthy skin donors	Healthy skin	Italy	7/22 were healthy synovium, 3/12 with inflammatory dermatosis, 2/12 with dermatitis on lymphatic vessels	
		23/105	22%		Solid organ transplant recipients and pneumologists and hematologists division	Pathological skin			
		0/39	0%			Bronchial lavages			
Szelei et al., 2010, EID	DNA	3/98	3%		Healthy pigs	Porcine Plasma samples	GB 2001-02		
		11/11	100%		Concentrates used by persons with representing of bone marrow donors	Porcine Factor VIII concentrates			
Eis-Hubinger 2010	DNA	0/775	0%		donations, HIV-1 neg, 2 pools HCV+, 6 HCV+, 3 HIV+, 15 HBV+, 26 B19+	Plasma pools	Germany		
		0/50	0%			Nucleic acid extracts			
Panning et al., 2010	DNA	24/279	9%	Geno 3	Infants collected previously for another trial	Blood	Ghana		
Adlhoeh et al., 2010	DNA	51/156	33%		Wild boars	Liver, serum and bile	Germany	Parvovirus B19 was found in a higher % of older	>10 ⁶ genome equivalents
		38/60	63%		Chimpanzees	Plasma + faecal (27)			
		2/11	18%		Gorillas	Plasma + faecal (27)			
Sharp et al., 2010, VI	IgG	0/91	0%		Old World monkeys (various species)	Plasma	Cameroon	DNA found for chimp and baboon variant is closer to human PAKV4 than to Phov or B19V.	
Sharp et al., 2010 EID	IgG	0/360	0%		General popl Blood donors	Plasma	UK and France	all were HCV negative	
		62/167	37%		Blood donors	Plasma	Burkina-Faso	all were HCV negative	
		59/238	25%		General population	Plasma	Cameroon	all were HCV negative	
		78/221	35%		General population (2 were HIV+)	Plasma	DRC	all were HCV negative	
		62/170	36%		Blood donors HIV+	Plasma	South Africa	all were HCV negative	
		8/180	4%		Blood donors HIV neg	Plasma		all were HCV negative	
Corcioli et al., 2010, J Virol	DNA	2/26	8%	Geno 2	Adults with B19-unrelated blood disorders (immunodepressed)	Bone marrow	Italy	7/10 were from the immunodepressed group - reactivation	3.8x10 ⁶ copies/ml
		1/25	4%		Adults with B19-unrelated skin lesions	Skin			
		1/21	5%		Healthy adults who had surgery for synovium	Synovium			
		8/39	21%		non-patients who gave blood for synovium (21/39 were	Plasma			
		17/35	49%			Myocardium			
		7/17	41%			Myocardium			
		4/17	24%		Autopsies from non-HIV patients with no known viral myocarditis or dilated cardiomyopathy	Lung			
		7/17	41%			Liver			
		3/17	18%			Kidney			
		0/17	0%			Blood			
		0/93	0%		29 bone marrow transplant recipients at different times after infusion	Urine			

Study	PARV4 DNA/ IgG/ IgM PBMC	Prevalence of PARV4	Percentage	PARV4 Genotype	Sample population various coagulation factor products and recombinant products from patients with various diseases (various coagulation factor products)	Tissue	Origin of samples	Characteristic of positive samples	PARV4 DNA levels
Madrow et al, 2010, Vox Sanguinis	DNA	0/39	0%				Central Europe, USA, Germany	no Hep E, Hep A, HBsV but some B19 detected	
Lahtinen et al 2011, EID	IgG	0/115	0%		controls: uni students	Plasma	Finland	only mildly positive	
	IgM	1/115	1%		controls: uni students	Plasma		compared to two negative group they reported more IDUse, persistent	
	IgG	61/78	78%		151 samples from 78 HIV IgG+ IDUs (also HCV+ mostly)	Plasma			
	IgM	4/78	5%		152 samples from 78 HIV IgG+ IDUs (also HCV+ mostly)	Plasma			
	IgG	69/200	35%		HCV IgG+ patients	Plasma			
Touinssi et al, 2011, J Med Viro	IgM	3/200	2%		HCV IgG+ patients	Plasma	France		
	DNA	57/185	31%	Geno 1	Haemodialysis patients, 22 HCV+, 75 HBV+, 4 HIV+, 5 HBsAg+	Plasma		low: <500 copies/ml of plasma	
		6/22	27%		HCV+ Haemodialysis patients	Plasma			
		29/75	39%		HBV+ Haemodialysis patients	Plasma			Genotype 1
		0/4	0%		HIV+ Haemodialysis patients	Plasma			
		3/5	60%		HBSAg+ Haemodialysis patients	Plasma			
		14/104	13%		Lung transplant recipients, 2 HCV+	Plasma		low: <500 copies/ml of plasma	
Yang et al, 2011, J Clin Virol	DNA	6/350	2%	Geno 2	HIV+ IDUs, pioneer study	Plasma	Taiwan		immunoblot study
	IgG	51/69	74%		HIV+ IDUs	Plasma		2/69 had IgM but no IgG	
	IgM	28/69	41%			Plasma		2/66 had IgM but no IgG	
	IgG	47/66	71%		HIV+ MSMs	Plasma			
	IgM	11/66	17%			Plasma		1/68 had IgM but no IgG	
	IgG	40/68	59%		HIV+ Heterosexuals	Plasma			
	IgM	12/68	18%			Plasma			
	IgG IgM	10/51 IgG (5 also IgM)	20%		HIV neg Rheumatoid factor +	?			
Simmons et al, 2011, JID	IgG	24/55	44%		Healthcare workers		UK		
	PBMC	23/88	26%		HCV+	PBMC			Elispot
	PBMC	3/13	23%		HIV+	PBMC			Elispot
	IgG	22/84	26%		HCV+	Plasma			ELISA
	IgG	2/13	15%		HIV+	Plasma			ELISA
	PBMC + IgG	0/7	0%		B19 acutes	PBMC + plasma			Elispot and Elisa
	PBMC + IgG	0/8	0%		Healthy	PBMC + plasma			Elispot and Elisa
Mueller et al, 2011 Curcumin organ transplant	PARV4 as a risk in xenotransplantation								
Benjamin et al 2011 EID	DNA	2/12	17%	Geno 2	Children < 16yrs	CSF + 1 plasma	South India	1 patient 7 copies per ml in CSF, 3.2x10 ⁵ for the other. Patient 1 also	

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