HLA, Vpu and ADCC correlates of a unique Chinese HIV-1 Positive village cohort



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The recent relative success of the Thai Vaccine trial and failure of the STEP trial have prompted renewed calls for a 'back to basics' approach. This premise dictates a greater and clearer understanding of the various immune parameters at play in HIV infection. The overall aim of this project was to look at different immune parameters at play in our unique Chinese SM HIV-1 cohort, which has an unusually high proportion of slow progressors. The unique dynamics of the SM cohort make it ideal for studying immune parameters.

As the HLA Class I region has been shown to have the strongest genetic association with durable control of HIV, we set out to investigate the HLA correlates for our unique SM cohort. We recruited a neighbouring Han village cohort for comparisons, and employed stringent statistical tests for our analysis. Our analysis revealed that there was no enrichment of any specific HLA alleles in the SM cohort. This may be due to the limited study size, or that the protective effect has waned over time.

Next we were interested in the immune pressure exerted on Vpu protein, which has been shown recently to be critical in HIV release from the cell, and not many studies have focused on Vpu. We did not find any statistically significant associations in our analysis of HIV-HLA associated polymorphisms in Vpu, and our screening ELISPOTS using overlapping peptides did not reveal any T-cell responses to the protein. However, our analysis for NK-mediated pressure, identified a single amino acid position in the Vpu sequence which was significantly associated with the presence of a specific KIR gene ($p = 0.0047$): In amino acid position 14 of Vpu, HIV-1 sequences (59%) derived from KIR2DS1+ individuals encoded for a valine (V) whereas the consensus amino acid alanine (A) at this position is found in the vast majority (76%) of KIR2DS1- individuals. This is extremely interesting as Vpu position 14 has been shown to be critical for interaction with the host restriction factor, tetherin.

We next explored further the role of NK cells in relation to antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cell-mediated virus inhibition (ADCVI). Using an adapted ADCC-ICS assay, we found ADCC responses to Env in 37% of the SM cohort, with five subjects also having responses to Vpu peptides. We devised an Enzyme-linked Immunosorbent Assay (ELISA) which allowed us to map the Vpu ADCC epitopes in the SM cohort. We also wanted to know whether the Env/Vpu ADCC responders also displayed ADCVI activity. We optimised the ADCVI assay in our lab using HIV NL4-3 as our infecting strain of virus. All 5 Env/Vpu responders had very similar inhibition of virus – nearing 95 %, in the ADCVI assay. We followed up on these 5 Env/Vpu ADCVI responders and tried to get more plasma – unfortunately we were only able to get plasma from one subject – SM 017 as the rest could not be traced. This patient – SM 017 2011-demonstrated the prozone effect, and we hypothesise that the ADCC antibody titres have increased in SM 017 over the years, to augment the ADCC response *in vivo*.

In conclusion, in this study, we analyse host genetic, virological, and immunologic factors to enrich our understanding of HIV/AIDS parameters in a unique Chinese slow progressors cohort. We build up on the HLA database for slow progressors, and demonstrate a KIR association with Vpu amino acid position 14, something which has not been reported before. We demonstrate ADCC responses in the SM cohort, and also optimised an ELISA-based assay to reduce amount of plasma used for the ADCC-ICS assay. We plan now to isolate the antibodies from a unique patient, and possibly, identify the first Vpu ADCC antibody.

Acknowledgements

The journey for finishing off this thesis has seen more than its shares of ups and downs, throwing up all sorts of challenges, both in and off the lab environment. The whole experience has taught me a lot about science, as well as people conducting science around you, so this DPhil has been a truly illuminating experience.

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REFERENCES

List of Abbreviations

aa	amino acid
AIDS	Acquired Immunodeficiency Syndrome
CA	p24 Capsid protein
CMI	Cell-mediated immunity
CTL	cytotoxic T lymphocytes
DMSO	dimethyl sulphoxide
ELISpot assays	IFN-γ enzyme-linked immunospot assay
FCS	Foetal Calf Serum
ART	Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HLA	human leukocyte antigen
ICS	intracellular cytokine staining
IFN-γ	interferon-γ
LTNPs	long term non-progressors
LTRs	Long Terminal Repeat sequences
MA	p17 Matrix protein
MHC	major histocompatibility complex
NK	natural killer cells
R0	Serum-free RPMI 1640
R10	RPMI 1640 supplement with 10% Foetal Calf Serum, 2mM L-glutamine and 50U/ml penicillin/streptomycin
R15	RPMI 1640 supplement with 15% Foetal Calf Serum, 2mM L-glutamine and 50U/ml penicillin/streptomycin
rpm	revolutions per minute
RRE	Rev-Responsive Element
TM	Transmembrane Domain
SFU	spot-forming units
SM cohort	Shuang Miao village cohort
SPs	Slow progressors
ADCC	Antibody-dependent Cell-mediated Cytotoxicity
ADCVI	Antibody-dependent Cell-mediated Viral Inhibition
Ala	Alanine
NC	Nucleocapsid

Chapter 1: Introduction

1.1 Global HIV Epidemiology

It was back in the summer of 1981 that the clinicians in New York and California observed that amongst young, previously healthy, homosexual men, there was a clustering of diseases not normally seen, such as Kaposi's sarcoma and opportunistic infections such as Pneumocystis Carini Pneumonia and unexplained, persistent lymphadenopathy (1981, 1982, Ziegler et al., 1984). The syndrome was named Acquired Immunodeficiency Syndrome (AIDS) and within the first year of its identification, the disease was also identified in hemophiliacs, injection drug users, blood transfusion recipients and infants born to infected mothers. Soon after this, a heterosexual epidemic of AIDS primarily affecting women was reported in Central Africa (Piot et al., 1984, Quinn et al., 1986, Hudson, 1996).

The second decade was dominated by further spread of the epidemic to other areas of the world, including the southern cone of Africa, Asia and the countries in Eastern Europe and Russia. However, the death rate started to plateau and decline in the developed world following the advent of highly active antiretroviral drugs in the 1990s. The situation though continued to get worse in the developing world, where the rates of death due to HIV/AIDS and mother-to-child transmission continued to follow on an increasing trajectory, with almost 2.4 million deaths and more than 3 million new infections, with two-thirds of these in sub-Saharan Africa (Piot et al., 2001, Gayle and Hill, 2001) reported in 2001.

UNAIDS estimates in 2011 put the total number of people living with HIV infection at a total of 34.2 million compared to 29.1 million in 2001; with 2.5 million people infected in 2011, which is a 22% decline from the number in 2001, and a death toll of 1.7 million, a decline of 26% from 2005, when the number of AIDS deaths peaked at 2.3 million (UNAIDS, 2012). Likewise, the number of new infections amongst neonates and infants decreased from 2003 peak of 570,000 to 330,000 in 2011.

Sub-Saharan Africa remains the most severely affected, accounting for 69% of the people living with HIV worldwide, with nearly 1 in every 20 adults (4.9%) living with HIV infection. Although the regional prevalence of HIV infection is nearly 25 times higher in sub-Saharan Africa than in Asia, almost 5 million people are living with HIV in South, South-East and East Asia combined. After sub-Saharan Africa, the regions most heavily affected are the Caribbean and Eastern Europe and Central Asia, where 1.0% of adults were living with HIV in 2011.

The UNAIDS 2012 global report gives the details of the current state of the threat faced by HIV/AIDS (Figure 1.1.1). Southern Africa is particularly badly affected by the epidemic – HIV infection has become hyperendemic there, with an overall prevalence amongst adults of up to 31% in Swaziland, 25% in Botswana and around 17% in South Africa. Even more astoundingly, the prevalence amongst women between the ages of 30 and 34 years is 54%. Around the world, men who have sex with men, female sex workers, injection drug users, truck drivers, fishermen, and military personnel are disproportionately affected (Beyrer et al., 2012, Baral et al., 2012, Mathers et al., 2008, Pandey et al., 2008, Abebe et al., 2003).

Figure 1.1.2 summarises the key findings (Global AIDS report, 2012), and Figure 1.1.3 summarises the infection data, by region (Global AIDS report, 2012).

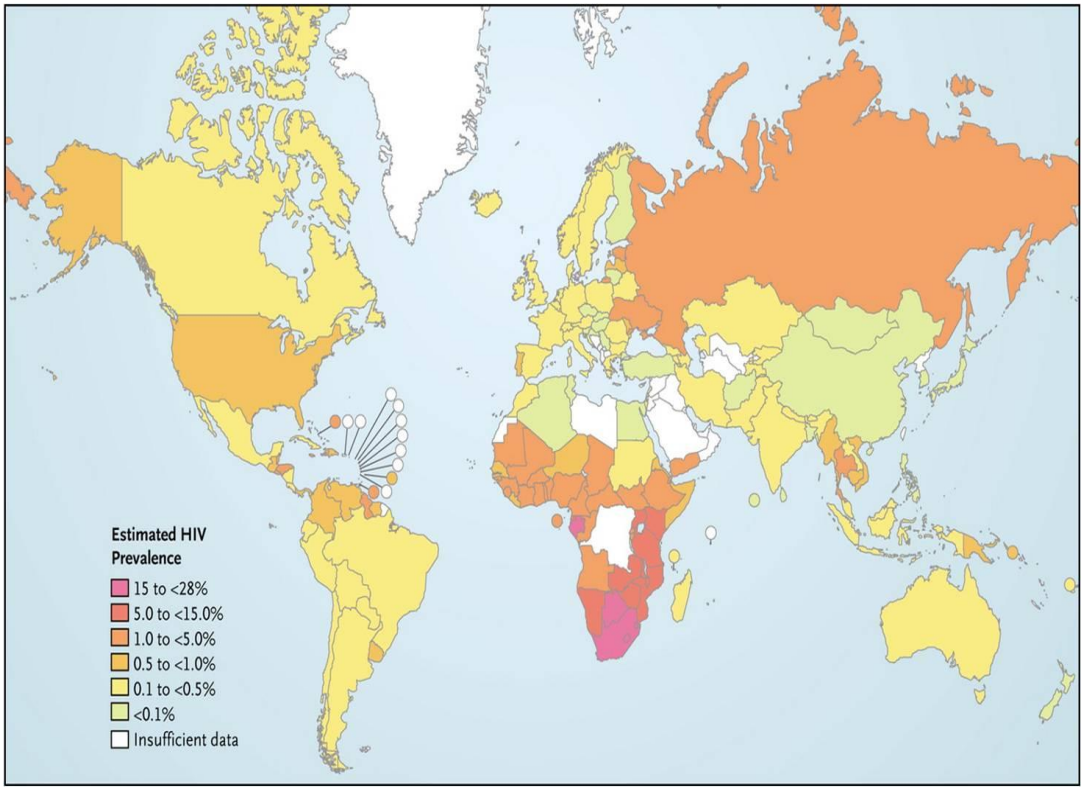


Figure 1.1.1 Global HIV map (UNAIDS 2012 Report)

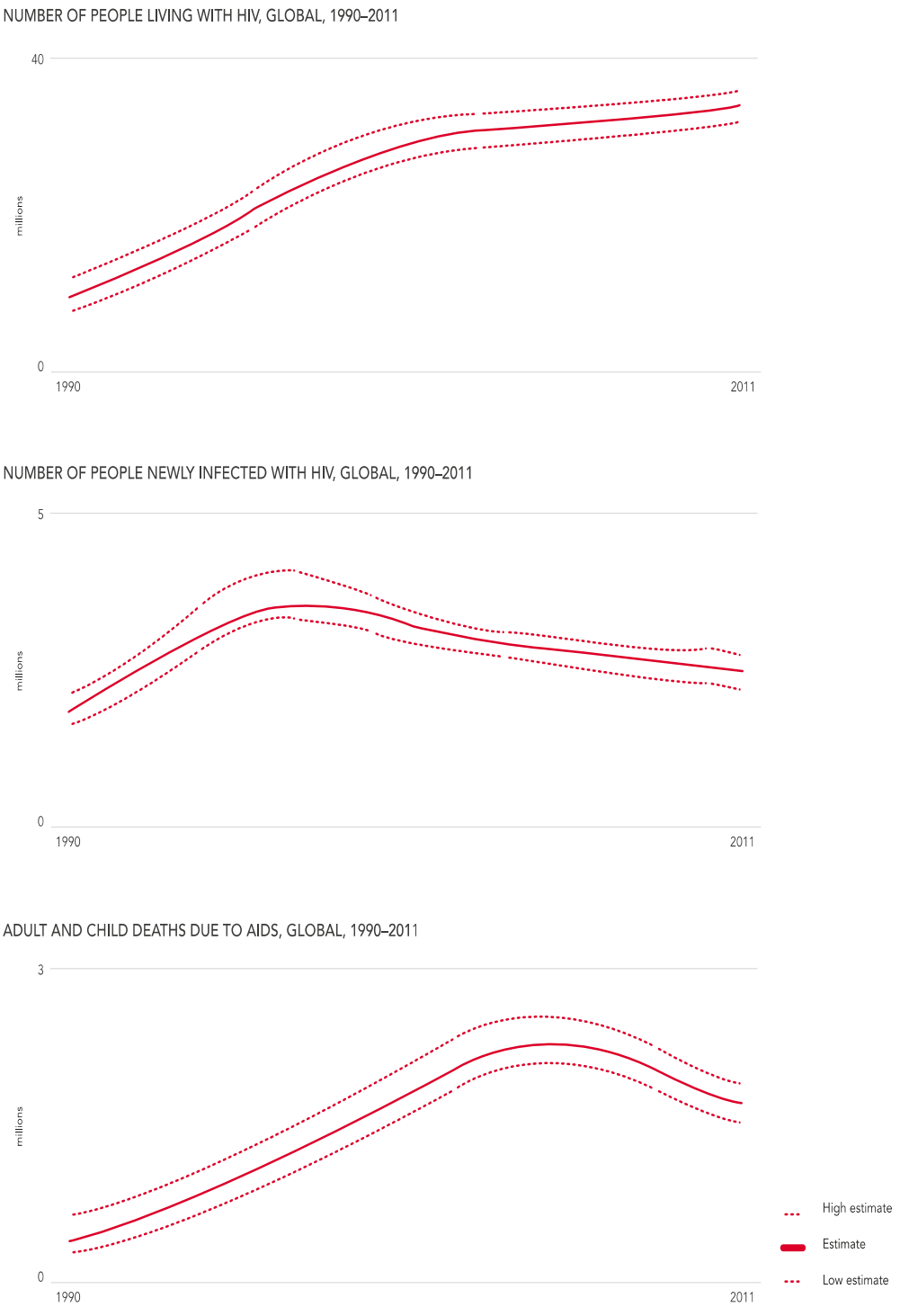


Figure 1.1.2 Global HIV trends, 1990-2011

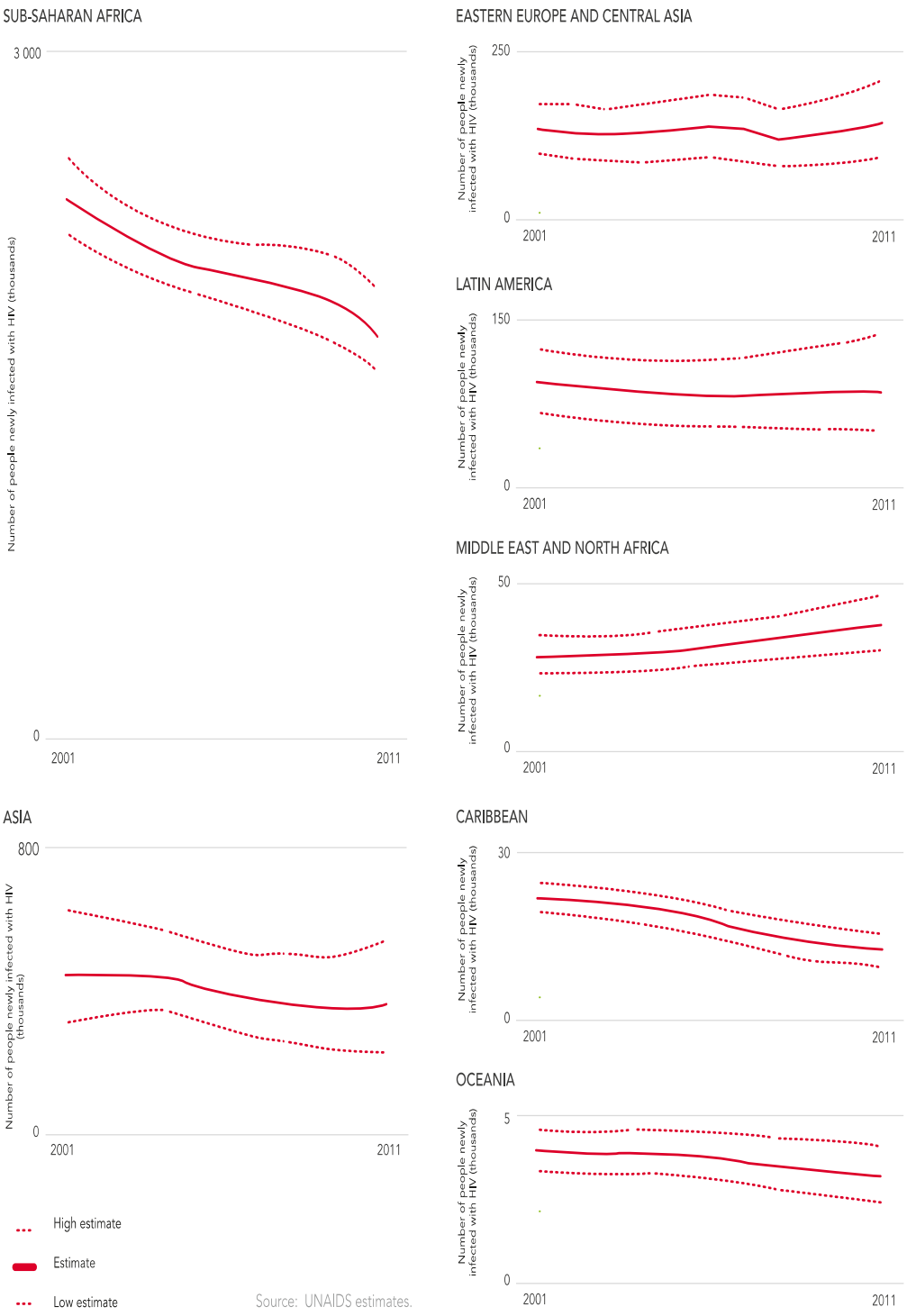


Figure 1.1.3 HIV infections, by region

1.1.1 HIV-1 infection in China

China's large and ethnically varied population, its vast geographical expanse, booming economic growth, and changing cultural norms, are all shaping the growing HIV/AIDS epidemic. AIDS was first reported in Beijing in 1985, and UNAIDS estimates show that at the end of 2011 (2012 China AIDS Response Progress Report, Ministry of Health of the People's Republic of China), the estimated number of people living with HIV in China at 780,000 people. Of these, 28.6% were women; there were 154,000 cases of AIDS; overall prevalence was 0.058%. The estimated number of new infections in 2011 was 48,000 and the estimated number of deaths 28,000. Of the 780,000 people estimated to be living with HIV, 46.5% were infected through heterosexual transmission, 17.4% through homosexual transmission, 28.4% through injecting drug use, 6.6% were former blood donors or transfusion recipients, and 1.1% were infected through mother-to-child transmission.

The HIV epidemic in China could broadly be split into four phases (Ruxrungtham et al., 2004, Portsmouth et al., 2003, Sheng and Cao, 2008). The first phase (1985-1988) was marked by a small number of AIDS cases, for instance, four haemophiliac patients in Zhenjiang Province were infected through imported factor VIII ((Lu F, 2003) in the early stages when the epidemic was starting. The second phase (1989-1993) began with the identification of HIV infection in 146 drug users in Yunnan Province in October 1989, this was the first instance of a local and isolated outbreak (Lu F, 2003). In the early 90s, HIV infection spread beyond Yunnan to other provinces, and a considerable number of HIV cases were reported amongst drug

users and commercial plasma donors from various regions. By, 1998, HIV infection had been reported in all 31, provinces, regions and municipalities (Lu F, 2003). The year 2001 marked the current fourth phase, still ongoing, when the Chinese Government published a detailed plan to tackle HIV/AIDS, with the silence around HIV in China coming to an end. Various initiatives were launched with the aim of providing widespread access to treatment and curbing the spread of HIV/AIDS in China.

It is worth mentioning here a few details about former plasma donors and HIV spread in China, as our study cohort falls within this category. A shortage of blood products in the 1980s and early 1990s led to a large market for illegal commercial plasma and blood collections. Hundreds of commercial plasma collection centres were set up in central and eastern China. The donors generally comprised farmers from poorer localities, and plasma donation was much more common than whole blood donation, as more frequent donations could be made. It is not clear exactly how plasma donors became infected with HIV, but commercial plasma donation with unhygienic re-infusion of red blood cells, often pooled from several donors with the same blood group, was common in rural communities in the early 1990s (Qian et al., 2005). The outbreak of HIV/AIDS amongst former plasma donors was mainly in Henan, Hebei, Hubei, Anhui, Shanxi and Jilin provinces, with the rates of HIV incidence varying between 10% (the lowest area) and 60% (the highest area) (Zhang et al., 2008, Zhang et al., 2004, Zhang Kl and Ma, 2002).

Currently, according to the 2012 UNAIDS country report on China, the epidemic exhibits five major characteristics: 1) National prevalence remains low, but the epidemic is severe in some areas;2) the number of people living with HIV continues to increase, but new infections have been contained at a low level; 3) gradual progression of HIV to AIDS resulting in an increase of AIDS-related deaths; 4) sexual transmission is the primary mode of transmission, and continues to increase; 5) China's epidemics are diverse and evolving.

In the recent past, the proportion of reported cases due to homosexual and heterosexual transmission is increasing every year. The proportion of cases resulting from heterosexual transmission has increased from 33.1% in 2006 to 76.3% in 2011, and the proportion due to homosexual transmission jumped from 2.5% in 2006 to 13.7% in 2011. Sentinel surveillance data has also revealed that HIV prevalence amongst MSM has showed an increasing trend, but the number of people who use drugs testing positive for HIV has been falling since 2005.

Figure 1.1.1.2 shows the geographical distribution of HIV/AIDS cases, as of 31st December 2011 (2012 China AIDS Response Progress Report, Ministry of Health of the People's Republic of China) and the breakdown of newly reported HIV cases.

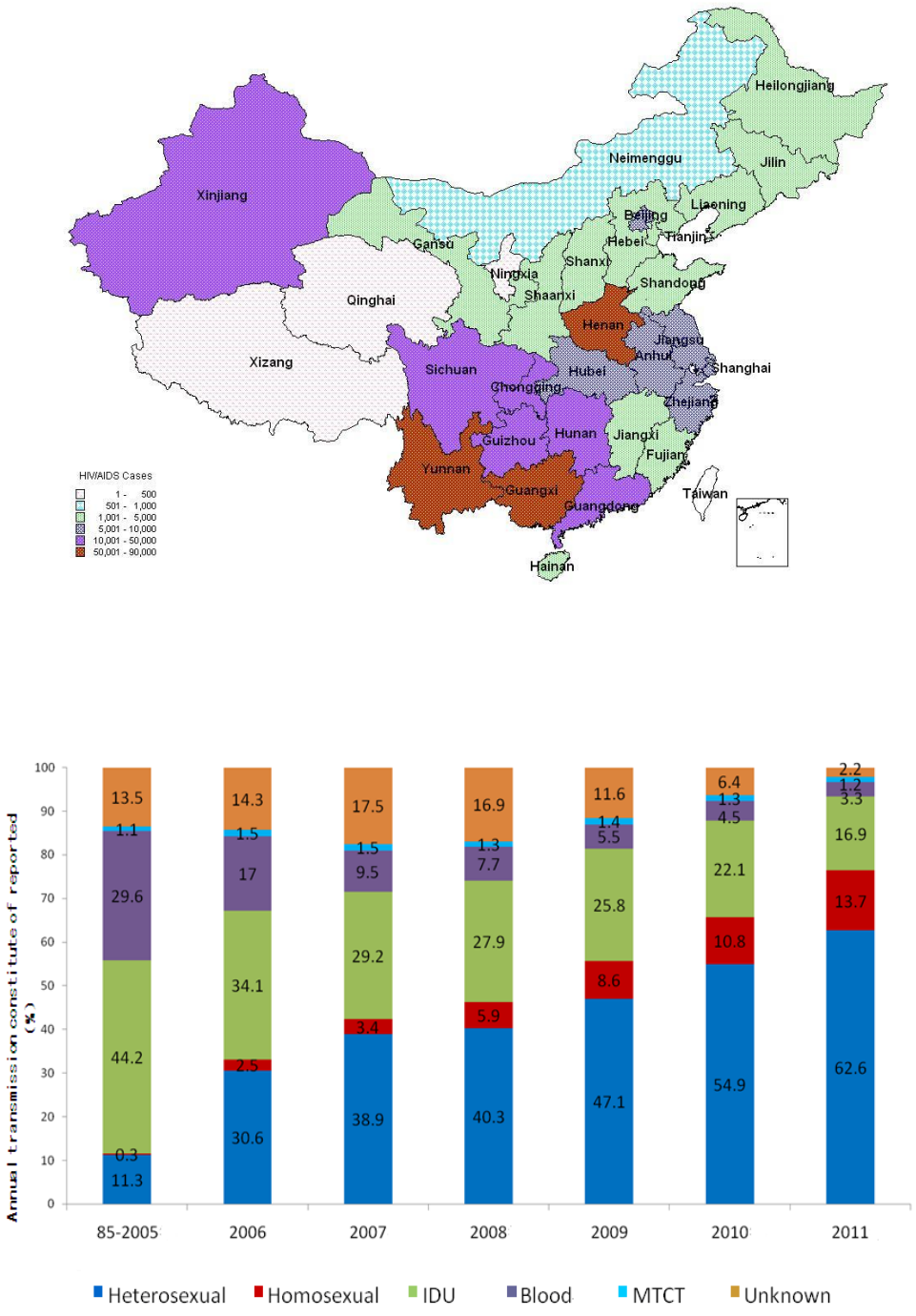


Figure 1.1.1.2 The distribution of HIV/AIDS cases in China and the breakdown of newly reported HIV cases

1.2 The structure and organization of the HIV-1 genome

1.2.1 The morphological structure of HIV

HIV is a single stranded RNA virus belonging to the lentivirus subfamily of retroviruses. Similar to other retroviruses, HIV virions contain a virus capsid, which consists of (a) the major capsid protein, p24; (b) the nucleocapsid protein, p7; (c) the diploid single-stranded RNA genome, and (d) the three viral enzymes: protease, reverse transcriptase and integrase (Barre-Sinoussi, 1996, Sundquist and Krausslich, 2012).

Reverse Transcriptase (RT) is the hallmark of a retrovirus: the function of this enzyme is to transcribe the genomic RNA into double-stranded DNA. This DNA copy of the retroviral genome is described as a 'provirus'. After integration into the genome of the host, the provirus serves as a template for cellular DNA-dependent RNA polymerases to generate new viral RNA genomes in addition to shorter subgenomic messenger RNAs. The unspliced and singly-spliced viral RNAs are translated into the protein components of the viral core and the envelope proteins and the multispliced viral RNAs into the accessory/regulatory proteins.

The viral capsid is surrounded by a matrix protein (p17), which is located underneath the virion envelope. The virion envelope comprises a lipid bilayer membrane, which is derived from the host cell, and a virally encoded trimeric envelope protein complex, each subunit of which is made up of two non-covalently-linked membrane

proteins, gp120 (the ‘outer’ envelope protein) and gp41 (the ‘transmembrane’ protein) (Barre-Sinoussi, 1996, Sundquist and Krausslich, 2012).

Figure 1.2.1 below depicts the structure of the HIV virion ((reproduced from http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/Pages/hivVirionLargeImage.aspx)

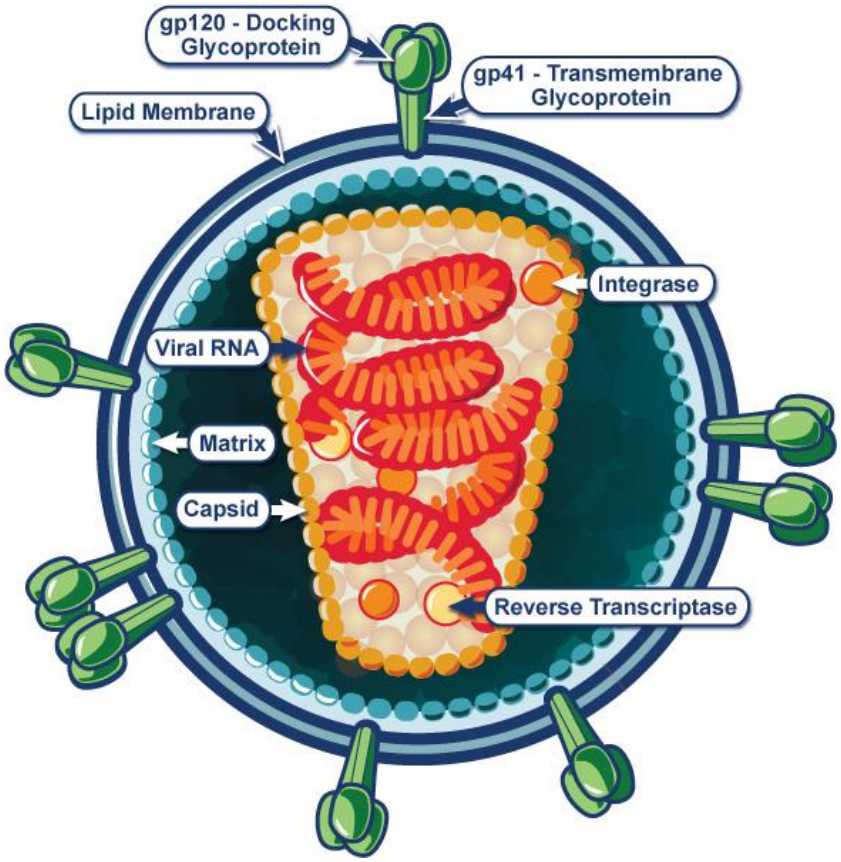


Figure 1.2.1 The structure of the HIV virion

1.2.2 The organization of the HIV-1 genome

The retroviral genome encodes approximately 9500 RNA bases, which consists of nine open reading frames flanked by long terminal repeat sequences (LTRs), which contain binding sites for gene regulatory proteins that control expression of viral proteins. Three out of the nine open reading frames encode the Gag, Pol, and Env polyproteins, which are subsequently proteolysed into individual proteins. The four Gag proteins. p17 matrix protein (MA), p24 capsid protein (CA), p7 nucleocapsid protein (NC), and p6, and the two Env proteins, gp120 surface protein and gp41 transmembrane protein, are structural components that constitute the core of the virion and the outer membrane envelope. The three Pol proteins, protease (PR), reverse transcriptase (RT), and integrase (IN) provide essential enzymatic functions and are also encapsulated within the particle. In addition to this, HIV-1 encodes six additional proteins, often denoted as accessory/regulatory proteins. Three of these encode proteins Vif, Vpr, and Nef, which are found in the viral particle. Two other accessory proteins, Tat and Rev, constitute essential gene regulatory functions, and the last protein, Vpu, which is the focus of this thesis, has been indirectly associated with assembly of the virion and lately, counteracting the host restriction factor, tetherin (Sundquist and Krausslich, 2012, Malim and Bieniasz, 2012, Malim and Emerman, 2008).

Figure 1.2.2 depicts the organization of the HIV-1 genome.

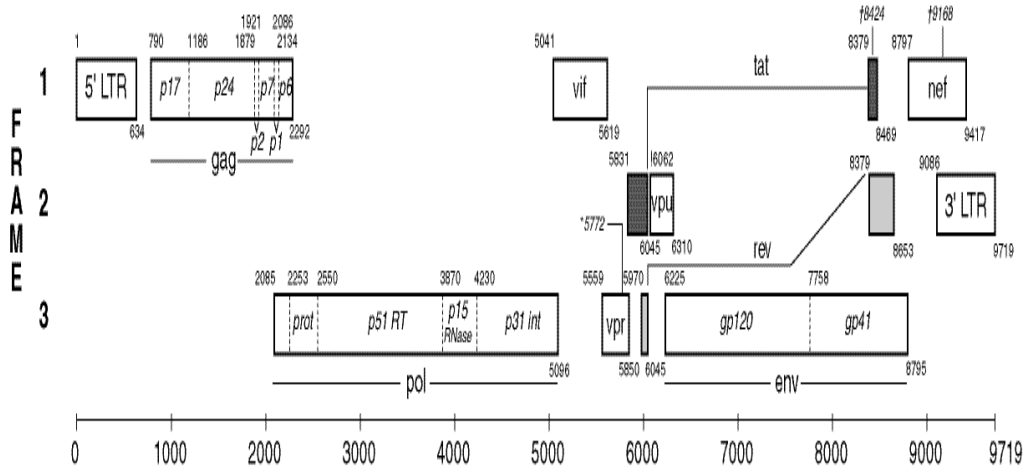


Figure 1.2.2 The organization of the HIV-1 genome, strain HXB2. (reproduced from http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html)

Gag

Gag gene contains around 1500 nucleotides, which encodes four separate proteins. Gag is translated as a polyprotein precursor that encodes matrix (p17), capsid (p24), nucleocapsid (p7) and p6 proteins. The production of functional proteins is mediated by viral cleavage of the Gag polyprotein precursor (Meng and Lever, 2013, Jouvenet et al., 2011, Ganser-Pornillos et al., 2008). In the mature viral particle, the 132-residue matrix protein lines the inner surface of the virion membrane. It is critical for targeting Gag precursor polyproteins to the plasma membrane prior to viral assembly.

In addition, Matrix protein is also involved in the nuclear transport of the viral genome (Gallay et al., 1995, Bukrinskaya et al., 1996). Capsid protein forms the core of the virus particle. The C-terminal domain functions primarily in assembly(Gamble et al., 1997), while the N-terminal domain is critical for infectivity, apparently by participating in viral uncoating through its association with a cellular chaperone, cyclophilin A (CypA) (Luban, 1996, Braaten et al., 1997). Nucleocapsid protein p7 is the third component of the Gag polyprotein and coats the genomic RNA inside the virion core. The primary function of nucleocapsid is to bind specifically to the packaging signal and deliver full-length viral RNAs into the assembling virion (Berkowitz et al., 1995). p6 is critical for incorporation of Vpr during viral assembly (Kondo and Gottlinger, 1996, Lu et al., 1995b), and is also required for the efficient release of budding virion from an infected cell.

Pol

The Pol gene encodes three proteins involved in viral replication: protease, reverse transcriptase and integrase. After assembly, the virus particle buds from the membrane surface and is released. The immature particles formed are non-infectious, and the Gag and Gag-Pol polyprotein precursors must be cleaved by protease, and conformational rearrangement must occur within the particle in order to make mature infectious viruses (Kaplan et al., 1994a, Kaplan et al., 1994b).

Before the viral genome can be integrated into the host genome, it must be reverse transcribed into double-stranded DNA. The copying of the HIV genome into DNA is one of the key stages of the HIV life cycle. Reverse transcriptase catalyses both RNA-dependent and DNA-dependent DNA polymerisation reactions, and contains an RNAse H domain that cleaves the RNA portion of RNA-DNA hybrids generated during the polymerisation reaction (Oude Essink et al., 1996). The diploid single-stranded RNA genome is converted to a single double-stranded DNA copy. Viral DNA can be completely synthesised within 6 hours after viral entry, although the DNA may remain unintegrated for prolonged periods (Zack et al., 1990). The HIV-1 DNA polymerase lacks a proof-reading function, and is therefore replication error-prone which leads to the introduction of random incorporation of mutations into each new copy of the viral genome (Roberts et al., 1988, Roberts and Kunkel, 1988). Consequently, HIV is able to evade rapidly the host immune response, and develop resistance to antiviral drugs. Reverse transcriptase was therefore an early target in the development of antiviral drugs, and two general classes of reverse transcriptase inhibitors have been developed: the nucleoside analogues that are presumed to bind to the polymerase active site, and the non-nucleoside inhibitors. As a consequence of the high mutation rate of reverse transcriptase, resistance to these compounds develops rapidly, however, potent treatment regimens result from the combination of reverse transcriptase and protease inhibitors (Freed, 2001).

Following reverse transcription, integrase catalyses a series of reactions to integrate the viral genome into a host chromosome. Firstly, integrase removes the two 3' nucleotides from each strand of the linear viral DNA, leaving overhanging CA_{OH} ends (Katz and Skalka, 1994). Secondly, the processed 3' ends are covalently joined

to the 5’ ends of the target DNA. Finally, unpaired nucleotides at the viral 5’ ends are removed and the ends are joined to the target site 3’ ends, generating an integrated provirus flanked by five base-pair direct repeats of the target site DNA.

Env

Env encodes the envelope glycoprotein of HIV-1 that is expressed as a 160 kD glycoprotein (gp160). The envelope glycoprotein is cleaved into two subunits: the exterior gp120 protein and the transmembrane protein, gp41. The two HIV envelope glycoproteins are noncovalently linked and assemble into an oligomer, usually trimer, of the gp120-gp41 heterodimer that is expressed at the cell surface, and then, following viral budding, at the virion surface (Lu et al., 1995a, Kowalski et al., 1987).

After translation, gp160 is broken down into gp120 and gp41, which remain non-covalently linked to form a single subunit of a trimeric “spike” on the virion surface. The C-terminal subunit, gp41, consists of a cytoplasmic domain (ultimately inside the viral membrane), a membrane spanning domain and an extracellular domain, which is responsible for the conformational change needed for fusion. The N-terminal subunit, gp120, is completely outside the viral membrane. Although the protein has a complex fold it can also be viewed as being linearly organized into five conserved regions (C1-C5) interspersed with five variable regions (V1-V5) (Arrildt et al., 2012). The host receptor CD4 interacts with residues in the conserved

regions of gp120 on either side of V4, and the coreceptor CCR5 interacts both with a GPGR/Q motif at the apex of the V3 loop and at its base.

Tat

Tat is the viral trans-activator of transcription that binds the transactivation response RNA element at the 5'-end of all viral transcripts to promote transcriptional elongation (Marcello et al., 2001, Van Lint et al., 2013). Tat is an early regulatory protein that has a variable length of 86–104 aa, encoded by two exons. Mutational analysis of HIV-1 Tat has identified two important functional domains: an activation domain that mediates its interactions with cellular machinery and an arginine-rich region that is required for binding to the transactivation responsive element (TAR) RNA (Hwang et al., 2003).

Tat enhances the processing of transcribing polymerases. It increases the production of viral mRNA approximately 100-fold, and consequently is essential for viral replication (Jones and Peterlin, 1994). It promotes the elongation phase of HIV-1 transcription, so that full-length transcripts can be produced (Kao et al., 1987). So, in summary, the functions proposed for HIV-1 Tat include chromatin remodelling, phosphorylation of RNA polymerase II that is involved in the transcription of the

full-length viral mRNAs, transactivation of viral genes and binding to a specific structure of HIV-1 mRNAs (Ammosova et al., 2006, Richman et al., 2009, Wong et al., 2005).

Rev

HIV-1 uses alternative splicing of its full-length transcript to generate the array of mRNAs required for efficient expression of all viral genes. In addition, the full-length transcript serves both as a source of new viral genomes and also as a translational template. An inevitable corollary of this expression strategy is that HIV-1 RNAs that contain functional introns must be selectively exported from the nucleus. In contrast, the majority of unspliced cellular RNAs are ordinarily retained in the nucleus and either spliced to completion or degraded. To evade this problem, HIV uses a protein called Rev that binds to a cis-acting RNA target (the Rev-Response Element or RRE) present in all unspliced viral transcripts and targets them for nuclear export (Emerman and Malim, 1998, Goh et al., 1998). A molecule of rev can act as a 'shuttle' moving in and out of the nucleus, theoretically taking a new set of RNA each time it leaves the nucleus. In the absence of Rev protein, only regulatory proteins made from the multiply spliced transcripts are made. Therefore, Rev plays a critical role in the replication cycle of HIV-1, permitting new virus particles to be made (Haseltine, 1991).

Variation in HIV-1 Rev has been shown to down-regulate the expression of viral late genes and alter sensitivity to Gag-specific cytotoxic T lymphocytes (CTL) (Bobbitt et al., 2003). In addition, CTL epitopes have been identified within HIV-1 Rev (Addo et al., 2001). Therefore, genetic changes within Rev may facilitate immune evasion directly by altering CTL epitopes in Rev, and/or indirectly through altering Rev nuclear export activity and expression of structural proteins.

Nef

Nef was initially classified as a negative regulator of HIV infection and was thus named as 'negative factor' (Terwilliger et al., 1986, Ahmad and Venkatesan, 1988, Ameisen et al., 1989). However, subsequent studies demonstrated that Nef plays a critical role in several steps of HIV replication (Cheng-Mayer et al., 1989, Li et al., 2005b, Li et al., 2005a).

Similar to other accessory proteins, Nef contains no enzymatic activity nor does it serve any structural functions. Instead, it is a master in rearranging the signaling and trafficking pathways of the infected host cell, in a way that is beneficial for viral replication and spread. Nef does this by physically interacting with specific cellular proteins involved in these pathways, due to its multiple effector domains (Arien and Verhasselt, 2008, Foster et al., 2011). As a consequence of these interactions, Nef allows HIV to promote viral spread through a two-pronged strategy: by evading the anti-viral immune responses of the host, and by directly increasing viral replication.

The evasion of anti-viral responses is accomplished by down regulating the CD4 molecules (Garcia and Miller, 1991), and also the expression of MHC Class I molecules, which helps to evade the CTL-mediated killing (Schwartz et al., 1996). Viral replication is mediated by enhancing the infectivity of virions by possibly remodeling of the host actin cytoskeleton to promote the cell-to-cell transmission of virions (Haller et al., 2011, Sol-Foulon et al., 2002).

Vpr

Viral Protein R (Vpr) is a small protein incorporated into viral particles through an interaction with p6 (Sato et al., 1996). It is a 96 amino acid, 14 kDa protein that is highly conserved in both HIV-1 and simian immunodeficiency virus. Different studies have demonstrated that Vpr is a multi-functional protein. It mediates many processes that aid in HIV-1 infection, evasion of the immune system, and persistence in the host. The molecular functions of Vpr include nuclear import of viral pre-integration complex (PIC), induction of G_2 cell cycle arrest, modulation of T-cell apoptosis, transcriptional coactivation of viral and host genes, and regulation of nuclear factor kappa B (NF-κB) activity(Le Rouzic and Benichou, 2005) .

Vif

Viral infectivity factor (Vif) protein is a 192 amino acid that expressed at high levels in the cytoplasm of infected cells. Vif is critical for the reproduction of HIV-1 in peripheral blood lymphocytes, macrophages, and certain cell lines known as 'nonpermissive' cells (Strebel et al., 1987). Mutation in Vif can have a profound defect on virus infectivity. Compared with mature wild-type virions, Vif mutant viruses have similar protein and RNA contents, but grossly altered core structures, suggesting that Vif may play a role in viral assembly and/or maturation (Cohen et al., 1996). In 2003 later studies revealed another critical role for Vif: it binds directly to APOBEC3G, a host cellular enzyme which inhibits HIV infection in the non-permissive cell, and counteracts its anti-HIV activity by promoting its degradation (Marin et al., 2003, Sheehy et al., 2003).

Vpu

The accessory protein, Viral Protein U, is an 81 amino acid protein that has come under a lot of investigation lately due to its role in restricting the host restriction factor, tetherin. Residues 1-27 of Vpu constitute the N-terminal hydrophobic membrane anchor followed by 54 residues that extend into the cytoplasm. A highly conserved region spanning residues 47-58 contains a pair of serine residues that are constitutively phosphorylated by casein kinase II.

Structural analysis of synthetic peptides representing the cytoplasmic domain of Vpu detected two discrete α-helical structures encompassing amino acids 35-50 and 58-70, respectively, separated by a flexible segment containing the two conserved phosphorylated serine residues (Cohen et al., 1988, Strebel et al., 1988, Terwilliger et al., 1989, Schubert et al., 1994, Zheng et al., 2003, Nomaguchi et al., 2008, Ruiz et al., 2010).

The traditional function designated to Vpu has been degradation of CD4 (Terwilliger, Cohen et al. 1989; Willey, Maldarelli et al. 1992; Willey, Maldarelli et al. 1992). More importantly, Vpu has been in the limelight since the discovery of the host restriction factor tetherin, and its role in counteracting tetherin activity (Neil, Zang et al. 2008). In response to Vpu, tetherin is downregulated from the surface of the HIV infected cell (Van Damme, Goff et al. 2008). After the discovery of tetherin, there have been a spate of studies which have tried to map the residues in tetherin's TM domain, and their critical roles in association with Vpu (Gupta, Hue et al. 2009; McNatt, Zang et al. 2009; Rong, Zhang et al. 2009). More recently, a detailed analysis using cysteine-scanning mutagenesis coupled with oxidation-induced cross-linking demonstrated that Vpu and Tetherin TM domains bind directly to each other in the membranes of living cells, and also mapped the TM domain residues that contact each other (McNatt, Zang et al. 2013). Full details about Vpu and tetherin are detailed in Chapter 4.

1.3 HIV-1 lifecycle

HIV infection begins with the attachment of the virus to the cell surface aided by an interaction between the extracellular domain of HIV-1 gp120 and cellular receptors (Moore and Ho, 1993). The major receptor for HIV-1 is CD4, and CCR5 and CXCR4 are the main coreceptors in vivo (Clapham and McKnight, 2002). After binding to the coreceptor, viral and cellular membranes fuse and the viral core is released into the cytoplasm. Viral uncoating involves cellular factors and the viral proteins MA, Nef and Vif. The viral RNA genome is retrotranscribed into a full-length double-stranded DNA by the viral reverse transcriptase enzyme (Harrich and Hooker, 2002, Herschhorn and Hizi, 2010). The fidelity of reverse transcription is affected by the presence of cellular host restriction protein APOBEC3G. HIV-1 Vif counteracts the antiretroviral effect of the cellular protein APOBEC3G by reducing its expression and incorporation into progeny viruses. The preintegration complex docks to the nuclear membrane, directed by HIV-1 Vpr (Popov et al., 1998), and enters the nucleus through the nuclear pore (Le Rouzic and Benichou, 2005). Linear double- stranded DNA in the preintegration complex is inserted into the host chromosome as a result of the activity of viral integrase (Van Maele and Debyser, 2005).

In the early phase of the replication cycle, only multiple-spliced mRNAs are produced and the regulatory proteins Tat, Nef and Rev are expressed. When enough amounts of Rev are produced, non-spliced or single-spliced species are generated and transported to the polysomes, leading to the production of the other viral proteins and genomic RNA (Pollard and Malim, 1998). The Env and Gag-Pol genes

are translated. After translation, the Env proteins migrate and are inserted into the plasma membrane. Gag and Gag-Pol polyproteins also move to the cellular membrane and start to assemble, directed by the Gag polyprotein (Sandefur et al., 2000, Derdowski et al., 2004). Later on, this complex buds through the plasma membrane, producing an immature virion (Nguyen and Hildreth, 2000).

Worth mentioning here is the role played by Vpu, which counteracts the host restriction factor tetherin, which as the name implies, tethers the virus to the cell membrane and restricts release (Hotter et al., 2013) Budding triggers the activation of the PR that autocatalytically cleaves the Gag and Gag-Pol polyprotein, releasing the structural proteins and enzymes. The individual proteins undergo further interactions, with CA and NC forming the nucleocapsid, and MA remaining associated with the viral envelope (Briggs et al., 2003).

Figure 1.3 summarises the HIV-1 replication (Engelman and Cherepanov, 2012).

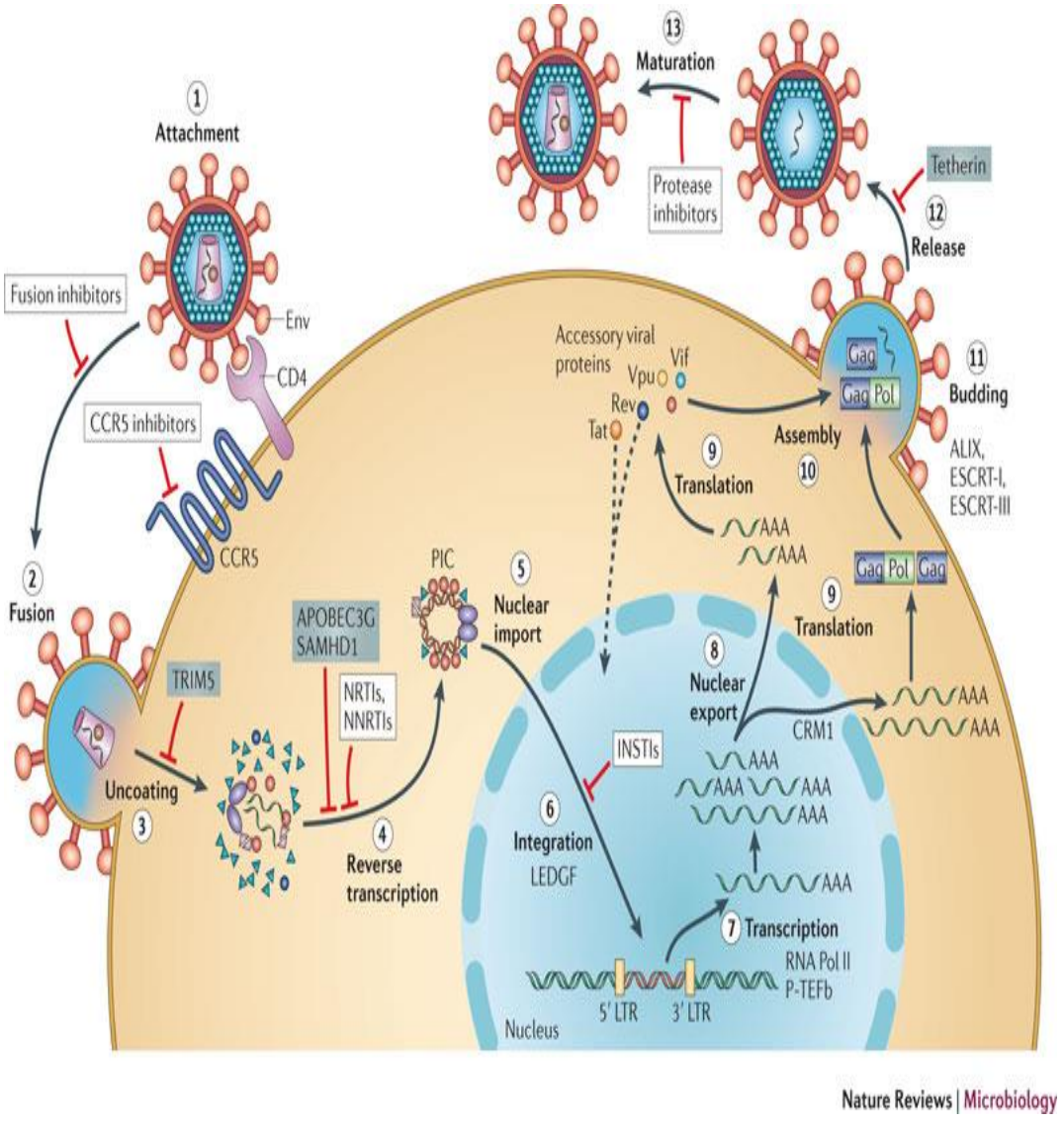


Figure 1.3 The HIV-1 replication cycle

(Engelman and Cherepanov, 2012)

Figure 1.3 Legends:

The infection begins when the envelope (Env) glycoprotein spikes engage the receptor CD4 and the membrane-spanning co-receptor CC-chemokine receptor 5 (CCR5) (step 1), leading to fusion of the viral and cellular membranes and entry of the viral particle into the cell (step 2). Partial core shell uncoating (step 3) facilitates reverse transcription (step 4), which in turn yields the pre-integration complex (PIC). Following import into the cell nucleus (step 5), PIC-associated integrase orchestrates the formation of the integrated provirus, aided by the host chromatin-binding protein lens epithelium-derived growth factor (LEDGF) (step 6). Proviral transcription (step 7), mediated by host RNA polymerase II (RNA Pol II) and positive transcription elongation factor b (P-TEFb), yields viral mRNAs of different sizes, the larger of which require energy-dependent export to leave the nucleus via host protein CRM1 (step 8). mRNAs serve as templates for protein production (step 9), and genome-length RNA is incorporated into viral particles with protein components (step 10). Viral-particle budding (step 11) and release (step 12) from the cell is mediated by ESCRT (endosomal sorting complex required for transport) complexes and ALIX and is accompanied or soon followed by protease-mediated maturation (step 13) to create an infectious viral particle

1.4 Natural History of HIV-1 infection

The clinical course of HIV-1 infection can be divided into three general phases or stages: (1) Primary or acute infection; (2) Latency or asymptomatic phase and (3) AIDS-defining illness (Abrams et al., 1984, Pantaleo and Fauci, 1996, Douek et al., 2009). During the course of acute infection, acute viral replication results in high levels of viraemia and the patient often presents with an acute viral illness resembling infectious mononucleosis within 2 to 4 weeks post-infection (Clark et al., 1991, Cooper et al., 1985). After this initial peak of viremia, the plasma RNA level decreases dramatically, stabilising at an equilibrium level, referred to as the 'viral set point'. This viral set point strongly predicts how rapidly an individual will develop AIDS (Mellors et al., 1995).

After the initial peak in viraemia, the HIV-specific humoral and cell-mediated immune responses are readily detected (Borrow et al., 1994, Koup and Ho, 1994, Koup et al., 1994, Pantaleo et al., 1994). Despite the early appearance of a vigorous immune response, this immune response ultimately fails to eliminate the virus since the transition to the chronic phase of infection occurs in most individuals (Pantaleo et al., 1993a, Pantaleo et al., 1993b). During the latent phase, HIV actively and continuously replicates, especially in the lymphoid organs.

During the prolonged asymptomatic phase there is a simultaneous decline in peripheral $CD4^+$ T cell count and slowly rising viraemia. When the $CD4^+$ T cell count falls below 200 cells/ul, there is an increased susceptibility to opportunistic infections or neoplasms, which essentially characterise acquired immunodeficiency

syndrome (AIDS). The median time from infection to AIDS is normally around 10 years; however, progression to AIDS can also occur within 2-3 years in a minority of individuals, termed rapid progressors. By contrast, a small percentage (less than 2-5% in different cohorts) HIV-infected individuals do not experience progression of disease for over 10 years, termed as long-term nonprogressors (Buchbinder et al., 1994, Stewart et al., 1997, Lobritz et al., 2011, Shacklett, 2006)

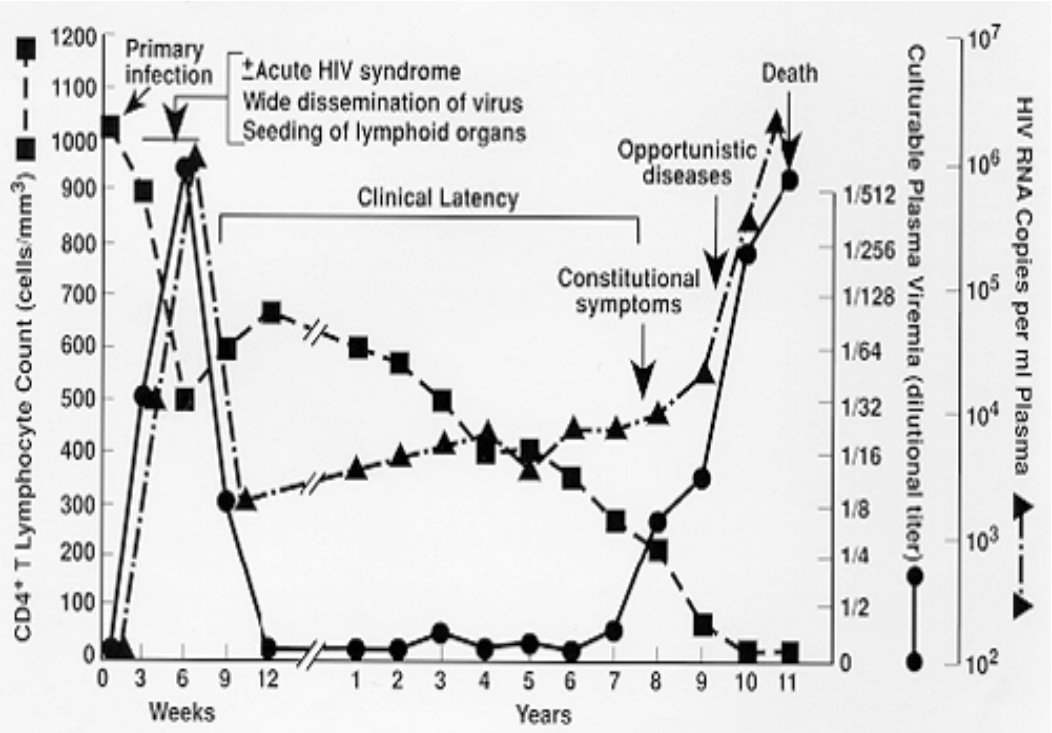


Figure 1.4 The typical course of HIV infection (Pantaleo et al., 1993b)

1.5 Pathogenesis of HIV infection

The pathogenic mechanisms behind HIV infection are multifactorial and quite complex. It was realised from the start that an apparent paradox existed whereby the immune system was aberrantly activated at the same time (Dalgleish et al., 1984) that individual exhibited immune deficiency (Fauci, 1988). This was later demonstrated to be a consequence of the unregulated secretion of various cytokines, and the intensive cell signalling induced by the viral envelope (Fauci, 1993). Depletion of CD4+ T cells was recognized as the hallmark of the disease at an early stage (Gottlieb, 2006, Masur et al., 1981), and it was demonstrated in 1984 that the CD4 molecule is the primary receptor for the virus on a subset of T cells and monocytes (Dalgleish et al., 1984, Klatzmann et al., 1984).

Studies in the 1990s revealed the roles played by the chemokine receptors CXCR4 and CCR5 in the efficient binding and entry of two different viral phenotypes of HIV-1, referred to as X4 and R5, respectively (Fauci, 1996, D'Souza and Harden, 1996). The critical importance of CCR5 coreceptor in the pathogenesis of HIV infection was demonstrated by the finding that cells from subjects homozygous for a 32 base pair deletion in the CCR5 gene could not be infected *in vitro* with R5 viruses, and therefore such individuals are extremely resistant to HIV infection (O'Brien and Moore, 2000). Figure 1.5.1 summarises the pathogenic events in untreated HIV-mediated disease (Fauci, 1996).

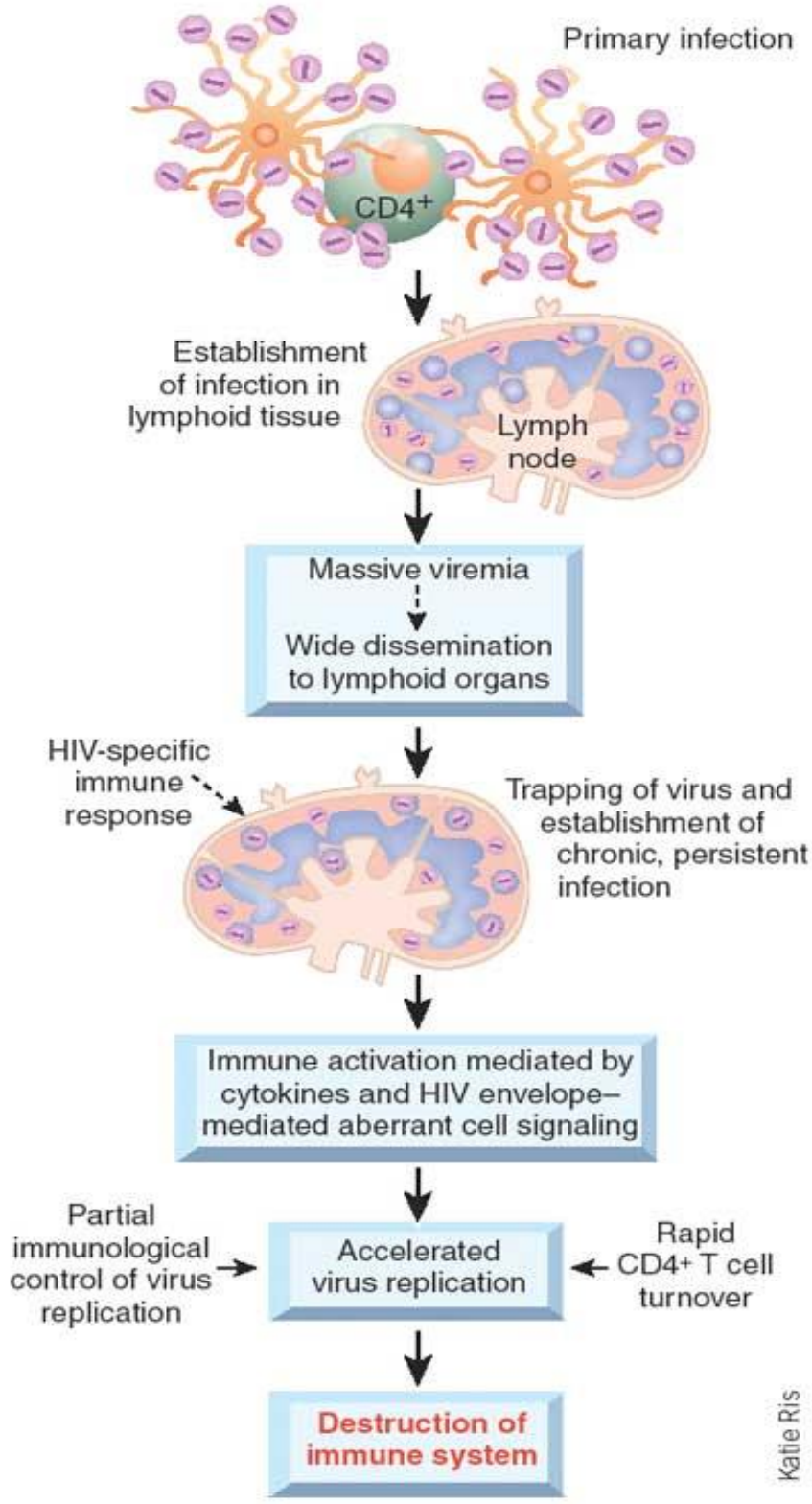


Figure 1.5.1 The pathogenic events in untreated HIV infection. Figure legend follows

Figure 1.5.1 Legend:

HIV enters the body and binds to Langerhans or dendritic cells, which carry the virus to $CD4^+$ T cells. Infected $CD4^+$ T cells home to lymphoid tissue, where the infection is established. Virus replication accelerates, and massive viraemia leads to the wide dissemination of virus throughout the body's lymphoid tissue. An HIV-specific immune response occurs and virus is trapped on the follicular dendritic cells of germinal centres in the lymphoid tissue. At this point, chronic, persistent infection is established despite an immunological response to the virus. Immune activation is an important driver of HIV replication and is mediated by the secretion of various cytokines and by aberrant cell signalling caused by interaction of the viral envelope with cellular receptors. Because there is usually only partial immunological control of virus replication, continual and accelerated production of virus ensues. This is associated with a rapid turnover of $CD4^+$ T cells. Ultimately, lymphocyte depletion occurs, along with destruction of the architecture of lymphoid tissue.

Thereafter, studies demonstrated that lymphoid tissue is indeed the chief target and reservoir of HIV infection (Embretson et al., 1993, Pantaleo et al., 1993a). The virus continues to multiply, even though the infected subject appears clinically well.

Classic viral dynamic studies (Ho et al., 1995, Wei et al., 1995) helped to characterise the huge turnover of virus in HIV disease, and the delicate balance between virus production and T cell dynamics. It was noted that there is a direct relationship between virus replication and disease progression, which led to the demonstration that the viral set point strongly predicts disease progression (Mellors et al., 1995). Neutralising antibodies, potent HIV-specific CD8+ cytotoxic T cell responses and HIV-specific CD4+ T cells are present in many individuals infected with HIV at various stages of HIV infection (Fauci, 1996). However, the CD8+ cytotoxic T cells can select for escape mutants, and the most effective neutralising antibodies are directed at cryptic epitopes against which it is difficult to produce antibodies. The cellular and humoral immune responses to HIV are discussed in detail in the following sections.

Figure 1.5.2 summarises some of the key questions which are yet to be completely delineated in HIV infection (Virgin and Walker, 2010).

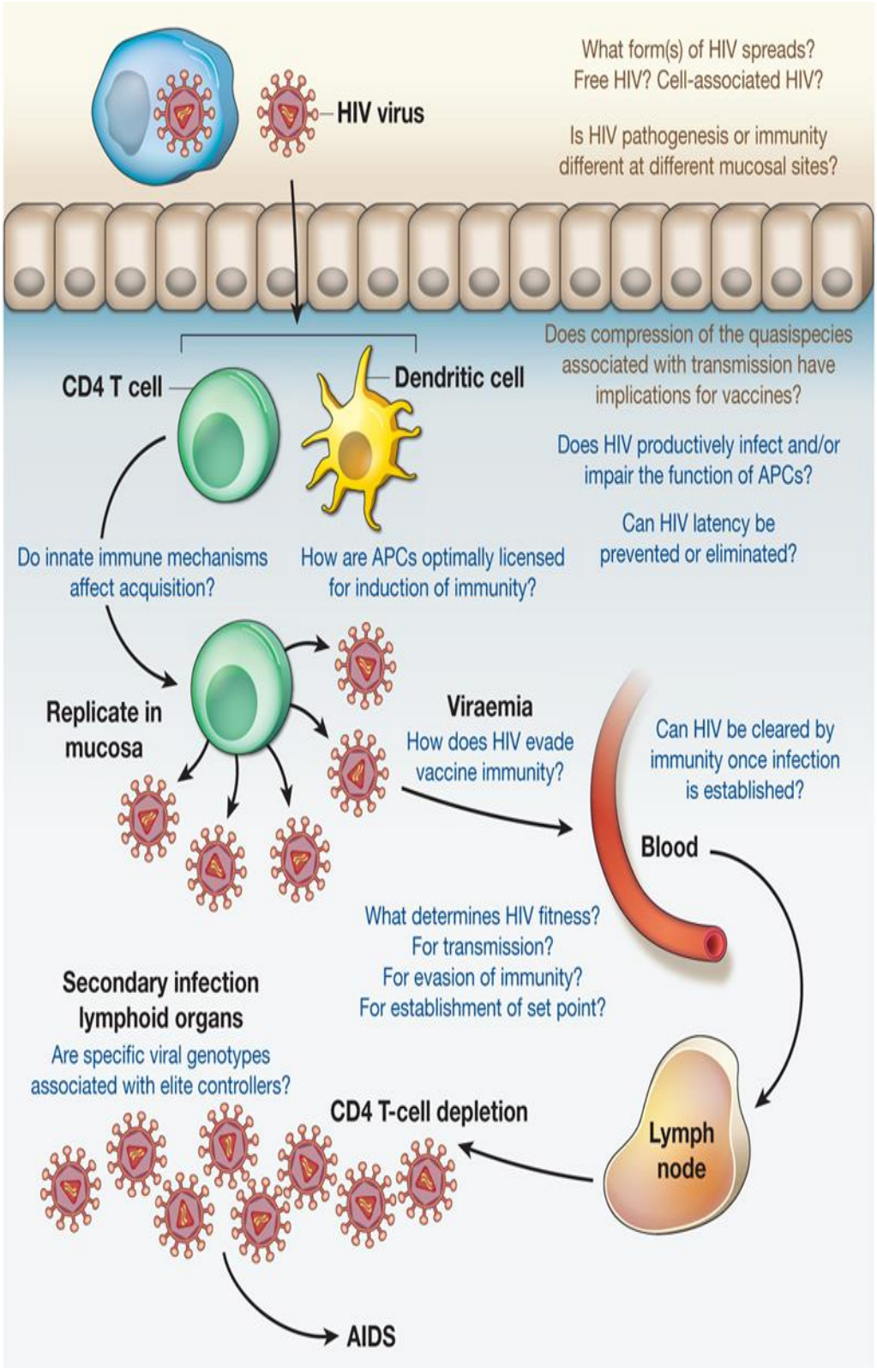


Figure 1.5.2 The key questions still to be tackled in HIV pathogenesis

1.6 Cellular Immune responses in HIV infection

1.6.1 Helper T cell response

The hallmark of HIV-1 infection is progressive depletion of CD4+ T cells. The decrease in CD4+ T cell count affects not only the CD4+ T cell response, but also coincides with a decline in CTL activity (Lane and Fauci, 1985, Murray et al., 1984, Clerici and Shearer, 1993). T cell help is needed for the generation of the neutralising antibody response as well as the cytotoxic T cell response. In addition, it has been demonstrated that HIV preferentially infects and replicates in CD4+ T cells specific for HIV antigens (Douek et al., 2002). Therefore, the biggest hole in the immune repertoire of most HIV infected persons is the selective lack of HIV-specific T helper cells.

Evidence for the role of CD4+ T cells in the control of HIV-1 replication has been accumulating since it was first demonstrated that HIV-specific CD4+ T cells, as measured by intracellular interferon-gamma expression, persisted in patients with chronic HIV infection, but the frequency of HIV-specific CD4+ T cells was greater in long-term nonprogressor patients than in chronic HIV-1 infection (Pitcher et al., 1999). In addition, there was selective detection of IL-2 secreting CD4+ T cells in aviraemic but not viraemic individuals, implying that persistent antigen stimulation led to the loss of IL-2 secreting functions in HIV-specific CD4+ T cells (Rosenberg et al., 1997, Boaz et al., 2002). The inability to secrete IL-2 was coupled with the inability of the CD4+ T cell to proliferate. Studies have also showed that in the very small fraction of infected patients in whom viraemia is spontaneously controlled

without the need for antiretroviral therapy, referred to as ‘elite controllers’, control of viraemia is associated with a strong virus-specific T helper cell response (Rosenberg et al., 1997). A strong CD4+ T cell response in primary HIV infection was found to be linked with slower disease progression and better control of viraemia, and those with a poor CD4 response in primary HIV infection failed to control viremia effectively (Gloster et al., 2004).

A subset of CD4+ T helper cells, Th17 cells, have been identified and are believed to be critical for regulating gut mucosal immune responses against extracellular microbial pathogens, and may serve as a link between innate and adaptive immune responses (Dandekar et al., 2010, Wynn, 2005). Th17 cells secrete interleukin 17 (IL-17) and other cytokines that play a critical role in maintaining mucosal integrity and control of bacterial and fungal infections (Korn et al., 2009). Th17 cells were shown to be perturbed during HIV infection, and are significantly depleted in the gastrointestinal tract of HIV-infected individuals (Elhed and Unutmaz, 2010). Therefore, based on studies on HIV-1 and other viral infections, CD4+ T cells are a critical component of the host’s immune response against HIV.

1.6.2 HIV-specific cytotoxic T lymphocyte (CTL) response

The cytotoxic T lymphocyte (CTL) response is another crucial component of the adaptive immune response to HIV infection (Walker et al., 1987, Walker et al., 1988, Nixon et al., 1988). Virus-specific CTLs can lyse HIV infected cells by recognising viral peptides (usually 8-11 amino acids in length) presented on the surface of the infected cells in association with HLA class I molecules. This recognition triggers the CTLs to lyse the infected cell, and/or secrete cytokines that may have an antiviral effect, such as interferon-gamma and tumour necrosis factor.

In the acute phase of infection, the CTL response appears early and coincides with a rapid drop in HIV-1 plasma viral load (Koup et al., 1994, Koup and Ho, 1994). The most compelling evidence for the critical importance of CD8+ CTLs in containing HIV-1 replication comes from studies in SIV-infected rhesus monkeys. In vivo depletion of CD8+ cells in the monkeys led to a substantial increase in the replication of SIV (Schmitz et al., 1999, Jin et al., 1999). Figure 1.6.2 on the next page illustrates this effects of CTLs on viraemia (Letvin and Walker, 2003). There is substantial evidence that CD8+ T cells play an important role in controlling HIV-1 infection (McMichael and Rowland-Jones, 2001). During chronic infection, there is an inverse correlation between the magnitude of a certain dominant CTL response and HIV-1 plasma viral load, for instance, the HLA-A*0201-restricted gag-specific CTL (Ogg et al., 1998).

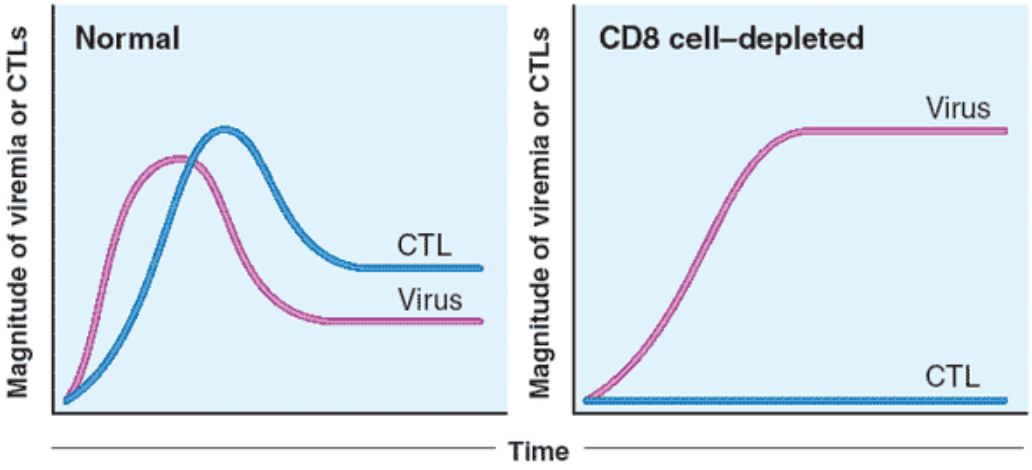


Figure 1.6.2 Effects of CTLs on viremia

Studies of viral escape mutations within immunodominant epitopes furthermore provide the strongest biological evidence for the *in vivo* efficacy of the CTL response during HIV infection. Escape occurs even through single amino-acid mutations in an epitope, at sites essential for MHC binding or T-cell-receptor recognition, but may also be influenced by mutations in flanking regions that affect antigen processing. Viral escape mutations have been associated with the failure to control viraemia in both primary and chronic HIV-1 infection (Borrow et al., 1997, Goulder et al., 1997, Koenig et al., 1995). HIV-1 infection immune-driven evolution occurs as a continuous process occurring throughout the disease course. HIV-1 evolution follows generally predictable patterns when specific immune pressures are applied. The kinetics and patterns of HIV-1 evolution are broadly predictable based on HLA restriction. The majority of persons expressing the "protective" HLA-B*57 allele (Carrington and O'Brien, 2003), for example, will select for a T to N mutation at position three of the TW10 epitope in the p24 Gag protein within the

first weeks after infection (Goulder and Watkins, 2004) and (Leslie et al., 2004). In B*27-expressing individuals, the first mutation that arises in the immunodominant Gag epitope KK10 is an L to M change at position six, followed years later by an R to K change at position two (Kelleher et al., 2001).

However, a multitude of factors influence the frequency and kinetics of viral escape, making the clinical consequences of immune-driven HIV-1 evolution challenging to quantify. Even among individuals expressing the same HLA allele, substantial differences in the magnitude and frequency of epitope targeting are often observed, rendering it problematic to study the clinical consequences of escape in HIV infection. A second complication is the issue of "immunodominance", an incompletely understood phenomenon describing the fact that, despite the expression of up to six HLA class I alleles (and the potential to target multiple epitopes per allele), an individual's CTL response is often initially directed against a single or few epitopes with a single HLA restriction (Yewdell and Bennink, 1999). Also, it is important to bear in mind that escape probably does not occur in isolation, because mutations are likely to be selected in the context of a variety of compensatory changes and at the same time that reversion of transmitted mutations is occurring.

1.7 Antibody responses to HIV-1

During primary HIV infection, antibodies against HIV-1 are detected in most individuals 6-12 weeks post-infection (Pantaleo et al., 1993b). The first antibodies detected recognise linear determinants in HIV-1 structural proteins, such as p24 and p17 in Gag. Thereafter, antibodies to HIV-1 Env and Pol epitopes appear, and also to the HIV-1 regulatory and accessory proteins. Antibody can prevent viral infection through both neutralising and non-neutralising activities. Neutralising antibody is the term used to denote an antibody that prevents infection through its Fab region recognising and blocking functions that mediate virus entry to cells. Protective non-neutralising antibody is the term used to describe an antibody that protects through the action of its Fc region initiating destruction of the offending pathogen by the innate immune response, which may involve the NK cells, and others.

During the first two decades of HIV-1 research, only four bnAbs (broadly neutralising antibodies) capable of neutralizing multiple primary HIV-1 isolates were identified. These antibodies target three sites on the HIV-1 envelope glycoprotein: glycan site on gp120 recognized by 2G12 (Trkola et al., 1996), the CD4 binding site (CD4bs) of gp120 recognized by b12 (Burton et al., 1994), and the membrane proximal external region (MPER) site of gp41 recognized by 2F5 (Muster et al., 1993) and 4E10(Stiegler et al., 2001).

The native form of HIV-1 envelope is thought to be responsible for the inefficiency of neutralising antibodies. The viral envelope is a heavily glycosylated trimeric structure with the CD4 binding site partially masked by the hypervariable V1 and

V2 loop structure (Wyatt et al., 1995). The conserved coreceptor (CCR5) binding site is protected from neutralising antibodies by the V3 loop, and is only accessible for a short time after ligation-induced conformational changes of the gp120 structure (Chen et al., 2005). Consistent with their unusual features, innumerable attempts to elicit these antibodies by vaccination were not successful.

Recently about two dozen bnAbs have been isolated from HIV-1 patients using direct neutralisation screening of individual B cells, designed probe-based B cell isolation, in combination with deep sequencing (Bonsignori et al., 2012a, Kwong and Mascola, 2012). The newly identified antibodies include the two mAbs targeting the CD4bs, VRC01 and VRC02, which were identified by isolation of single mAb-producing B cells against an antigenically resurfaced HIV-1 gp120 specific for the structurally conserved site of initial CD4 receptor binding (Zhou et al., 2010). The new bNAbs have been found to target four major "sites of vulnerability" on the HIV-1 envelope spike: CD4-binding site (CD4bs), the N160 glycan-dependent site associated with the V1/V2 loops, the N332 glycan-dependent site at the base of the V3 loop, and the MPER on gp41.

Figure 1.7 summarises the antibody target sites on the HIV-1 envelope spike (Klein et al., 2013).

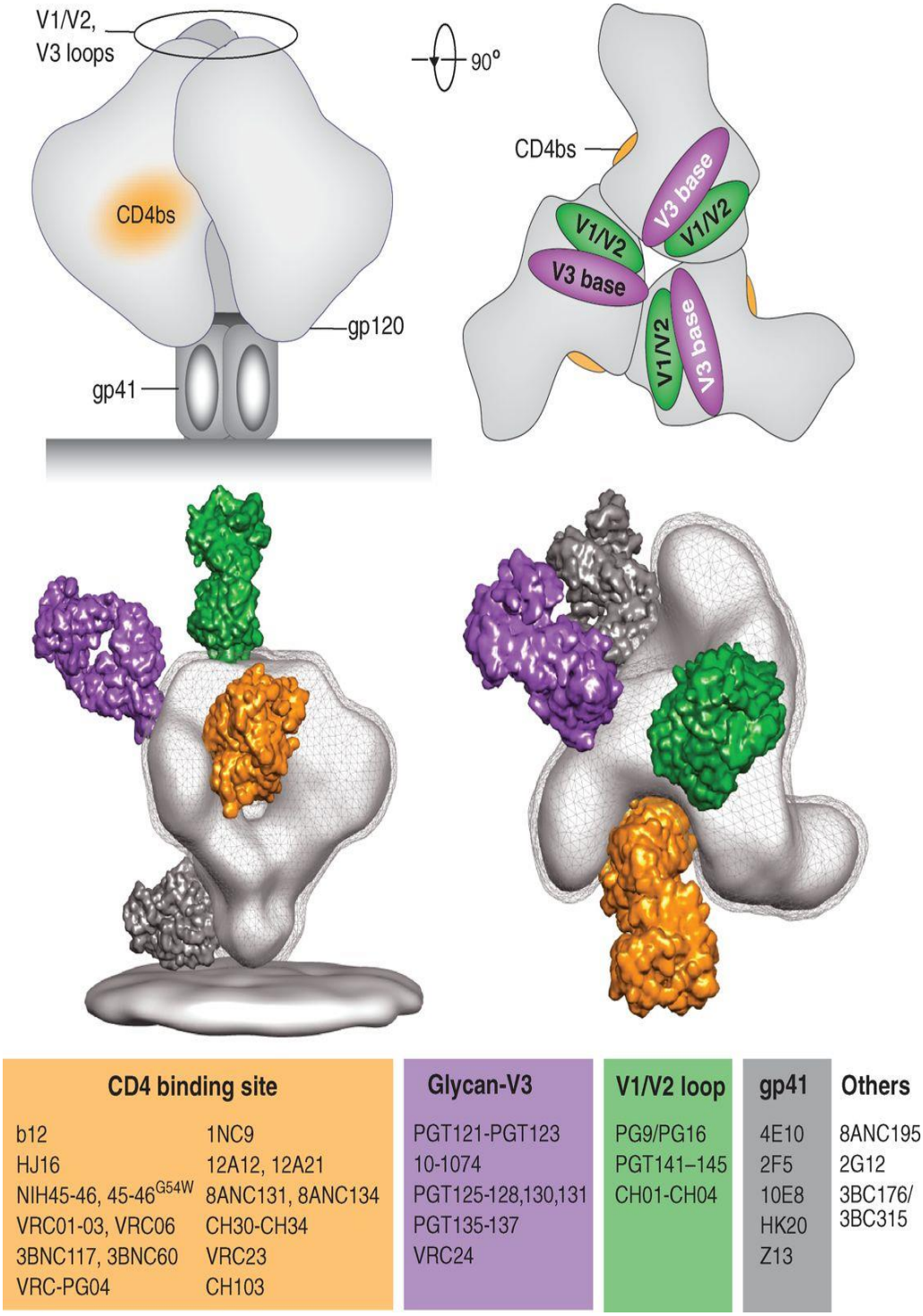


Figure 1.7 Antibody target sites on the HIV-1 envelope spike

Figure 1.7 Legend:

Schematic representation of the HIV-1 envelope spike. Each of the monomer units of the trimer is composed of a gp120 and gp41 transmembrane protein. The four best-characterised broadly neutralising target sites are highlighted and include the CD4-binding site, the glycan-associated epitopes on the base of the V3 loop, the V1/V2 loop, and the MPER on gp41.

The recently concluded RV144 ALVAC-HIV prime / AIDSVAX B/E boost clinical trial demonstrated the first evidence of of vaccine-induced protection from acquisition of human immunodeficiency virus type 1 (HIV-1) infection (Rerks-Ngarm et al., 2009). In secondary analysis, low plasma IgA Env antibody levels in association with high levels of antibody-dependent cellular cytotoxicity (ADCC) were inversely correlated with infection risk (Haynes et al., 2012). Therefore, it has been hypothesised that the observed protection in RV144 is due, in a subset of vaccinees, to ADCC-mediating antibodies.

1.7.1 Antibody-dependent cell-mediated cytotoxicity (ADCC) in HIV

Antibodies that coat an HIV-infected cell can recruit innate immune cells to either kill the HIV-infected cell or otherwise inhibit viral replication. The killing of the HIV-infected cell is referred to as antibody-dependent cellular cytotoxicity (ADCC). The inhibition of viral replication as a result of the death of the HIV-infected cell or other mechanisms is referred to as antibody-dependent cell-mediated virus inhibition (ADCVI). ADCC assays have three components: Target cells that express HIV antigens (CD4+ T cell lines or primary CD4+ T cells), effector cells such as natural killer (NK) cells, and a source of antibody such as serum, plasma, or monoclonal antibodies.

An efficient ADCC response requires three components: infected target cells that are expressing the foreign antigen on their surface, antigen specific antibodies of the IgG isotype, and lastly, effector cells that express Fc-gamma receptors (Evans, Thomson-Honnebier et al. 1989; Weinhold 1991; Moretta, Marcenaro et al. 2008). A major population of ADCC mediating effector cells are the Fc-gamma RIII (CD16) bearing NK cells. Other cells including macrophages, eosinophils and neutrophils can also mediate ADCC, though these cell types normally require a larger antibody titre and higher antigen expression by the target cells (Szelc, Mitcheltree et al. 1992; Jenkins, Mills et al. 1993).

ADCC responses are detected first early in the course of primary HIV-1 infection (Forthal, Landucci et al. 2001). The titres of ADCC antibodies are the highest and the magnitude of the responses the greatest during early infection (Sawyer, Katzenstein et al. 1990). It is known that CD16 which is a key receptor for the induction of ADCC, binds with the highest affinity to the Fc portion of IgG1 and IgG3, and these are the isotypes more frequently induced in HIV-1 infection (Cavacini, Kuhrt et al. 2003). HIV-1 infection can lead to the decreased expression of CD16 by the NK cells, and the overall dysfunction of NK cell-mediated immune activity over time, which is therefore associated with progression of disease (Ratcliffe, Lukey et al. 1994; Alter, Teigen et al. 2005).

Different studies have tried to gauge ADCC activity in different population cohorts. Long-term survivors (LTS) from the Multicenter AIDS (MACS) study had high serum ADCC titres times at most visits, and rapid progressors had little or no serum ADCC activity (Baum, Cassutt et al. 1996). HIV-infected women have been shown to have, in addition to having serum activity, specific ADCC antibodies in their genital fluids (Battle-Miller, Eby et al. 2002). In addition, it was shown that women with cervicovaginal lavage ADCC activity had lower genital tract viral loads than did women with serum ADCC activity only (Nag, Kim et al. 2004). Analysis of Elite Controllers from a multicentre French trial demonstrated that Elite Controllers had higher titres of ADCC antibodies against HIV than viremic individuals (Lambotte, Ferrari et al. 2009).

1.8 Host factors that influence the progression of disease

The course of HIV infection varies widely amongst infected individuals. The median time from infection to the development of AIDS is normally 10 years, but a small proportion of patients, probably no more than 2% to 5%, have been characterised as long-term nonprogressors (LTNPs) (Cao et al., 1995, Pantaleo et al., 1995). The unique immune dynamics of LTNPs provide a rare opportunity to understand the diverse mechanisms at play that halt HIV disease progression. However, a myriad of factors render the exact co-relates of elucidating protection variables very hard to decipher in LTNPs– viral features of specific cohorts might contribute to slow progression (Zaunders et al., 2011), or intrinsic host factors (Lehner et al., 2011), or immunological parameters could promote control of infection (Poropatich and Sullivan, 2011).

The classic example of viral features contributing to non-progression is the LTNP virus attenuation documented in the Sydney Blood Bank Cohort (Crotti et al., 2006, Learmont et al., 1999). This Cohorts' members got infected from blood transfusions from the same donor infected with an HIV-1 strain that housed a large deletion in the long-terminal repeat, extending to a conserved and biologically significant area in the *nef* gene. Six of these subjects went on to become LTNPs and maintained stable CD4+ T cell levels and low viral loads for more than a decade, with only three recipients having maintained undetectable plasma viral loads to-date (Zaunders et al., 2011). There is not enough evidence to demonstrate patterns in attenuating mutations from LTNP virus isolates that could contribute in a major way to virus control. While the nef and vpr mutations cause viral attenuation in some LTNPs and

explain their infection with a less pathogenic virus, screening of larger cohorts reveals that such mutations are infrequently present (Poropatich and Sullivan, 2011). Instead of being solely responsible for HIV-1 disease non-progression, inhibitory mutations in HIV-1 genes probably act in concert with other virological and immunological factors to facilitate control of infection. Also, it is difficult to separate out attenuating mutations that have happened *after* infection, from those that were present at infection.

A number of host factors have also been implicated in disease non-progression in the case of LTNPs (Sobieszczyk et al., 2011). The main way in which host genetics has been statistically associated with controlling HIV-1 progression are genetic polymorphisms that interfere with virus binding to susceptible target cells, either through impaired receptor expression on the cell-surface or an increase in production of competitive inhibitors. Chemokine receptor polymorphisms, the most common example being the CCR5-Δ32 polymorphism (Hutter and Ganepola, 2011), have been implicated for HIV non-progression. A number of studies (Blankson et al., 2007, Cohen et al., 1997) show that LTNPs carry a copy of the 32 bp deletion in the gene, called the CCR5-Δ32 polymorphism, which results in a premature stop codon in the CCR5 gene that causes it to be produced as a truncated protein that is trapped in the endoplasmic reticulum. The frequency of this polymorphism varies amongst different ethnicities. Study results are conflicting with regards to CCRF-Δ32's association with control of HIV-1 infection. Those that argue that it is associated with disease non-progression show that HIV-infected subjects heterozygous for the allele have a significantly slower progression to AIDS, whereas several more studies have found that the polymorphism is absent in their

LTNP cohorts. Variations in CRCR6, CCR2, CX3CR1, CCR2-64I and DARC have also been implicated in the rate of HIV-1 disease progression (Lehner et al., 2008). Although multiple protective effects have not been collectively observed in the same LTNP subject, the combinational effect of some of these polymorphisms would probably provide strong protection from disease progression.

It is likely that immunologic control of the vast turnover of HIV-1 progeny plays a critical role in delayed HIV progression amongst LTNPs. Predominance of specific Human Leukocyte Antigen (HLA) Class I genotypes among LTNPs could lead to a more robust CTL response in these individuals (Bashirova et al., 2011, Carlson and Brumme, 2008, Martin and Carrington, 2005). The exact mechanisms resulting in slow disease progression during HIV infection are still obscure. Most of what is currently known in the field is based on the protective effect of HLA-B*57 and –B*27 (Goulder et al., 1997, Martin and Carrington, 2005). Patients expressing these alleles generated a potent and polyfunctional T-cell response specific to conserved Gag epitopes (TW10 and KK10 respectively) which select escape mutations that induce a fitness cost to the virus (Altfeld et al., 2006, Brumme et al., 2008, Streeck et al., 2007). How these HLA alleles contribute to the control of HIV replication is not well understood, and immune correlates of protection against HIV are still not defined. This again warrants more detailed analysis of further studies recruiting LTNPs in an expanded setting where distinct HLA alleles are associated with protection.

1.9 Specific aims of this study

The overall aim of this project was to look at different immune parameters at play in our unique Chinese SM HIV-1 cohort, which has an unusually high proportion of slow progressors. The unique dynamics of the SM cohort make it ideal for studying immune parameters. Epidemiological and phylogenetic analyses strongly suggest that all cohort members were infected by virus from an unusually narrow source by the same route during the same time period. Due to the very narrow source virus in the cohort, genetic divergence in the subjects reveals how much viral diversity at the population level is attributable to host factors. The aim of these studies is to explore the interplay between immune pressure exerted by CTLs and by NK cells.

Therefore, firstly, we investigated whether there were any specific HLAs that are enriched in our SM cohort. We recruited a control negative cohort, sharing similar Han lineage, for this purpose, and then compared the HLA distributions between the two cohorts using extensive logistic regression analysis.

Next we were interested to determine the effect of CTL on Vpu in our cohort. Vpu has recently been shown to be critical in HIV release from the cell, and not many studies have focused on Vpu. We used our unique SM cohort to investigate whether there was any significant CTL immune pressure being exerted on Vpu in the unique SM setting, and whether any sequence mutations had been selected. In addition to CTL-mediated immune pressure, we were also interested to determine if there was any evidence for NK-mediated selection pressure, by analysing the KIR profile for our SM cohort in relation to the Vpu sequences.

Finally, we conducted analysis of ADCC and ADCVI responses in the SM cohort. We initially screened for ADCC responses directed against Env and Vpu, and then devised a tailored assay for elucidating Vpu ADCC epitopes in our SM cohort. Then we tested to determine whether these patients also exhibited ADCVI activity.

This thesis presents studies of several immune parameters in the SM village cohort, with the aim of inferring the different immune pressures being exerted by the different arms of the immune system.

Chapter 2: Materials and Methods

2.1 Study Cohorts

Two patient populations were used for this study – the HIV Positive Cohort (231 subjects) were recruited from a village in Henan Province, China. HIV negative – control samples (257 subjects) were collected from a neighbouring village where low risk factors for HIV were present.

The HIV Positive Cohort samples were collected from former plasma donors with chronic HIV-1 infection, living in SM village, Henan province, China. This SM village is a close-knit and geographically isolated rural community, with most of the residents having lived there for several generations and have intermarried between families. Between 1993 and 1995, many residents in this village joined a scheme for paid plasma donation, many of whom donated their plasma repeatedly. Most members of the cohort were not aware that they had been infected with HIV-1 until 2004 when large-scale HIV screening programs were initiated in China. We estimate that 407 former plasma donors in SM village acquired HIV infection, based on the identification of 258 HIV-1 infected adults in 2005 and reports of 149 premature adult deaths with symptoms compatible with HIV-1 disease before 2004. HIV-1 infection was not detected in persons residing in the village during 1993-95 that did not donate plasma, suggesting that infection was not easily transmissible through the village by routes not associated with plasma donation. Epidemiological and phylogenetic analyses strongly suggest that all cohort members were infected with

an unusually narrow source virus by the same route during the same time period. Due to the very narrow source virus in the cohort, genetic divergence in the subjects reveals how much viral diversity at the population level is attributable to host factors.

The HIV Negative Cohort comprised age and sex-matched individuals from a neighbouring village which did not take part in the plasma donation scheme. Samples had been collected from the Negative Cohort. Overall, HLA results were conclusively available for 257 subjects from the control cohort.

2.2 Quantification of CD4 counts and viral loads

200µl of heparin-anticoagulated fresh whole blood was analysed for CD3+T/CD4+T/CD8+T lymphocytes percentages and absolute counts by Flow Cytometry using the BD MultiTest IMK kit (BD, USA) and MultiSetTM software (BD, USA). Within the BD MultiTest IMK kit, there were BD Multitest CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC antibodies and BD Trucount tubes. The procedure followed three steps: first, multitest antibodies were mixed with the sample and incubated for 15 minutes in the dark at room temperature, and then samples were transferred to BD Trucount tubes. Then, 450 µl of 1× BD Multitest lysing solution was added to the tube and incubated for another 15 minutes in the dark at room temperature. Finally, samples were sent to Flow Cytometry to calculate CD3+T/CD4+T/CD8+T lymphocyte percentages and absolute counts by using MultiSetTM software.

HIV-1 plasma viral load was quantified by Nucleic Acid Sequence Based Amplification (NASBA). NASBA is a two-step process: an initial enzymatic amplification of the nucleic acid targets, followed by detection of the amplicons. There are several advantages of integrating NASBA with the liposomal nanovesicle diagnostic technology: firstly, NASBA is a completely isothermic reaction (i.e., does not require an expensive thermocycler which facilitates miniaturization). Secondly, the amplicons are single stranded and thus do not require an additional denaturation step prior to detection. Finally, oligo nucleotide probes, coupled to liposome nanovesicles, can hybridize and detect the amplicons at the same temperature used to perform NASBA (i.e., no additional temperature controlling equipment is needed). The lower limit of detection of the assay was 50 RNA copies/ml and samples below the level of detection were assigned an arbitrary value of 30 copies/ml. Plasma HIV-1 viral load was measured in Wu's lab in Beijing Youan Hospital. Please refer to Table 3-1 and 3-2 for a summary of patients' characteristics.

2.3 Extraction of genomic DNA and HLA typing

Genomic DNA was isolated from 3 ml whole blood using the PureGene DNA Isolation kit (Gentra Systems, Minneapolis, MN, USA). 3 ml whole blood was suspended in 9 ml red cell lysis solution in 15 ml Falcon tube for 15 minutes. The tube was inverted several times during this period. Then, cells were centrifuged at 2000 rpm for 10 minutes after the colour of the solution changed to a transparent red. The supernatant was discarded and the remaining cells were washed twice with phosphate buffered saline (PBS) (Oxoid, UK). Approximately 3-5 million PBMCs

were obtained and resuspended in 600 µl cell lysis solution and stored. Samples within cell lysis solution were mixed with 200 µl of protein precipitation buffer, vortexed well and centrifuged at 13,000 g for 15 minutes. The supernatant was decanted into a clean 2 ml vial containing 600 µl of 100 percent isopropanol (Sigma, UK). After the tube was gently inverted for 50 times, genomic DNA was noted floating in the solution and then the tube was centrifuged at 13,000 g for 3 minutes to pellet the genomic DNA. The supernatant in the tube was discarded and genomic DNA was washed with 70 percent ethanol (Sigma, UK). After discarding the ethanol, centrifuging the tube, and removing all the solution in the tube, the genomic DNA was dissolved in 50 µl hydration solution.

Low-resolution HLA molecular typing was performed using Sequence Specific Primers at HIU, WIMM, Oxford by Tim Rostron at the Sequencing Lab.

2.4 Statistical Analysis for HLA correlates

There are 2 main possible methods of analysing the HLA data of SM and control cohorts. The first method, which has traditionally been used in the past, involves splitting the data into a series of 2x2 tables, each consisting of whether or not the subjects carry the allele in question, and carry out a series of Chi-squared or Fischer's tests for differences between cases or controls. This approach requires that each test would be needed to be corrected for multiple tests using the false discovery rate (similar for corrections to the Bonferroni method). The problem with this approach is that when it is used to test allele frequencies it can give spurious results (Sasieni, 1997), as the corrections can potentially create a bias, and make it difficult

to identify genuine associations. Plus, the tests are not independent because each subject has to have 2 alleles from the set.

The second method takes into account the problems associated with multiple testing. The logistic regression analysis (Prentice et al., 1984) enables a global test of association and avoids the problem of multiple testing and dependence between the 2x2 tables. We collaborated with Dr. Ly-Mee Yu (Senior Medical Statistician, Head of NHS Support Team) and Dr Jane Holmes at the Centre for Statistics in Medicine for helping us with the logistic regression analysis. Below are the details of the model used, and the simulation study conducted to reaffirm the robustness of the analytical model.

The Model

Let y_{ij} denote case-control status of an individual with alleles a_i and a_j, and θ_{ij} the probability of being a case. Then,

$$y_{ij} \sim Bernoulli(\theta_{ij})$$

$$\text{logit}(\theta_{ij}) = \mu + \beta_i + \beta_j$$

Assume β_1 is the reference group (matrix is singular so we need a reference group). Then β_j is interpreted as the change in log odds ratio between alleles j and 1 (either $a_1\ a_1$ to $a_1\ a_j$ or $a_1\ a_j$ to $a_j\ a_j$). To see if any alleles are associated with disease status

we can carry out a global test of whether $\beta = 0$ for all β. The test is a likelihood ratio test:

LRS=deviance under the null-deviance under the full model

where deviance = - 2*log likelihood. LRS ~ χ^2 with degrees of freedom given by the number of β parameters fitted in the full model (number of alleles – 1). (Note: we could not use the likelihood ratio test if our model had parameters for all alleles and no mean term, i.e. no reference group, as then the null model would not be nested within the full model.) It does not matter which allele is taken as the reference category for the overall test of difference. The p-value is the same regardless of which allele is taken as reference, but since the model measures contrasts, the significance of the individual regression parameters will differ. This highlights the fact that a global model can find no overall difference even though some parameter contrasts are found to be significant. If the global model found evidence of departure from the null hypothesis of no difference, then we could fit separate models to the alleles and correct for multiple testing. But the model to fit would not be an individual allele analysis but would be a model of either

- No allele, 1 allele, 2 alleles
- No allele, at least one allele

Simulation study

For each of HLA-A, B and C, a simulation study was carried out to compare the different tests. Under a null model, we would expect to find a positive finding when in fact there is no association 5% of the time when using a 95% test.

Simulation

1. Exclude subjects who have a rarer allele (subject excluded if they had an allele that occurred < 10 times) so that there is no issue with small cell counts
2. Assign case/control status to each individual randomly – now we have a null model as there can be no true association
3. Fit a global logistic regression model and test for association
4. Fit allele model: Correct p-values using FDR and Correct p-values
5. Repeat steps 2 – 4 10,000 times and count up the number of times each test is wrong (i.e. finds a significant association when there isn't one – we are expecting this to be 5%)

Results

	Logistic regression	**Allele with FDR**	**Allele with Bonferroni**	**Allele, proportion uncorrected significant p-values**	**Alleles excluded from analysis**
HLA-A	0.057	0.029	0.024	0.034	20, 23, 66, 68, 69, 74
HLA-B	0.062	0.023	0.023	0.036	05, 14, 18, 45, 47, 49, 50, 53, 56, 59, 67, 81
HLA-C	0.056	0.043	0.029	0.037	05, 16, 18

2.5 Extraction of RNA for Vpu Analysis

HIV RNA was extracted from Plasma samples from the HIV Positive Patients in our Chinese Cohort recruited in 2008 using the QIAamp Viral RNA Mini-Kit. We used random sampling for the recruitment of the 90 subjects used for the Vpu analysis. Stored Plasma samples were used for RNA extraction using the QIAamp Viral RNA Kit (Qiagen). Briefly, 140 µl of plasma was added to the prepared Buffer AVL-carrier RNA in a microcentrifuge tube. After pulse-vortexing for 15 sec and an incubation at room temperature for 10 min, 560 µl of ethanol was added to the sample, followed by pulse-vortexing for 15 sec. Then using the supplied QIAamp Mini column (in a 2ml collection tube), the solution is centrifuged at 8000 rpm, with the filtrate being discarded. Then 500 µl of Buffer AW1 is added to the QIAamp Mini column and centrifuged. Subsequently, 500 µl of Buffer AW2 is added and centrifuged at full speed for 3 min. At the end, 60 µl of Buffer AVE equilibrated to room temperature is added and centrifuged to deposit the RNA as the filtrate in the centrifuge tube.

2.6 Primers and Cycling Conditions for Vpu analysis

Inner and Outer Primers were designed using the software Oligo (Version 7).

Outer Primers Vpu F_{out} (AGATTAGAGCCCTGGAAGCATCC)and

Vpu R_{out} (TACACAGGCATGTGTGGCCC); and Inner Primers

Vpu F_{in} (CATCTCCTATGGCAGGAAGAAG) and Vpu R_{in} (GTACCCCATAATAGACTGTGACCC) were employed in a Nested PCR Protocol. Nested PCR was used to ensure specificity of the target Vpu part of the

genome, whose short sequence length demands that the sequence generated is free from any potential errors and any mutations taking place are picked up precisely at every possible locus.

Cycling conditions after optimisation for the first round were: 94°C for 2 min; 25 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 45 sec; 72°C for 10 min. Thermocycling conditions for the second round were 94°C for 2 min; 20 cycles of 94°C for 15 sec, 54°C for 30 sec, 72°C for 30 sec; 72°C for 7 min. The amplified products were detected on a 1.8 % agarose gel and visualized by ethidium bromide staining.

PCR optimisation included changing the annealing temperature, and trying to progressively decrease the amount of enzyme used per reaction. As the viral load from the plasma samples varied quite a lot, it was quite a challenge at times to extract the RNA from samples with very low viral loads, requiring repeated rounds of extraction from samples and Nested PCR. At the end after optimisation and removal of variables, the reaction mixture of 25 μl for both first and second round PCR contained 1.0 mM MgCl2, 200 μM dNTPs, and 0.5 U of enzyme. The first round PCR was performed with 1.0 μM of primers (Vpu F_{out} and Vpu R_{out}) and the second round employed 1.5 μM of primers (Vpu F_{in} and Vpu R_{in}).

After visualisation of the bands on a 1.8% agarose gel, the PCR products were purified using a DNA Purification Kit before being sent for sequencing.

2.7 Vpu Sequence and phylogenetic Analysis

Sequencing was done by Tim Rostron at the Sequencing Lab at the WIMM. After the sequences were received back, they were cleaned using the software BioEdit (Version 7.0.9). Thereafter, the software ClustalW was used for multiple sequence alignment. The maximum-likelihood method was used for the phylogenetic analysis. A series of individual programmes from PHYLIP and PAML were employed to calculate the maximum-likelihood. The DNAML programme from PHYLIP (http://evolution.genetics.washington.edu/phylip/ progs.data.dna.html) was used to estimate phylogenies from nucleotide sequences by maximum likelihood. Through computing the probability of an alignment for a fixed tree, the maximum-likelihood tree was selected and identified. Finally, the maximum-likelihood tree was presented by using treeview software which was a simple program for displaying phylogenies on Apple Macintosh and Windows PCs and downloaded from http://taxonomy.zoology.gla.ac.uk/rod/treeview.html.#

After alignment of the sequences and making sure they were all in-frame, the sequences were translated using the ExPaASY Proteomics Server, which is available at:

http://web.expasy.org/translate/

The translated sequences were then compared and mutations noted in the different patients for the full domain of the Vpu protein. Firstly, a univariate analysis of correlations between the presence or absence of HLA alleles and the presence or absence of amino acids at every position in the HIV-1 Vpu region, using already published methods was conducted (Dong, Zhang et al. 2011), correcting for multiple comparisons. Next, for those amino acids for which there was at least one correlated

HLA allele, we conducted multivariate analysis with logistic regression for each residue to assess the statistical significance of association between the presence of a polymorphism (defined as any substitution of the consensus amino acid) and the HLA allele associated with it.

The translated sequences were also used to generate a consensus VPU translated sequence for our Chinese Cohort. This consensus was generated using the software BioEdit and also the consensus generator tool available on the Los Alamos website. The consensus was then used to formulate 9 peptides that span all of VPU. These peptides were then used for the screening ELISPOT analysis.

2.8 IFN-γ ELISPOT

Freshly isolated PBMCs or frozen PBMCs were used in ex vivo IFN-γ enzyme-linked immunospot assay (ELISpot) assays to detect antigen-specific T cell responses. After thawing, the viability of these frozen cells was determined by tryphan blue dye exclusion. Cell suspension was mixed with 0.04% trypan blue dye, and live and dead cells were then counted on a haemocytometer. Next these cells were left overnight at 37 degrees, and the following morning the cells were stained again and their viability checked again with tryphan blue staining. Thereafter, the cells were then used for ELISPOT experiments. Assays were done in ninety six-well MultiScreen filter plates (MAIP S45, Millipore, MA) coated with capture antibody, 15 µg/ml of anti-IFN-γ monoclonal antibody (1-DIK, Mabtech, Sweden), overnight at 4℃. The second day, coated 96-well MultiScreen filter plates were washed with RPMI 1640 six times and blocked with blocking solution, 10% FCS-RPMI (R10), 200 µl/well and left for 1 hour at room

temperature. Frozen PBMCs were used.. Cells were counted and made up at the appropriate dilutions (1 x 10^6 fresh PBMCs/ml, or 2 x 10^6 frozen PBMCs/ml) in R10. After discarding the blocking solution, PBMCs were aliquoted into plates at 0.2 x 10^6 frozen PBMCs/well in a volume of 100 µl and stimulated with sterile stimulator peptide (overlapping peptides or optimal epitopes) at a final concentration of 20 µM. R10 alone and 20 µg/ml phytohemagglutinin (PHA, final concentration 2µg/ml) (Murex Biotech Ltd, Dartford, Kent, UK) were used as negative and positive controls, respectively. After 16 hours incubation at 37 centigrade, 5% CO2, plates were washed six times with phosphate buffered saline (PBS)-0.05% (v/v) Tween-20 (Sigma, UK) and 50 µl of detector antibody at a concentration of 1 µg/ml, biotinylated anti-IFN-γ mAb (Mabtech, Sweden), was added and incubated for 3 hours at room temperature. Plates were then washed six times with PBS/0.05% (v/v) Tween-20 and coated with conjugate antibody, streptavidin-conjugated alkaline phosphatase (Mabtech, Sweden), and incubated at room temperature for 1 hour. Finally, plates were washed six times with PBS/0.05% Tween-20 and developed with alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA) to detect individual cytokine-producing cells. As for the definition of a positive response, a cut-off classification was chosen in which responses were regarded as positive if they had at least three times the mean number of SFU in the two control wells and had to be >50 SFU/10^6 PBMC (Addo et al., 2003), in order to compare any results to published literature. None of our responders had any responses to Vpu peptides when we did the screening ELISPOTs.

2.9 KIR genotyping

Genomic DNA was genotyped for the presence or absence of the following 15 KIR genes: 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, and 2DP1. Genotyping was performed by using PCR amplification with two pairs of primers specific for each locus (PCR-SSP) as previously described (Martin and Carrington, 2008). This technique uses specific primers to amplify two segments of different sizes from the same KIR gene to see if it is present. The fragments were then stained with ethidium bromide during electrophoresis in 2% agarose gel. Then the specific bands were visualised on a UV light box, an electronic picture of the gel was taken and scored for the presence or absence of specific bands. Assays with discrepant results (i.e. one primer pair is positive while the other is negative) were repeated and the gene was considered present if one of the reaction pairs was consistently positive. The purpose of using two pairs of primers to detect the same gene was to limit false negative results as much as possible. The absence of specific bands on both reactions was confirmed by repeating the typing to ensure that the gene was definitely absent. Each reaction also contained a pair of internal control primers amplifying a 796-bp fragment from the third intron of HLA-DRB1 gene to check for PCR efficiency. The amplification conditions are:

1 cycle	94 ºC	3:00				
5 cycles	94 ºC	0:15	65 ºC	0:15	72 ºC	0:30
21 cycles	94 ºC	0:15	60 ºC	0:15	72 ºC	0:30
5 cycles	0:15	55 ºC	1:00	72 ºC	2:00	
1 cycle	72 ºC	7:00				

2.10 Preparation of Peripheral Blood Mononuclear Cells

Venous blood was drawn into collection tubes containing heparin and separated by Ficoll-Hypaque density centrifugation. Whole blood was diluted in the same volume of serum-free RPMI 1640 (R0) (Sigma, UK), and carefully layered on Ficoll-Hypaque (Sigma, UK). The layered blood was centrifuged without braking for 20 minutes at 2000 rpm. The cells at the interface were carefully removed to a new 15ml centrifuge tube and washed twice with serum-free RPMI 1640 by centrifugation at 1500 rpm for 10 minutes. After that, cells were resuspended in 10 ml R10 medium (RPMI 1640 (Sigma) supplemented with 10% Foetal Calf Serum (FCS; Sigma, UK), 2 mM L-glutamine and 50 U/ml penicillin/streptomycin). 10 µl cell suspensions were mixed with an equal volume 0.2% (w/v) trypan blue for 2 minutes, and dropped into a haemocytometer to quantitate. The purified cells were either used immediately in an assay or frozen by using cold freezing media (Foetal Calf Serum with 10% dimethyl sulphoxide (DMSO)) and then frozen slowly in a -80℃ freezer using a Mr FrostyTM (Nalgene) box and then stored long term in liquid nitrogen for later use.

2.11 Thawing frozen Peripheral Blood Mononuclear Cells

Frozen PBMCs in a cryovial were put in a 37℃ water-bath until a small lump of ice remained. Then, the vial was sprayed and wiped with alcohol before being transferred into hood. PBMCs from the vial were transferred into a 15 ml Falcon tube using a micropipette. After that, R10 was added 5 drops at a time, stirring frequently for the first 5ml; then topped up to 10ml. Cells were then centrifuged at

1400 rpm for 5 minutes, the supernatant discarded, and the cells were resuspended in 5 ml R10. Finally, cells were counted using a haemocytometer.

2.12 A library of Env and Vpu Peptides for the ADCC assay

211 overlapping Env peptides and 19 Vpu peptides for HIV-1 Subtype B were ordered from the NIAID (the AIDS Reagent Program). Peptides were dissolved in DMSO (Sigma) at 100 mg/ml and diluted with RPMI 1640 (20 mg/ml, long-term stock stored at -80℃) before being individually filtered and diluted to be used at a final concentration of 3 µg/ml in the ADCC assays. As a high DMSO concentration is cytotoxic, the final DMSO concentration in any pool was always ≤0.04%.

2.13 ADCC-ICS assay

The ICS-based assay that we employed in our lab in Oxford is used to measure HIV Ab-mediated NK cell cytokine expression and degranulation, provided through a collaboration with the Kent Lab in Melbourne, Australia, which has pioneered the study of ADCC to HIV-1 peptides. In brief, using either 200 uL of fresh whole blood or 50 uL of patient anticoagulated plasma, patient samples are mixed with 150 uL of healthy donor blood and incubated with the pool of overlapping Vpu peptides for 5 hours in the presence of Brefeldin A and Monensin (Sigma). At the end of the incubation CD56+ CD3− or CD2+CD3− NK lymphocytes are studied for the expression of intracellular IFN-γ and surface CD107a. Fluorescent antibodies used in the ICS assays are CD3 (catalog no. 347344, fluorescent label PerCP); CD2 (catalog no. 556611, FITC); CD56 [catalog no. 555516, phycoerythrin (PE)]; CD8α

(catalog no. 335787 PE-Cy7); CD107a [catalog no. 624078, adenomatous polyposis coli (APC)]; and IFN-γ (catalog no. 557995, Alexa700). The Fluorescent antibodies used in the ICS assays are all available from BD Biosciences. The assay helps us to identify the regions of Vpu that are recognised by ADCC responses in the Chinese SM cohort. The assay uses neat plasma, but we modified the assay using dilutions of plasma in some analysis for a unique SM cohort Vpu/Env ADCC responder. This ICS based assay helps to overcome some of the variability issues associated with past ADCC assays, and the major advantage of this ICS based assay, in addition to its being less labour intensive and less time-consuming, is the ability to use small peptides or overlapping peptide pools as the antigen, which allows short linear epitopes of ADCC antibodies to be delineated (Chung et al., 2011b). Therefore, the difficulty in mapping epitopes with past standard assays, has been overcome.

2.14 ELISA for screening Vpu epitopes

We optimised an ELISA-based approach to identify Vpu epitopes in our SM cohort subjects. Briefly, this involved coating single wells of 96-well half-area, high-binding polystrene microplates with 50 ul of each peptide at 40 ug/ml in PBS and leaving them overnight at 4 degrees. The next day, the wells were then blocked at with 150 uL of 5% nonfat powdered milk in PBS at 37 degrees for I hour. Plasma at a dilution of 1:50, was then added to each well and incubated at 37 degrees for 1 hr. Wells were then washed five times with PBS 0.05%-Tween 20 (PBS-Tween), and 50 ul of horseradish peroxidase (HRP)-conjugated goat antihuman IgG antibody diluted in 1:1000 in 5% nonfat powdered milk in PBS was added to each well for further incubation at 37 degrees for 1 hr. The Plates were then washed 10 times with

PBS-Tween and developed with 50 ul of soluble tetramethylbenzidine reagent. The reaction was stopped by the addition of 50 ul of acidic solution, and the optical density at 450 nm was measured.

2.15 ADCVI assay

Target cells for the ADCVI assay were prepared by infecting polybrene-treated CEM.NKR-CCR5 cells (National Institutes of Health AIDS Research and Reference Program) with a laboratory strain of HIV-1 (HIV NL4-3) at a multiplicity of infection of 0.05. After 48 h, target cells were washed to remove cell-free virus. Effector cells (PBMCs from healthy donors) were added to target cells at an E:T ratio of 10:1. Test sera were added to target and effector cells to achieve a final dilution of 1/100. Seven days later, supernatant fluid was collected, and p24 was measured by ELISA (Cambridge BioScience). The percent inhibition due to ADCVI was calculated relative to pooled HIV-negative serum as follows: percent inhibition = 100(1 − ((p24p)/(p24n))), where (p24p) and (p24n) are concentrations of p24 in supernatant fluid from wells containing a source of HIV-positive or -negative Ab, respectively. Individual sera were assayed in triplicate using a single donor. Each serum was assayed in two separate assays with different donors, and the mean percent inhibition was calculated. In general, values from the two assays were in close agreement with each other. Specimens whose SD was ≥20% were repeated a third time, and the mean of all three assays was used. A32 antibody – courtesy of Prof. Barton Haynes at Duke, was used as a positive control in optimising our ADCVI assay in the laboratory.

Chapter 3: HLA correlates in a cohort of Slow Progressors from China: effects on HIV-1 disease progression.

3.1 Introduction

There have been major milestones in HIV research in recent years (Fauci, 2006). Yet, the ultimate goal for a vaccine remains as elusive as ever. The recent relative success of the Thai Vaccine trial (Rerks-Ngarm et al., 2009) and failure of the STEP trial (Buchbinder et al., 2008) have prompted renewed calls for a 'back to basics' approach. This premise dictates a greater and clearer understanding of the various immune parameters at play in HIV infection, for instance, why some individuals progress rapidly towards AIDS and others survive as long as 15 years without developing symptoms is still not clear.

Multiple host, environmental and virological factors account for the heterogeneity in HIV infection susceptibly and disease progression. Most importantly, host genetic factors have been shown to have a profound impact on antigen presentation and immune activation, thereby greatly influencing HIV pathogenesis and progression to AIDS. Two main families of host genes have been shown to influence the course of HIV infection: chemokine receptors and Human Leukocyte Antigens (HLA). More specifically, the HLA class I region on chromosome 6, the most polymorphic locus in humans (for which 4,269 different HLA-A, HLA-B, and HLA-C molecules have so far been described), has a stronger association with HIV disease outcome than any other groups of genes (Piacentini et al., 2009).

HLA Class I loci (HLA-A, -B and –C) produce cell surface molecules that present peptides to cytotoxic T-cells (CTLs) to stimulate clearance of virus-infected host cells(Klein and Figueroa, 1986). Class II are responsible for notifying helper T-cells of their role in antibody production, mostly against parasites or bacteria. The maximal allelic variation displayed by Class I is thought to be maintained by co-evolutionary processes whereby increased HLA diversity is favoured by the natural selection pressure of periodic infectious disease outbreaks to ensure maximal recognition of diverse pathogen repertoire (Parham and Ohta, 1996).

A number of genome wide association studies (GWAS) have shown that single-nucleotide polymorphisms (SNPs) within the HLA Class I region have the strongest genetic association with durable control of HIV (Dalmasso et al., 2008, Fellay et al., 2007, Limou et al., 2009). These initial GWAS studies clearly showed that the major genetic signal associated with a viral set point is exclusively within the HLA and there are no other genetic associations that reach statistical significance, adequately demonstrating the sense of the magnitude of the HLA effect.

More recently, a stronger view of the genetic impact of HLA on viral control arose from coupling GWAS data with sequencing within the HLA locus of multi-ethnic HIV-1 controllers and progressors (Pereyra et al., 2010). This showed that the most significant independent genetic associations with viral control relate to specific amino acids within the HLA-binding groove that are involved in binding to the viral peptide.

However, the exact mechanisms underlying how specific HLAs influence the course and outcome of HIV infection are still being unravelled, and need further scrutiny. Several alleles are associated with slow disease progression as their frequencies are increased in individuals who remain asymptomatic over 10 years after seroconversion with low or undetectable viraemia (Casado et al., 2010). Those long-term non progressors (LTNPs) maintain normal blood CD4 counts (> 500 cells/ml) and therefore survive longer than most individuals following HIV infection. HLA-B*5701 and HLA-B*2705 are the two main alleles consistently associated with protection in Caucasians. B*57 and B*27 individuals exhibit better (CTL) cytotoxic T cell responses than those with other HLA types which target conserved immunodominant HIV epitopes making it difficult for the virus to escape. In addition to those protective B alleles, Class I heterozygosity correlates with delayed onset of AIDS, probably as a result of enhanced diversity of viral peptides being presented to T and NK cells (Carrington et al., 1999).

To date, the majority of HIV-1 host genetic association studies have recruited patients of Western European ancestry, which are not representative of populations worst afflicted by the HIV pandemic (Gotch et al., 2000). This prompts further studies in these populations based not on just ethical reasons, but more crucially, expanding the population net increases likelihood for discovering new genetic links and potentials for therapy.

Long-Term non-progressors (LTNPs) normally make up between 2 and 5% of all HIV-seropositive individuals (Klein et al., 1998). Their unique immune dynamics provide a rare opportunity to understand the diverse mechanisms at play that halt

HIV disease progression. However, a myriad of factors render the exact correlates of immune protection variables at play in LTNPs very hard to decipher – viral features of specific cohorts might contribute to slow progression, or intrinsic host factors, or immunological parameters could promote control of infection.

The mechanisms resulting in slow disease progression during HIV infection are still obscure. Most of what is currently know in the field is based on the protective effect of HLA-B*57 and –B*27 (Goulder et al., 1997, Migueles et al., 2000). Patients expressing these alleles generate a potent and polyfunctional T-cell response specific to conserved Gag epitopes (KW10 and KK10 respectively), in which T-cell escape mutations would induce a fitness cost to the virus (Miura et al., 2009). How some HLA alleles contribute to the control of HIV replication is not well understood, and immune correlates of protection against HIV are still not defined. This again warrants more detailed analysis for further studies recruiting LTNPs from an expanded setting where different HLA alleles may be associated with protection. It is increasingly clear that the way around those hurdles is to study populations of various ethnicities. As host genetic factors are differently regulated in different populations, such a strategy could highlight the contribution of new factors in HIV control.

In line with this, we sought to identify new protective HLA alleles in our Han Chinese population cohort. We studied a cohort of closely related individuals in rural China who were infected with a very narrow range of clade B HIV-1 strains following plasma donation in the early 90s (Dong et al., 2011). Most members of this cohort did not get anti-retroviral therapy (ART) during the first ten years of

infection, therefore the confounding effect of ART on the outcome of HIV infection is virtually negligible. Information on the events that took place during that 10-year period is virtually non-existent, however there are records of several individuals having died after displaying symptoms compatible with AIDS. This suggests that from the original group of infected subjects, only Slow Progressors would have survived to date, which makes this cohort a unique setting for studies aimed at identifying factors influencing the course of HIV infection.

In order to identify HLA protection correlates in this Slow Progressors cohort, we also recruited individuals from a neighbouring village sharing similar Chinese Han lineage and hypothesised that the distribution of HLA alleles in this population would be similar to that of our Slow Progressors cohort before infection.

We therefore compared the HLA distribution of the two populations (HIV+ and HIV-) to identify which HLA alleles were enriched in subjects which had survived more than 10 years after HIV infection without ART. We hypothesised that the enrichment of some HLA alleles in the Slow Progressors (HIV+) cohort would be the result of either a survival advantage or increased susceptibility to HIV infection.

In conjunction with clinical data and using stringent statistical tests, we also sought to determine which HLAs might have influenced the clinical course of infection in this particularly unique setting. This is to our knowledge the most detailed and stringent study of HLA distribution in the Chinese HIV population in the Slow Progressors setting.

3.2 Results

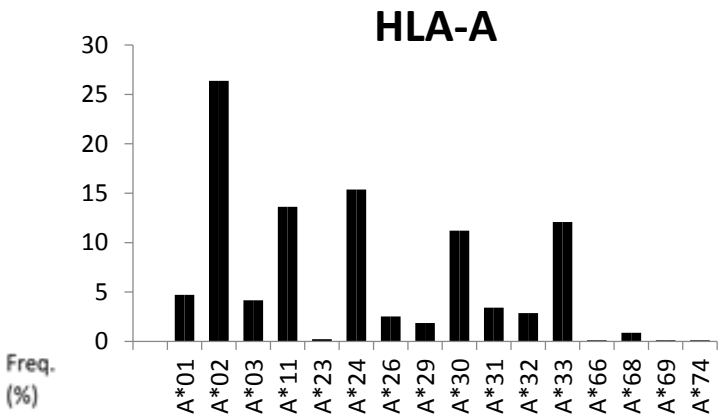
Frequencies were first computed combining both the HIV+ and HIV- cohorts to give us a glimpse of the HLA distribution in our sampled Han Chinese population. These are depicted in Figure 1 on the next page.

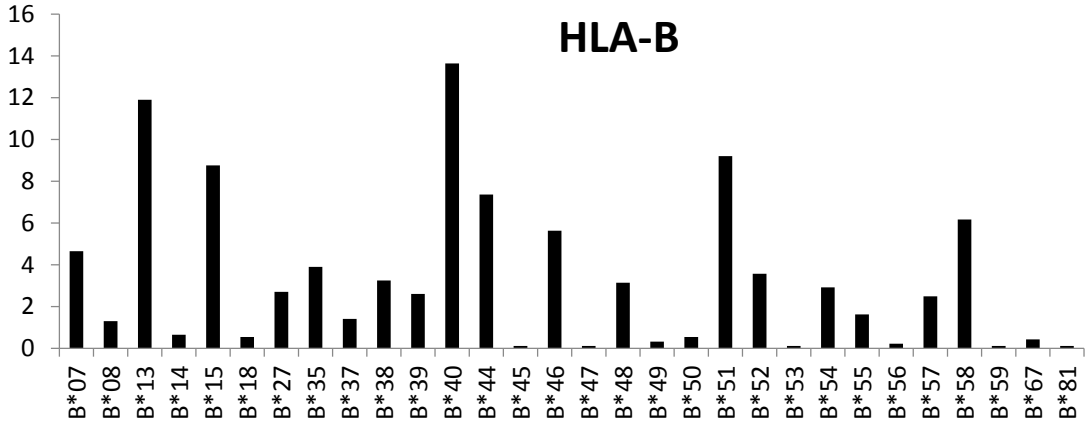
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After this, frequencies were then analysed for the two cohorts (HIV positive and HIV negative) separately. These are detailed in Table 1.

Figure 1:

Distribution of HLA-A, HLA-B and HLA-C in our sampled Chinese Han population (both HIV+ and HIV- cohorts). The y-axis represents the frequency % of the respective alleles which are listed on the x-axis





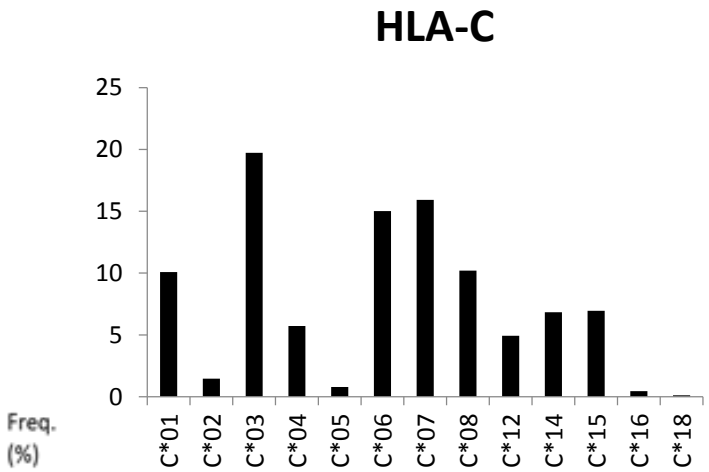


Table 1: Frequencies of HLA-A, HLA-B and HLA-C for HIV-negative and positive cohorts

Allele	HIV-negative population (N = 257)		HIV-positive population (N = 231)	
	Proportion	Number	Proportion	Number
A*01	0.0331	(17)	0.0606	(28)
A*02	0.2646	(136)	0.2944	(136)
A*03	0.0331	(17)	0.0476	(22)
A*11	0.1401	(72)	0.132	(61)
A*20	0	(0)	0.0022	(1)
A*23	0.0019	(1)	0.0022	(1)
A*24	0.1556	(80)	0.1537	(71)
A*26	0.0311	(16)	0.0152	(7)
A*29	0.0136	(7)	0.0195	(9)
A*30	0.1128	(58)	0.0974	(45)
A*31	0.0350	(18)	0.0281	(13)
A*32	0.0233	(12)	0.0325	(15)
A*33	0.1304	(67)	0.1039	(48)
A*66	0.0019	(1)	0	(0)
A*68	0.0117	(6)	0.0043	(2)
A*69	0.0019	(1)	0	(0)
A*74	0.0019	(1)	0	(0)
B*05	0	(0)	0.0022	(1)
B*07	0.0467	(24)	0.0411	(19)
B*08	0.0097	(5)	0.0173	(8)
B*13	0.1401	(72)	0.1017	(47)
B*14	0.0078	(4)	0.0043	(2)
B*15	0.1109	(57)	0.0714	(33)
B*18	0.0058	(3)	0.0043	(2)
B*27	0.0195	(10)	0.0346	(16)
B*35	0.0292	(15)	0.0476	(22)
B*37	0.0136	(7)	0.0152	(7)
B*38	0.0331	(17)	0.0281	(13)
B*39	0.0233	(12)	0.026	(12)
B*40	0.1342	(69)	0.1407	(65)
B*44	0.0895	(46)	0.0541	(25)
B*45	0.0019	(1)	0	(0)

B*46	0.0506	(26)	0.0606	(28)
B*47	0	(0)	0.0022	(1)
B*48	0.0214	(11)	0.039	(18)
B*49	0	(0)	0.0065	(3)
B*50	0.0039	(2)	0.0065	(3)
B*51	0.0837	(43)	0.1017	(47)
B*52	0.035	(18)	0.0346	(16)
B*53	0.0019	(1)	0	(0)
B*54	0.0253	(13)	0.0281	(13)
B*55	0.0175	(9)	0.013	(6)
B*56	0.0019	(1)	0.0022	(1)
B*57	0.0156	(8)	0.039	(18)
B*58	0.0603	(31)	0.0584	(27)
B*59	0.0019	(1)	0	(0)
B*67	0.0058	(3)	0.0022	(1)
B*81	0.0019	(1)	0	(0)
C*01	0.0973	(50)	0.1039	(48)
C*02	0.0097	(5)	0.0173	(8)
C*03	0.2237	(115)	0.1883	(87)
C*04	0.0545	(28)	0.0584	(27)
C*05	0.0078	(4)	0.0065	(3)
C*06	0.1498	(77)	0.1429	(66)
C*07	0.1693	(87)	0.1558	(72)
C*08	0.0817	(42)	0.1169	(54)
C*12	0.0467	(24)	0.0498	(23)
C*14	0.072	(37)	0.0584	(27)
C*15	0.07	(36)	0.0541	(25)
C*16	0.0078	(4)	0	(0)
C*18	0.0019	(1)	0	(0)

After this, we tested our hypothesis as to whether there were any significant differences between the distribution of the alleles between the HIV+ and HIV- populations. This analysis was conducted using a logistic regression model, which was specifically designed for this analysis and tested to check for stringency and accuracy (Simulation studies detailed in the Methods Section). Essentially, the model compares the different HLA alleles present in the two groups to determine if there are any statistically significant differences present. No statistically signiificant differences were found ($p<0.5$) using our regression model, which has been discusssed in detail in Chapter 2.

Table 2: Overall test of association (subjects with missing information on the gene being tested are excluded)

	HLA-A	HLA-B	HLA-C
Number of cases included	229	225	217
Number of controls included	255	255	255
Likelihood ratios statistic	20.15	37.96	14.53
Degrees of freedom	16	29	12
p-value	0.21	0.12	0.27

In order to make sure that relatively rare alleles were not biasing our results, we tried to combine the rare alleles and conducted the regression analysis again, as demonstrated in Table 3 below. Again, no significant differences were obtained ($p<0.5$).

Table 3: Combining rare alleles (alleles that appear less than 10 times). Overall test of association (subjects with missing information on the gene being tested are excluded)

	HLA-A	HLA-B	HLA-C
Number of cases included	229	225	217
Number of controls included	255	255	255
Number of rare alleles combined	6	11	3
Likelihood ratios statistic	14.80	25.55	10.15
Degrees of freedom	11	19	10
p-value	0.19	0.14	0.43

After comparing the Slow Progressors cohort with the control cohort, we were also interested to see if there would be any correlation betwen the HLA distribution, and progression of disease amongst the Slow Progressors.

We now analysed subjects in the Slow Progressors cohort, and CD4 counts were measured in a random sample of subjects in 2007 using convenient sampling. None of these 94 subjects had received ART.

Of these 94 subjects with information on CD4 count, HLA data was available for 84 subjects– these were then used to determine if there were any associations of specific HLAs with disease progression. Table 4 lists the CD4 counts and viral loads of these 84 subjects – no specific associations were noted. The statistical model fitted here is the same as in the case-control analysis, except now the y-variable is CD4 and the distribution is normal (i.e. linear regression instead of logistic regression). Again, we used the likelihood ratio test to test the hypothesis of no difference between the alleles. No correlation of any alleles was noted with the CD4 data of the Slow Progressors – Table 5.

Table 4: CD4 counts and Viral loads for HAART-naïve subjects in the SM cohort. Samples were collected in 2007.

Patient ID	Viral Loads	CD4 Counts	Patient ID	Viral Loads	CD4 Counts
7	35	239	325	21000	340
8	23000	370	327	130000	658
11	35	645	328	5800	976
14	100000	113	331	2200	157
19	650000	698	334	35	700
22	1400	995	335	79000	301
23	28000	550	338	55000	391
34	5200	250	342	150000	222
35	85000	424	345	14000	596
36	360	872	346	35	587
40	42000	248	349	820	378
43	350000	585	351	280000	250
47	88000	143	353	130000	169
56	35	724	356	200000	238
59	9000	332	358	35	736
60	3100	763	359	4000	649
64	86000	460	360	110000	334
65	830000	72	363	640	580
166	11000	590	364	35	1030
167	44000	761	373	23000	464
170	35	319	376	35	755
176	12000	621	377	50000	308
178	35	647	379	35	570
179	58000	56	381	140000	106
180	35	408	384	35	877
181	300000	252	403	160000	225
185	1900	497	411	35	1214
188	690	449	414	35	683
193	30000	239	417	170000	40
194	35	1039	425	35	590
196	8100	542	426	750	765
197	35	624	427	1100000	8
199	35	513	429	250000	87
209	35	508	434	35	1007
210	35	609	435	49000	238
211	35	911	437	140000	150
215	200000	514	441	43000	143
217	1400	392	446	14000	421
222	35	679	450	1000	406
232	35	1019	452	13000	502
321	12000	38	457	980	397
324	20000	174	459	180000	222

Table 5: Co-relating CD4 clinical date with alleleic differences. Overall test of association (subjects with missing information on the gene being tested are excluded)

	HLA-A	HLA-B	HLA-C
Number of subjects included	84	82	77
Likelihood ratios statistic	12.49	23.51	8.15
Degrees of freedom	11	22*	10
p-value	0.33	0.37	0.61

Next we analysed in detail the Elite Controllers amongst our Slow Progressors cohort. These were defined as individuals who had who had been infected for >10 years and had repeated undetectable viral loads (Assay lower limit of detection was 50 RNA copies/ml) in the absence of ART.

The limited sample size meant of the Elite Controller data set (22 individuals) meant that we could only use descriptive statistics for this analysis. This is represented in figure 2 on the following page.

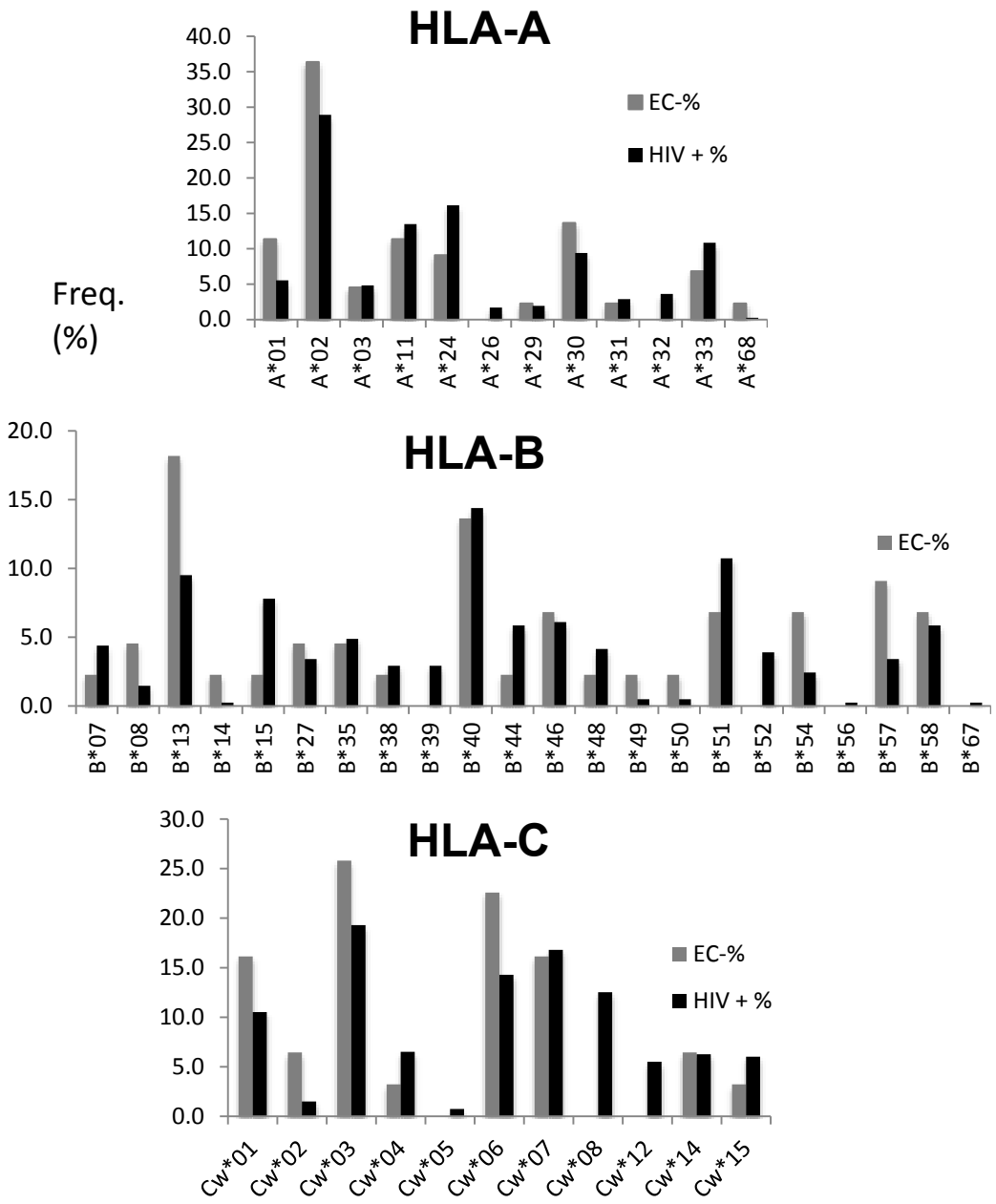


Figure 2:

Distribution of HLA-A, HLA-B and HLA-C in 22 Elite Controllers (EC) and the HIV Positive Cohort. The y-axis represents the frequency % of the respective alleles which are listed on the x-axis

3.3 Discussion

There has been very little reported about HIV LTNP and Slow Progressor Cohorts from China, with some recent studies looking at HIV-infected cohorts (Li et al., 2007, Huang et al., 2009b, Liu et al., 2012). Most studies in the past have focused on the HIV correlates of the Caucasian population (Poropatich and Sullivan, 2011, Beattie et al., 2002, Buchbinder and Vittinghoff, 1999), therefore limited information is available regarding other ethnic groups, particularly populations from the Far East. Our study helps to bridge this gap, and add to the HLA dataset available for China.

Combining all the HLA data from all our study gives us a glimpse of the HLA distribution in our sampled Chinese Han Population (Figure 1). Predominant HLA-A alleles in order of decreasing frequency are A*02, A*24 and A*11. For HLA-B, B*40 was the most common allele, with B*13, B*51 following behind. HLA-Cw*03 was by far the most commonly present HLA-C allele. Analysing the HIV+ and HIV- groups separately reveals a very similar picture – the only major difference being that HLA-B*13 is more common than B*40 in the Control Cohort.

The HLA profile data of our cohorts is similar to those of previous published reports on the HLA gene distribution amongst the Han nationality (Huang et al., 2009a, Huang et al., 2009b, Li et al., 2007). In our Chinese Han population cohorts, HLA-A*02 and A*24 and A*11 are the most frequently occurring alleles. Globally, HLA-A*2402 is the most commonly occurring HLA-A allele, followed

by A*0201 and A*1101 (Solberg et al., 2008). A*02 is also very prevalent in the northern parts of Western Europe, the western States of the United States, and relatively less common than A*24 in Africa (Solberg et al., 2008). In our study we found HLA-B*40 to be the most common allele in our Chinese Han population. This is in contrast to the global picture, where B*3501 and B*5101 are the most common HLA-B alleles. Comparing our Han population HLA data with other populations also reveals an interesting picture. In the Japanese population, most commonly occurring alleles having frequencies of over ten percent were found to be A*2402, A*3303, A*1101, A*0201, B*4403, B*5201, Cw*0102, Cw*1403, Cw*0304, Cw*0702, Cw*0801, and Cw*1202 (Tokunaga et al., 1997). Analysing different populations in Sub-Saharan Africa, in the Kenyan population, A*02, A*01 and A*68 are present in high frequencies, and B*1503, B*0801 and B*0702 are the most common HLA-B alleles in the Ugandan population (Cao et al., 2004). This heterogeneity in HLA distributions in different populations warrants expanding the net wider and further to elucidate different immune parameters at play in HIV infection in the context of different HLAs in different populations.

Comparing the two groups using our logistic regression analysis reveals no allelic differences between the two cohorts. This is not entirely surprising; as the numbers in this study were relatively small. Moreover, it is worth mentioning here the lost protective effect for HLA-B*51 which has been described for the Japanese population, because of a major escape mutation that has occurred in one of the most dominant HLA-B*51 restricted epitopes (Pol 283-289) in the predominant HIV-1 populations (Kawashima et al., 2009). Similar to the Japanese population, our HIV +ve Cohort has also undergone mutations in three dominant HLA-B*51 restricted

epitopes: Gag 327-345 (NI9) NANPDCKTI, Pol 743-751 (LI9) LPPVVAKEI and Pol 283-289 (TI8) TAFTIPSI (YongHong Zhang et al., 2011). A clear hierarchy in selection of epitope mutations was observed in the HIV +ve Cohort, with higher viral loads and lower CD4 counts observed in patients who had developed escape mutations in all three epitopes or who lacked T-cell responses specific to these epitopes(Zhang et al., 2011). Therefore, even if there were some specific T-cell restricted HLA responses, over the course of time, they may have been lost.

The presence of HLA-B*57 and B*27 has already emerged as uniformly favourable in Caucasian and African ancestry, not to mention, for both clade A and B viruses (Costello et al., 1999, Kaslow et al., 1996, Migueles et al., 2000). Even though our HIV positive Cohort has a relatively high number of LTNPs and SPs, we did not identify a subsequently high proportion of B*57 and B*27 in our Cohort. In addition, no significant protection correlates were found for these alleles. As was expected, B*57 was found to be enriched in the Elite Controller Group (Fig. 2). However, the fact that B*57 did not turn out to be significantly enriched confers credence to the argument that traditionally protective HLA alleles correlates may not hold for different populations, warranting more studies in diverse populations, to elucidate their protective HLA correlates.

We used statistical analysis to calculate allele associations with HIV-1 infection or resistance. Undeniably, this carries an intrinsic risk of error, although the statistical model we have employed is very stringent. At the same time, we have tried our utmost to limit any confounders in this study – primarily by recruiting the study

subjects very carefully. The uniqueness of the 'founder effect' in the HIV +ve Cohort provides an excellent opportunity for analysis discounting erroneous external factors. The Control Cohort was also selected very carefully, keeping the demographics as similar to the HIV +ve Cohort as possible. Potential confounders could include limitations of the sample sizes used for the two Cohorts. The HIV Positive Cohort has an unusually high proportion of LTNPs and SPs, but there are progressors as well as Elite Controllers in the Cohort. We have presumed that the Rapid Progressors have already died in the years after initial infection. Some members of the HIV Positive Cohort have already been on therapy and that has reduced the number of people who we could have used for clinical data analysis. The absence of any sequential time points for clinical data also is an impediment, as it would have been worthwhile to charter the progression amongst the Cohort. The logistic regression analysis is by far the strongest and most stringent statistical tool available for analyzing data of this sort, and provides reliable strategy.

Undeniably, the 2-digit typing employed for HLA-typing for this study is a considerable limiting factor. We used low-resolution typing using sequence specific primers which was the only modality available at the time, and this carries an inherent restriction. Improved typing technology via direct sequencing of exon 2 and 3, which encode the α_1 and α_2 extracellular domains is a substantial advance, with the first two digits specifying a group of alleles (for instance, HLA-B*15), and the third, fourth and/or fifth specifying distinct HLA molecules (for instance, HLA-B*15:01 through HLA-B*15:254, .given that there are 254 distinct HLA-B*15 molecules). It is known that distinct HLA molecules have varying effects on HIV disease progression – dramatic differences in outcome in HIV clade C virus

infection are associated with HLA-B*58:01 and HLA-B*58:02, both from the same supertype and differing by only three amino acids (Kiepiela et al., 2004, Ngumbela et al., 2008). It is also known that specific HLA molecules can have completely diverse outcomes depending on the HIV clade – HLA-B*35:01 is not linked with poor outcomes in C clade infection, where the dominant Gag epitope NPPIPVGDIY (Gag 253-262) is highly targeted; but importantly, in Clade B infection, HLA-B*35:01 is associated with rapid disease progression(Gao et al., 2001, Lazaryan et al., 2011, Pereyra et al., 2010), where this Gag epitope is not targeted. Therefore, it is certainly possible that potential HLA linkages at the distinct HLA molecule level may have been missed in our study. In this context, for future work, all new samples being collected for the Slow Progressors cohort are being typed using sequence-based typing to know the specific HLA loci for each subject.

Even though the strongest known genetic associations with HIV control are mediated by HLA Class I alleles, there are limited studies from the past which have also suggested that HLA Class II alleles, particularly at the HLA-DRB1 locus, could also have an effect on disease progression(Malhotra et al., 2001, Julg et al., 2011). More recently, distinct stratification has been observed in the effects of HLA-DRB1 alleles on HIV viraemia, with HLA-DRB1*15:02 significantly associated with low viremia and HLA-DRB1*03:01 significantly associated with high viraemia, in a large cohort of 1,085 treatment-naïve HIV-infected individuals of European ancestry (Ranasinghe et al., 2013). We did not analyse HLA Class II alleles in our Cohorts, and would, therefore, have missed any HLA-DRB1 locus associations. However, the added problem of lack of sequential clinical data for the Slow Progressors would have made it hard to make any associations of HLA Class II with

viremia, in the context of our Chinese Cohort. We propose that future studies related to this Slow Progressors or other Chinese Cohorts take this aspect into consideration and expand the HLA data to incorporate a detailed Class II analysis as well.

The HLA data presented here serves as a crucial first step for further studies designed to elucidate immunological and genetic parameters of protection in this unique Han HIV Positive Cohort. KIR analysis is already underway and further down the line we plan to examine the Elite Controllers in greater detail, scrutinizing the genomic sequences and trying to find if any T-Cell mediated selection pressure is taking place.

Chapter 4: Immune response correlates of the Vpu accessory gene in a cohort of Slow Progressors from China

4.1 Introduction

HIV-1 has evolved different mechanisms to counter host defence factors (Kirchhoff, 2010, Arhel and Kirchhoff, 2010, Chakrabarti and Simon, 2010). Amongst the accessory proteins, Vpu has recently come under intense investigation over its role in restricting virus release from the cell (Neil et al., 2008, Soll et al., 2010).

Vpu is an oligomeric, 81 amino acid type I integral membrane protein (Andrew and Strebel, 2010, Ruiz et al., 2010, Nomaguchi et al., 2008). Vpu consists of two major domains: an N-terminal hydrophobic membrane anchor domain (TM; transmembrane) (27 amino acid residues), and a cytoplasmic domain (54 residues) that contain a pair of serine residues, whose phosphorylation is critical for CD4 degradation in the endoplasmic reticulum.(Schubert et al., 1994, Tiganos et al., 1998).

The orthodox function designated to Vpu has been degradation of CD4 (Geleziunas et al., 1994, Levesque et al., 2004, Ruiz et al., 2010). In addition to Vpu, Nef and Env are also implicated in the viral effort to downregulate CD4 from the cell surface, albeit through entirely different mechanisms. Vpu induces proteosomal degradation of CD4 from the Endoplasmic Reticulum through a mechanism that involves the Endoplasmic-reticulum-associated protein degradation (ERAD) pathway (Willey et al., 1992, Binette et al., 2007, Magadan et al., 2010).

The second function of Vpu, which has gained considerable attention more recently, is the regulation of virion release from the cell (Neil et al., 2006, Neil et al., 2007, Neil et al., 2008). It had been postulated for quite some time that Vpu regulates the detachment of otherwise complete and infectious virions from the cell surface. This traced back to original observations that deletion of HIV-1 Vpu resulted in an almost five to ten fold decrease in the release of virus from infected T cells, without having any adverse effects on the function of other viral proteins (Strebel et al., 1988)

Various models had been put forward to explain Vpu's mechanism of action. However, the mechanism remained enigmatic until the discovery of tetherin, when the model that invoked the inactivation of a cellular inhibitor of virus release provided convincing evidence.

Tetherin, also known as or BST-2 or CD317, was identified as the interferon-inducible, Vpu-sensitive restriction factor responsible for the restriction of HIV-1 virus release (Martin-Serrano and Neil, 2011, Dube et al., 2010a, Evans et al., 2010). Tetherin is a 30-36 kDa type II transmembrane protein, consisting of 180 amino acids. The protein has both an N-terminal transmembrane domain and a C-terminal glycosyl-phosphatidinylinositol (GPI) anchor (Ruiz et al., 2010, Guatelli, 2009), a virtue which allows it to simultaneously associate with both viral and cellular membranes. As viruses attempt to bud from infected cells, tetherin becomes incorporated into the viral envelope and physically adheres the nascent virions to

the cell, thereby limiting virion release (Perez-Caballero et al., 2009, Hammonds et al., 2010, Fitzpatrick et al., 2010).

In response to Vpu, tetherin is downregulated from the surface of the HIV infected cell (Van Damme et al., 2008). Studies have also demonstrated that Vpu induces tetherin degradation dependent on the recruitment of a βTRCP2-SCF-Cullin1 ubiquitin ligase complex (Douglas et al., 2009, Goffinet et al., 2009, Iwabu et al., 2009).

Vpu localises predominantly to the tans-Golgi network (TGN) and recycling endosomes (Dube et al., 2009, Varthakavi et al., 2006). Vpu localization to the TGN correlates directly with its ability to counteract tetherin (Dube et al., 2009), and recent studies have demonstrated that tetherin also accumulates in the TGN in response to Vpu (Dube et al., 2010b, Gupta et al., 2009, Hauser et al., 2010, Dube et al., 2009). The TM domain of Vpu is necessary for its role in antagonising tetherin, and early studies have demonstrated this in great detail. Deletion of parts of the TM domain (Paul et al., 1998) in which hydrophobic amino acids from both ends (N and C terminal) or the middle of the domain are removed, generated proteins that are highly defective in their ability to enhance the release of virus-like Gag particles. Similarly, substitution of conserved amino acids from the N-terminal, middle or C-terminal parts of the TM domain was shown to generate proteins that lost their ability to enhance viral particle release (Tiganos et al., 1998).

After the discovery of tetherin, there have been a spate of studies which tried to map the residues in tetherin's TM domain, and their critical roles in association with Vpu (McNatt et al., 2009, Gupta et al., 2009, Rong et al., 2009a). More recently, a detailed analysis using cysteine-scanning mutagenesis coupled with oxidation-induced cross-linking demonstrated that Vpu and Tetherin TM domains bind directly to each other in the membranes of living cells, and also mapped the TM domain residues that contact each other (McNatt et al., 2013).

HIV type 1 infection is characterised by an early peak of viraemia that declines coincident with the development of strong Cytotoxic T lymphocytes (CTL) responses (Addo et al., 2002b, Borrow et al., 1994, Koup et al., 1994, McMichael et al., 2010). CTLs recognise antigenic peptides displayed by MHC Class I molecules on the infected cell surface and represent a major selective driving force driving HIV evolution. Most studies have focused on the assessment of immune responses directed against the structural HIV-1 proteins Gag, Pol and Env, as well as accessory protein Nef, with very limited data available about Vpu.

A variety of factors can have an impact on the recognition of viral peptides by CTL. The degree to which the sequence is conserved within a particular HIV-1 protein might have an influence on its recognition by CTL. It is known that Vpu is a highly variable protein, and based on published sequences, Vpu is the most variable HIV-1 protein, followed by Tat and Rev, while p24-Gag are the most highly conserved (Yusim et al., 2002, Hasan et al., 2012). CTL responses, in conjunction with their restricting HLA molecules, exert a selective pressure on the virus, which can lead

to sequence variation in the virus sequence in a predictable manner, according to the HLA Class I type of the donor.

A strong epidemiological link exists between specific HLA Class I molecules and different rates of HIV disease progression (Carrington and O'Brien, 2003), implying that the quality of the CTL response and the features of the targeted epitopes have a strong influence on the effectiveness of viral control (Goulder and Watkins, 2004). However, the strongest evidence supporting CTL as a major determinant of HIV-1 control may be mutational immune escape. This was first described in 1991, where the selection of viral escape mutations within critical CTL epitopes during primary (Phillips et al., 1991) (Borrow et al., 1997) and chronic HIV-1 infection (Goulder et al., 1997) identified immune-driven evolution as a continuous process that occurs throughout the course of the disease. Although escape mostly occurs within the CTL epitopes (therefore disrupting peptide-HLA binding or the recognition of the peptide/HLA complex by the T-cell receptor), escape is not necessarily restricted to the epitope boundaries. There can also be mutations in the epitope flanking regions, which can have an adverse effect on intracellular peptide processing and presentation (Allen et al., 2004), or there can be secondary or compensatory sequence variations that therefore stabilise the escape mutations that are being selected at another place in the virion (Schneidewind et al., 2007).

In addition to CTL, NK cells play a critical part in the host's immune response to HIV infection. It was found that reduction in viral replication during acute HIV infection often occurs before the induction of adaptive immune responses, like CTL responses, which strongly highlights the role played by the innate immune system

(Alter et al., 2007). Unlike CD8+ T cells, NK cells are a subset of large granular lymphocytes which do not express an antigen-specific marker, but rather display a variety of inhibitory and activating receptors on their surface that are involved in sensing changes in their ligands on the surface of the host's body cells (Lanier, 1998). NK cells survey the body for MHC Class I expression, using a network of receptors known as the Killer Immunoglobulin-like Receptors (KIRs), and are inhibited on interaction with MHC Class I.

KIRs consist of two (KIR2D) or three (KIR3D) extracellular domains and the intracellular tail can be either long (L), mediating inhibitory signals or short (S), in which case associated adapter proteins mediate activating signals. Different KIR molecules interact specifically with HLA-A, -B or -C. HLA-C allotypes have been divided into two groups where C1 (HLA-Cw1, -3, -7, -8) interact with KIR2DL2/L3/S2 and C2 (HLA-Cw2, -4, -5, -6) interact with KIR2DL1/S1.

HIV was the first viral infection in which KIRs were associated with disease outcome. It was shown that the activating receptor KIR3DS1 was associated with delayed progression to AIDS in conjunction with HLA-B alleles that have the Bw4 serological epitope with isoleucine at position 80 (Martin et al., 2002). This epistatic interaction protected against a decline in CD4 count, and hence the development of AIDS. In addition to 3DS1, it was later shown that additional inhibitory allotypes of 3DL1 are also associated with a slower HIV disease progression (Martin et al., 2007). These results suggest that NK cells may contribute to HIV control through at least two distinct mechanisms, one modulated by inhibitory receptors and a second

mediated by an activating receptor, and that the activating and inhibitory receptors may synergise to promote enhanced control of HIV viral replication.

More recently, efforts have been made to determine if NK cells may recognise and place pressure on the virus directly in vivo. Traditionally, the identification of 'footprints,' amino acid substitutions that accumulate specifically in the presence of specific HLA Class I molecules, has been regarded as a marker of CTL pressure (Allen et al., 2000, Telenti and Johnson, 2012, Matthews et al., 2009). However, it has been shown that similar footprints arise in the presence of distinct KIR genes (Alter et al., 2011). Their analysis led to the identification of 22 positions within the HIV-1 genome in which amino acid polymorphisms were significantly associated with the presence of a specific KIR gene. They then went on to assess the consequences of some of these KIR-associated amino acid sequence polymorphisms on NK cell mediated recognition of HIV-1 infected cells, showing that these KIR-associated HIV-1 sequence polymorphisms can enhance the binding of inhibitory KIRs to HIV-1 infected CD4+T cells, leading to reduced antiviral activity of KIR+ NK cells (Alter et al., 2011).

In this chapter, investigations of immune selection pressure on Vpu in the SM Cohort are described. The SM Cohort has a series of unique features, which we have described in Chapter 2 (Materials and Methods). Firstly, study participants were recruited from a close-knit rural community and most of them are genetically related. All the study participants were infected by a similar strain of the virus. Therefore, limiting viral and host variability, the SM Cohort provides an excellent

opportunity to further understand HIV pathogenesis through a simpler picture of the relationship between disease progression, viral evolution and host immune responses. The diversification of the virus from the same infecting strain provides the best opportunity to demonstrate correlations between the evolution of the virus and host immune function.

We therefore wanted to explore three things. Firstly, we wanted to look at Vpu diversity within our unique SM cohort. Secondly, we wanted to determine if there was any CTL pressure on Vpu in our setting which led to specific HLA Class-I restricted polymorphisms. Lastly, we wanted to determine if there were any KIR 'footprints' on Vpu in our SM Cohort.

4.2 Results

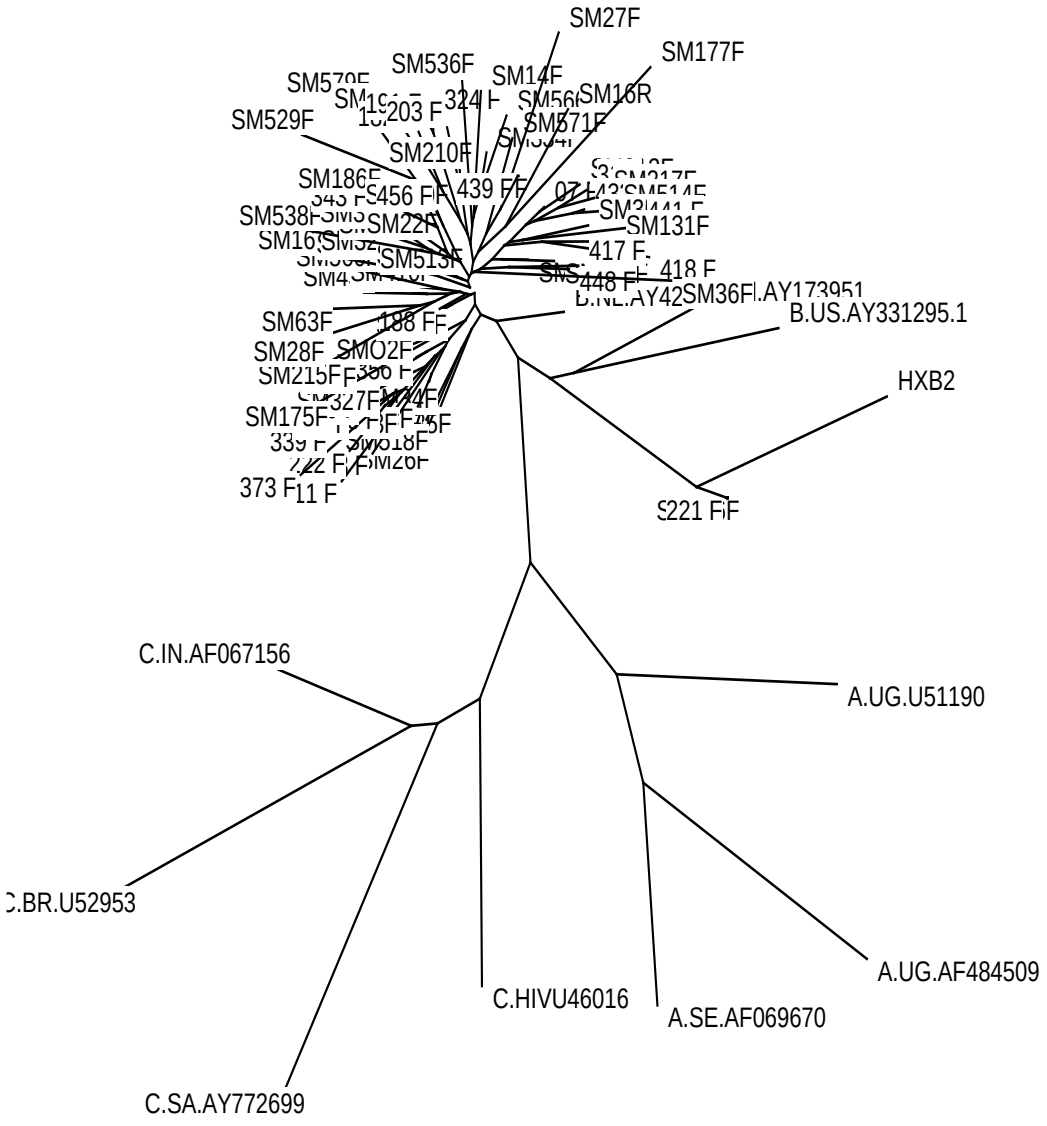
4.2.1 Genetic Variability of the VPU gene

We successfully amplified DNAs comprising the Vpu region from 90 samples from the SM Cohort which were collected using random convenient sampling in 2008.

A representative tree using the 90 patient sequences is depicted on the next page (Fig. 4-1). This phylogenetic tree also includes reference Chinese subtype B' sequences, taken from the HIV Database (http://hiv-web.lanl.gov/content/hiv-db/mainpage.html). The phylogenetic tree of the Vpu sequences along with the reference sequences was analysed using the Maximum-likelihood method, and as expected, our Vpu sequences bear close homology to the Chinese reference sequences of subtype B'.

Figure 4-1:

The phylogenetic tree of HIV-1 Vpu sequences from the SM cohort and reference sequences from the Los Alamos HIV Database.



Next we analysed the amino acid variability at each codon of Vpu by determining its Shannon entropy score (a simple quantitative measure of uncertainty or variability in a data set) the average of the entropy score was 0.41. This is illustrated in Figure 4-2 below. It is worth noting here for comparison purposes that Gag p24, a highly conserved protein, has an average entropy score of about 0.17 (Yusim et al., 2002).

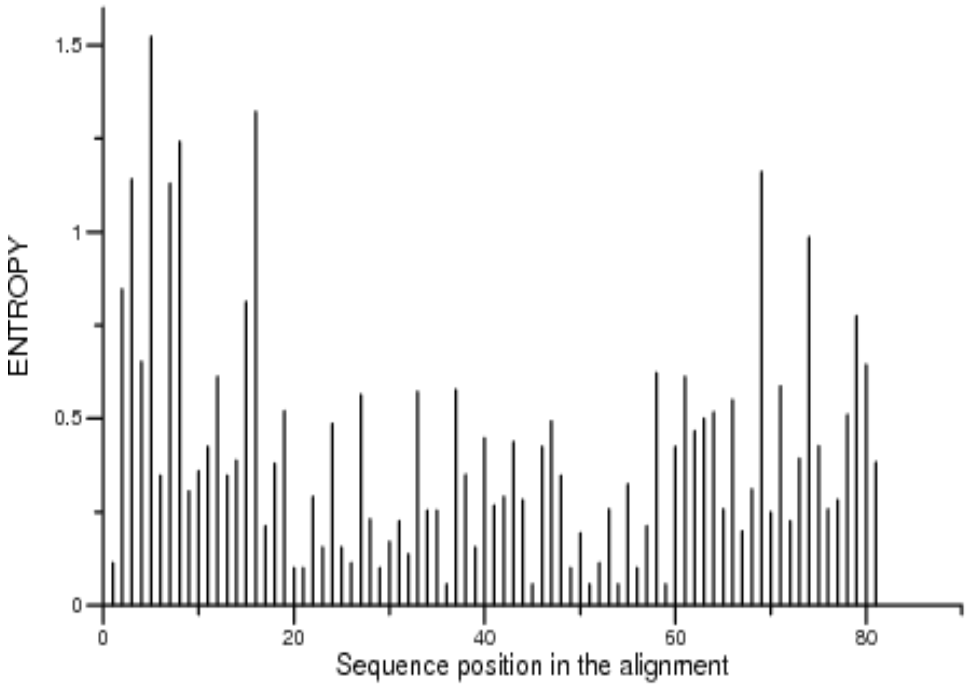


Figure 4-2:

The amino acid variability was analysed by determining its Shannon entropy score

We generated the consensus amino acid sequence for our Cohort, and this is compared to the Los Alamos Subtype B reference in Table 4-1 below. In fact, the consensus amino acid sequence of the Los Alamos reference and our SM Cohort dataset were identical except for 2 amino-acid residues: positions 3 and 23. These amino acid residues were variable, and are not associated with any known Vpu functional activity.

Table 4-1:

Comparing the Consensus Chinese Sequence with that of the Los Alamos Subtype B reference. The highlighted, bold positions denote variation in the amino acids.

Reference Sequence Subtype B, Los Alamos: MQ**S**LILAIVALVVAAIIAIVVW**S**IVFIEYRKILRQRKIDR LIDRIRERAEDSGNESEGDQEELSALGVEMGHHAPWDVDDL
Consensus Chinese Sequence for our Cohort: MQ**A**LILAIVALVVAAIIAIVVW**T**IVFIEYRKILRQRKIDR LIDRIRERAEDSGNESEGDQEELSALGVEMGHHAPWDVDDL

4.2.2 T-cell immune pressure on Vpu in the SM Cohort

Previous studies in the SM Cohort have already revealed that HLA Class I mediates a strong selective pressure on HIV-1 sequence variability, and therefore we sought to examine whether HLA-I-mediated selective pressure profoundly impacted the evolution of Vpu.

So firstly, using the Vpu translated sequences in relation to patient HLA data, we tried to identify positions in the VPU sequence that could be T-cell restricted. We first did a univariate analysis of correlations between the presence or absence of HLA alleles and the presence or absence of amino acids at every position in the HIV-1 Vpu region, using already published methods, correcting for multiple comparisons. Next, for those amino acids for which there was at least one correlated HLA allele, we conducted multivariate analysis with logistic regression for each residue to assess the statistical significance of the association between the presence of a polymorphism (defined as any substitution of the consensus amino acid) and the HLA allele associated with it.

In our dataset of 90 samples, after correcting for multiple comparisons, we were not able to find any statistically significant HIV-HLA associations in Vpu. This is not entirely surprising, because of a plethora of factors, discussed later.

We also conducted ELISPOT analysis using 6 random subjects from the SM Cohort (2 LTNPs, 2 SPs and 2 NPs) and used overlapping Vpu peptides generated using the consensus sequence. None of the subjects produced any meaningful responses. For these ELISPOT experiments, Gag peptides were used as positive controls. Figure 4-3 below represents ELISPOT data for SM 379 using Gag peptides as a positive control – none of the nine consensus-based Vpu peptides generated any response.

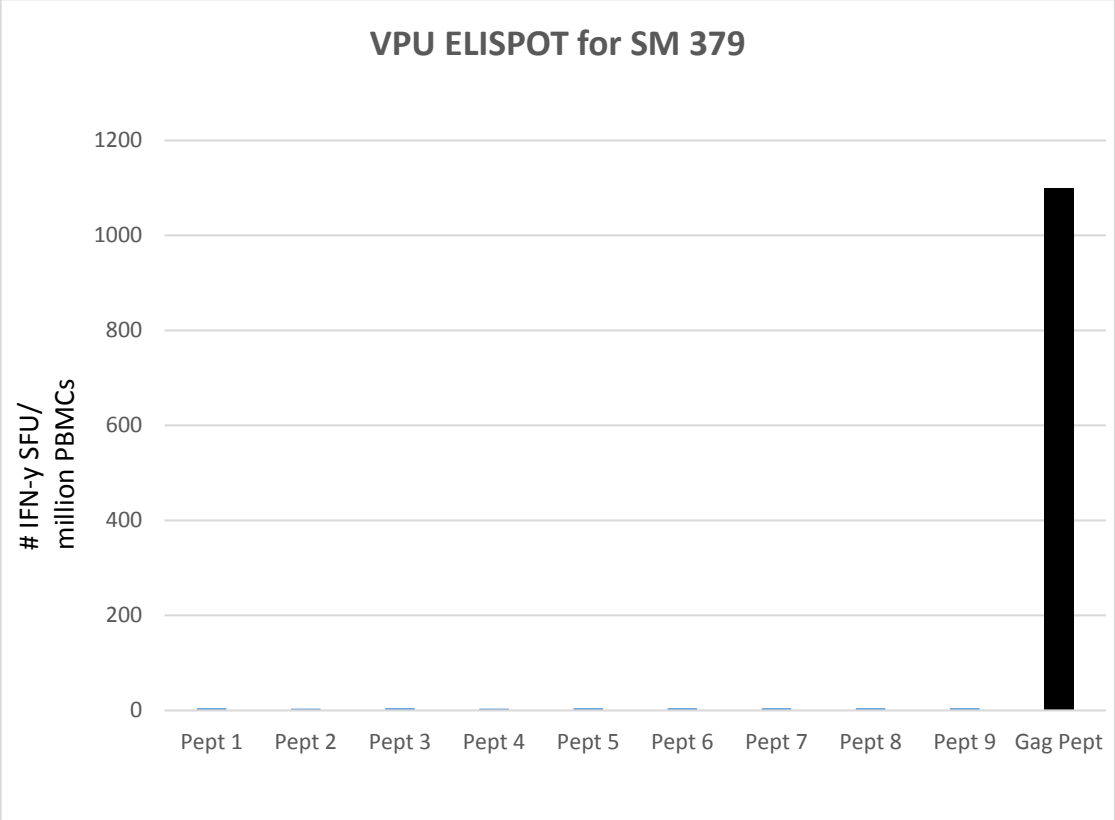


Figure 4-3:

ELISPOT data for SM 379 using consensus VPU peptides and Gag Peptides (Positive Control). No responses were seen to the VPU peptides.

4.2.3 NK-cell immune pressure on Vpu in the SM Cohort

After determining HLA-I mediated selection pressure, we were interested to find if there were any specific amino acid polymorphisms in the SM Cohort Vpu sequence that are significantly associated with the expression of specific KIR genes.

We used the Vpu translated sequences in conjunction with KIR data for the SM Cohort, and tried to identify any KIR-associated polymorphisms. This analysis was done in collaboration with Dr Mina John, using S-Plus 8.0 (Insightful Corporation, Seattle, USA). This method of statistical computing also utilised stratification analysis by Mantel-Haenszel tests to strengthen the associations inferred between viral polymorphisms at each amino acid residue and the KIR genes.

After a rigorous analysis, we found only 1 strong association: Vpu amino acid position 14 with KIR 2DSI. This is detailed in Table 4-2a and Table 4-2b on the next page.

Table 4-2a:

Frequency of Vpu amino acid polymorphisms using KIR2DS1+ and KIR2DS- subjects

Vpu position	Amin Acid	2DS1- (n)	2DS1+(n)
14	A	33	11
14	V	10	16

Table 4-2b:

KIR Footprints in HIV-1 Vpu sequences from the SM Cohort

Protein	Amino acid position	KIR-association	Consensus Amino acid	P-value
Vpu	14	2DSI	Ala	0.0047

4.2.4 Discussion

Initial studies have revealed that there is no enrichment of any specific HLAs in our SM Cohort that might confer protection against delayed progression to AIDS (Rai et al., 2013). We went on to explore potential selection pressure on Vpu in the SM Cohort, focusing first on the T cell correlates and then the NK cell correlates.

Sequencing Vpu from the Cohort reaffirmed that the infection in the SM Cohort is from a narrow source, and the Vpu sequences form a monophyletic subtype B′ cluster that cosegregated with other sequences associated with the plasma donation epidemic in central China, similar to what we have seen in Gag, Pol and Nef sequences from the SM Cohort (Dong et al., 2011).

It is known that Vpu is a highly variable protein, having the maximum entropy (maximum variability) amongst 101 aligned amino acid sequences comparing the different HIV M group proteins (Yusim et al., 2002). This finding was reaffirmed more recently from a Japanese study involving 240 chronically HIV-1 infected, treatment-naïve subjects, where the average of the entropy score reached 0.58. In our SM Cohort, the average of the entropy score was 0.41. This is in line with our expectations, as we know already that the genetically related SM Cohort was infected with a similar strain of HIV at approximately the same time.

The role of CTL in shaping population HIV-1 sequence diversity is influenced by a complex interaction amongst multiple conflicting selective forces, one instance being the delicate balance between the benefits of escape versus the associated costs to viral fitness (Liu et al., 2007). Most studies have focused on the assessment of immune responses directed against the structural HIV-1 proteins Gag, Pol and Env, as well as accessory protein Nef, with very limited data available about Vpu.

We explored HIV-HLA associated polymorphisms in Vpu, and did not find any statistically significant associations. This is not entirely surprising, as we have a very limited sample size, and it is possible that we might have missed some polymorphisms. In addition, our screening ELISPOTS using overlapping peptides did not reveal any T-cell responses to the protein, even though we used overlapping peptides using a consensus sequence based on our Chinese cohort. It is already known that Vpu is one of the least frequently targeted HIV-1 proteins. The high sequence variability might influence its recognition by CTL responses, and hence the lack of any ELISPOT responses. Another factor influencing the recognition of viral proteins by CTL may be the amount of expression of Vpu protein during viral infection, and this might also explain the lack of CTL responses. Other factors including antigen processing and presentation may also have critical roles. Further studies are needed to assess whether this infrequent recognition of Vpu reflects low immunogenicity of the protein *in vivo* or a lack of HLA Class I processing of Vpu.

The first report investigating CTL responses against all the accessory proteins (Altfeld et al., 2001) found detectable responses against HIV-1 Vpu in only one out of 60 (1.6%) screened individuals, with responses of 170 SFC/10^6 PBMC against Vpu-6 (IVFIEYRKILRQRKID), as opposed to responses against the other accessory proteins, Vpr and Vif (45% and 33% respectively of the tested individuals). In an extension of this study, they next screened 85 HIV-1 infected subjects at different stages of infection, and even though all the study subjects had detectable HIV-1 CTL responses (92% against Gag and 80% against Nef), only two (2.4%) subjects had detectable, but low-level CTL responses directed against HIV-1 Vpu (Addo et al., 2002a).

Furthermore, in order to characterise in detail the low-level CTL response, they generated Vpu-6 specific cytotoxic T cells using the limiting dilution assay, and determined the HLA Class I restriction of the Vpu-6-specific response using an autologous and partially matched antigen-presenting lymphoblastoid B cell line in a chromium release assay. The response against the Vpu-6 peptide was shown to be restricted by HLA-A*3303, and the nonamer peptide EYRKILRQR (ER9, Vpu 29-37) was determined to be the optimal sequence in the CTL epitope. This epitope was the first described optimal CTL epitope, as well as one of the first HIV-1 CTL epitopes restricted by HLA-A*3303.

More recently, a Japanese study involving 240 chronically HIV-1 infected, treatment-naïve subjects identified only three HIV-HLA associations in Vpu: a non-adapted association between HLA-C*03 and Glu-5, a non-adapted association

between HLA-A*33:03 and Arg-37, and an adapted association between HLA-A*33:03 and Lys-37. They defined 'non-adapted' amino acids as those enriched in the absence of the restricting HLA of interest, and 'adapted' amino acids as those enriched in the presence of the HLA allele. They only used sequencing analysis to generate these results, and did not test their HIV-HLA associations in CTL studies (particularly relevant for the HLA-C*03 association with Glu-5, as no one else has reported this before).

We did not encounter any HIV-HLA associations in our study. This might be a reflection of the limited sample size utilised in our study. Our SM Cohort is also very unique when it comes to viral and genetic variability, and it is possible that any protective HIV-HLA Vpu associations, along with the protective HLAs have been lost over time. In addition, the timeline of the infection, which probably happened in the SM Cohort, between 1994 and 1995, and the collection of samples for this study in 2008, means that the virus had adequate time to evolve and escape into a fitter form. It is also possible that CTL pressure does not have much influence on Vpu, which already is a very variable accessory protein. We were limited for the PBMCs we had for our study subjects, and could carry out a screen for CTL responses against HIV-1 Vpu in only 6 subjects. We used overlapping 18mers spanning all of Vpu based on the consensus Vpu sequence derived from our 90 subjects, to screen for any potential ELISPOT responses, so the notion that we could have missed responses because the autologous virus could be different from the protein sequence does not hold true. Therefore, our data add to the already widely-accepted notion that the influence of CTL immune selection on evolution of Vpu at the population level may be reduced compared to other highly variable HIV-1

proteins, possibly because Vpu is only produced in small quantities, or it may not be accessible to the Class I processing pathways, unlike the other HIV-1 proteins.

Next we tried to determine if there were any KIR-associated polymorphisms within the HIV-1 Vpu sequences in the SM Cohort. We used an approach that utilises stratification analysis by Mantel-Haenszel tests to strengthen the associations inferred between viral polymorphisms at each amino acid residue and the KIR genes. This analysis led to the identification of a single amino acid position in the Vpu sequence which was significantly associated with the presence of a specific KIR gene ($p = 0.047$).

In amino acid position 14 of Vpu, HIV-1 sequences (59%) derived from KIR2DS1+ individuals encoded for a valine (V) whereas the consensus amino acid alanine (A) at this position is found in the vast majority (76%) of KIR2DS1- individuals. Our stringent analytical calculations, which take into account multiple comparisons, showed a high level of significance ($p=0.047$) for this association.

There is a dearth of literature about KIR2DS1 and HIV infection. NK cells expressing KIR2DS1, an activating receptor, have been shown to have a decreasing frequency with increasing HIV-1 load in a cohort of chronically HIV-1 infected, treatment-naïve adult South Africans, suggesting that modulation of receptor expression may play a role in HIV-1 pathogenesis (Wong et al., 2010). However, the mechanisms through which KIR2DS1 might mediate this effect still need to be unravelled.

It is extremely interesting that our analysis pointed out a Vpu amino acid position that has been deemed critical to Vpu's function as pertains to tetherin antagonism. Tetherin or BST-2 mediated restriction of HIV-1 was known to be due to residues in tetherin's transmembrane domain for Vpu (Gupta et al., 2009, McNatt et al., 2009, Rong et al., 2009a). Building up on this, an elegant study (Vigan and Neil, 2010) that conducted screening mutagenesis through a region of the transmembrane domain of Vpu (residues 5 to 28) for their ability to rescue Vpu-defective HIV-1 from tetherin mediated restriction, revealed that major defects associated with changes at Alanine (A) at Vpu position 14 and Tryptophan (W) at position 22, and to a lesser extent, Alanine (A) at position 18, are associated with an inability to antagonize tetherin-mediated restriction of virus release, so these amino acids are postulated to contribute to the critical interaction between tetherin and Vpu. Therefore, it is very interesting that we found a KIR association for an amino acid position that is critical for Vpu's role in antagonising the host's immune response to the virus. These data are preliminary and more functional studies are needed to delineate the response in greater detail, and to charter its significance. These experiments would entail constructing HIV-1 viral variants that encode both Alanine and Valine at Vpu position 14, and then determine the functional consequences of these KIR2DS1-associated polymorphisms, assessing thereafter the ability of these viral variants to replicate in primary CD4+ T cells in vitro, and subsequently assessing whether the different viral variants have any impact on NK cell recognition and/or antiviral activity. Also, it would be interesting to devise an experiment with mutated viral constructs having Alanine and Valine at position 14, and then test them for their ability to antagonise tetherin, and the degree of tetherin antagonism.

Even though the data presented here is preliminary, however, it seems reasonable to propose that this critical amino acid position – Ala14 is under a lot of immune pressure from the host so it can undergo mutation, and therefore not counteract the activity of tetherin in the host cell. Both Alanine and the other amino acid at Vpu position 14, Valine have non-polar side chains. However, it may represent the immune pressure exerted by the host's NK cells and therefore the virus can evade such NK cell mediated immune pressure by selecting for sequence polymorphisms which do not have a detrimental effect, and can still counteract tetherin's activity.

Our unique SM cohort sheds some interesting lights on the host's immune response to Vpu. We did not encounter any HLA Class-I associated mutations in Vpu in our cohort, which reaffirms the findings from other cohorts that HLA-I-mediated selective pressure towards Vpu does not substantially drive Vpu variability at the population level in various cohort. However, we did find that there was a polymorphism at Vpu position 14 that was significantly associated with KIR2DS1. This is all the more interesting as this amino acid position has been shown to be critical for Vpu's activity against the recently identified host restriction factor, tetherin. More detailed functional studies are now needed to elucidate in detail this finding.

Chapter 5: ADCC and Vpu responses in a cohort of Slow Progressors from China

4.1 Introduction

NK cell activation and expansion has been described in humans with primary HIV infections, and precedes the appearance of virus-specific CTL responses (Alter et al., 2004). Virus-induced cytokines i.e. IL-12, type I interferon, IL-15 and many others, are usually responsible for the early NK cell activation and expansion (Lanier and Phillips, 1992). Activated NK cells activate dendritic cells, secrete interferon-gamma, and act as adjuvants by killing cells infected with the virus, and by releasing intracellular proteins from the infected cells.

Many studies have demonstrated that NK cells could potentially play a critical role in controlling HIV replication (Carrington and Alter, 2012, Borrow, 2011). NK cells have been shown to suppress HIV replication in cell cultures (Forthal et al., 2001, Kottilil et al., 2003). Also, high NK cell activity has been demonstrated in exposed, uninfected intravascular drug users from Vietnam, whose KIR repertoire is predominantly of an activating type (Ravet et al., 2007, Scott-Algara et al., 2003).

In addition to this, it has been shown from earlier studies that high NK cell activity also delays progression of HIV infection towards AIDS (Douglas et al., 2001, Ironson et al., 2001). It has been shown that decreases in NK cell cytotoxicity in addition to the NK cell counts in the circulation of the infected persons were

associated with their rapid $CD4^+$ T cell depletions and rapid progression towards AIDS (Ahmad et al., 2001b).

Animal models of HIV infection also support a succinct role for NK cells in controlling HIV infection. It has been suggested that one of the keys to successful lentivirus control may reside in part in a different regulation of NK-cell triggering receptor expression – this would explain why HIV-1 infection in chimpanzees, the closest human relative, rarely leads to disease progression (Rutjens et al., 2007, Heeney and Plotkin, 2006). More recently, an association has been identified between the copy number of activating KIR genes in rhesus monkeys and the control of SIV replication during primary infection, underscoring the potential importance of NK cell responses to control SIV spread (Hellmann et al., 2011).

Genetic studies from populations infected with HIV-1 infected individuals have provided strong evidence that NK cells might play a role in the control of HIV-1 replication and control. Initial studies revealed that HIV-1 infected individuals with HLA Class I alleles encoding for molecules carrying the Bw4 serological motif experience much better control of HIV-1 viremia and slower disease progression (Flores-Villanueva et al., 2001). Subsequently, it was shown that HIV-1 infected individuals expressing KIR3DS1 and a subset of HLA-Bw4 alleles encoding for an isoleucine at position 80 experienced significantly slower disease progression compared to individuals encoding for either HLA-Bw4-80I or KIR3DS1 (Martin et al., 2002). Further studies showed that the protection conferred by HLA-Bw-4 alleles is greatly affected by the KIR3DL1 genotype (Martin et al., 2007). Individuals with genotypes encoding for HLA-Bw4 and highly expressed KIR3DL1

allotypes had significantly lower levels of HIV-1 replication compared to individuals with encoding for the same HLA-Bw4 alleles but KIR3DL1 subtypes associated with low expression (Martin et al., 2007). Since then, different studies have reported associations between the HLA-Bw4/KIR3DL1 genotype and protection from HIV-1 infection (Boulet et al., 2008, Boulet et al., 2010, Kamya et al., 2011).

Even though population genetic studies have shown strong indications suggesting that KIR/HLA interactions play a critical role in HIV-1 disease progression, the functional correlates are much less well understood. The expansion of NK cells during acute HIV-1 infection, especially before the development of detectable adaptive immunity, appears to be regulated to some extent by HLA and KIR genotypes (Alter et al., 2009, Alter et al., 2007). Apart from the potential KIR-modulated recognition of HIV-1 infected cells by NK cells, less polymorphic receptors' such as NKG2D, may play a role in NK-cell mediated killing of infected cells. The HIV-1 Nef protein seems to downregulate not just HLA-A and HLA-B, but also NKG2D ligands expressed on HIV-infected cells (Cerboni et al., 2007, Sowrirajan and Barker, 2011). Overall, several different NK cell receptors have been shown to be associated with HIV-1 disease progression, implying that NK cells might contribute greatly to the control of HIV-1 infection.

The first phase 3 clinical trial designed to stimulate cell-mediated immunity, the STEP trial, was terminated prematurely because of evidence that prior immunity against the adenovirus vector was not protective and may even have made the vaccinee even more susceptible to HIV infection (Steinbrook, 2007, Moore et al.,

2008). The most recent clinical trial in Thailand RV 144 combined two vaccines designed to stimulate the production of antibodies and cell-mediated immunity. This vaccine protected a modest almost one-third of the individuals who were vaccinated (Rerks-Ngarm et al., 2009). Despite inducing only narrow neutralising antibody responses and very modest cytotoxic T-lymphocyte responses, non-neutralising antibodies were induced by this regimen (Karnasuta et al., 2005), and therefore such antibodies might have played a role in the protection observed in this trial (Haynes et al., 2012). Immunogenicity studies carried out on samples from vaccinees that were involved in the phase I/II trials of the RV144 prime boost strategy have highlighted a potential role for antibody-dependent cellular cytotoxicity (ADCC) in protection against HIV-1 infection (Karnasuta et al., 2005).

The antibodies in antibody-dependent cellular cytotoxicity (ADCC) bind through their variable antigen-binding sites to the viral antigen on the surface of the cells infected by the virus, and through their crystallisable fragments (Fc) to CD16 on NK cells (Ahmad and Menezes, 1996, Iannello and Ahmad, 2005). The antibodies cross-link CD16 on the NK cells and therefore trigger their cytolytic activity. This therefore results in the killing of the virus-infected cells, and the secretion of different cytokines and chemokines from the NK cells. ADCC is the classical example of cooperation between the innate and adaptive immune responses in the body's response to viral infections.

An efficient ADCC response requires three components: infected target cells that are expressing the foreign antigen on their surface, antigen specific antibodies of the IgG isotype, and lastly, effector cells that express Fc-gamma receptors (Moretta et

al., 2008, Evans et al., 1989, Weinhold, 1991). A major population of ADCC mediating effector cells are the Fc-gamma RIII (CD16) bearing NK cells. Other cells' including macrophages, eosinophils and neutrophils can also mediate ADCC, though these cell types normally require a larger antibody titre and higher antigen expression by the target cells (Szelc et al., 1992, Jenkins et al., 1993). The strength of an ADCC response is highly dependent on the affinity of the interaction between the effector cell receptor and the antibody, as ADCC has been shown to be amplified by increasing the affinity of the Fc portion of an ADCC antibody (2G12) for CD16 (Klein et al., 2010).

ADCC responses are detected first early in the course of primary HIV-1 infection (Forthal et al., 2001). The titres of ADCC antibodies are the highest and the magnitude of the responses the greatest during early infection (Sawyer et al., 1990). It is known that CD16 which is a key receptor for the induction of ADCC, binds with the highest affinity to the Fc portion of IgG1 and IgG3, and these are the isotypes more frequently induced in HIV-1 infection (Cavacini et al., 2003). HIV-1 infection can lead to the decreased expression of CD16 by the NK cells, and the overall dysfunction of NK cell-mediated immune activity over time, which is therefore associated with progression of disease (Ratcliffe et al., 1994, Alter et al., 2005).

Different studies have tried to gauge ADCC activity in different population cohorts. Rapid Progressors from the Multicenter AIDS (MACS) study had significantly lower titres of Abs that mediate ADCC against HIV-1 gp120 than those of nonrapid progressors at corresponding visits or those of nonprogressors at any visit.(Baum et

al., 1996). HIV-infected women have been shown to have, in addition to having serum activity, specific ADCC antibodies in their genital fluids (Battle-Miller et al., 2002). In addition, it was shown that women with cervicovaginal lavage ADCC activity had lower genital tract viral loads than did women with serum ADCC activity only (Nag et al., 2004). Analysis of Elite Controllers from a multicentre French trial demonstrated that Elite Controllers had higher titres of ADCC antibodies against HIV than viremic individuals (Lambotte et al., 2009).

Animal studies have also demonstrated a beneficial function of ADCC. ADCC responses correlate with slower disease progression or lower viral loads in SIV-infected macaques (Banks et al., 2002, Gomez-Roman et al., 2005, Xiao et al., 2010). Passive transfer of antibodies to macaques helped to establish the critical importance of the Fc portion of IgGs in protecting against infection (Forthal et al., 2006, Hessell et al., 2007, Hessell et al., 2009). More recently, it has been shown that a nonfucosylated variant of the broadly neutralising human IgG1 anti-HIV monoclonal antibody b12 in macaques possesses enhanced affinity for the cellular leukocyte receptor FcγRIIIa and enhanced FcγRIIIa-mediated ADCC in vitro relative to the wild-type antibody (Moldt et al., 2012).

In the past, the primary ADCC assays relied on the killing of radiolabelled target cells either infected with HIV or pulsed with whole HIV proteins (Ahmad et al., 2001a, Lyerly et al., 1987). The assay involved the labelling of antigen-coated or virus-infected target cells with Chromium (^{51} Cr). The target cells were then incubated with the patient serum containing antibodies, and either patient or donor peripheral blood mononuclear cells (PBMCs), and the killing of target cells

determined by the release of ^{51} Cr (^{51} Cr-release assay) (Lyerly et al., 1987). These assays are extremely difficult to standardise and are time consuming, and have the limitation of involving significant radioactivity. Fluorescence based ADCC killing assays have also been studied with relative success in recent years (Gomez-Roman et al., 2006). This entailed labelling the membrane and the intracellular contents of the target cell lines with fluorescent dyes - a membrane dye (PKH-26) and a viability dye (CFSE) prior to the addition of antibody and effector cells. Flow-cytometric analysis of the loss of CFSE fluorescence is used to determine killing (Gomez-Roman et al., 2006). This assay is much more quantitative than the Chromium release assay, however it still relies on the use of artificial cell lines labelled with virus/whole proteins as target cells and healthy donor PBMCs as the effectors. The nature of the target cells, the level and nature of the expression of the antigens on the target cells, in addition to the effector-to-target ratio used, may all contribute to variability in these assays.

Therefore, an alternative intracellular cytokine staining (ICS) based was developed (Stratov et al., 2008). This assay involves incubating patient whole blood or donor whole blood along with the patient plasma with antigen and then staining with fluorescently labelled monoclonal antibodies (Chung et al., 2009). Flow cytometric analysis of the NK cell population is performed and the ADCC responses is recorded as the percentage of activated NK cells based on intracellular IFN-gamma and degranulation marker CD107a expression(Stratov et al., 2008). This ICS based assay helps to overcome some of the variability issues associated with past ADCC assays, and the major advantage of this ICS based assay, in addition to its being less labour intensive and less time-consuming, is the ability to use small peptides or

overlapping peptide pools as the antigen, which allows short linear epitopes of ADCC antibodies to be delineated (Chung et al., 2011b).

We were interested in the ADCC responses in our SM cohort. The uniqueness of the SM cohort has been discussed in detail in Chapter 2 (Materials and Methods) – the subjects are very closely-related in terms of their genetics and all the study participants were infected by a similar strain of the virus. Therefore, we wanted to determine the influence of NK-cell mediated ADCC in our unique setting. We therefore wanted to explore three things. Firstly, we wanted to determine the sphere of ADCC activity in the SM cohort, with a particular focus on Vpu. Secondly, we wanted to identify any potential Vpu epitopes that mediate ADCC responses. Lastly, we wanted to determine if the ADCC responses in the SM cohort show any correlation with disease progression.

5.2 Results

5.2.1 Optimisation of the ADCC-ICS Assay

We employed the recently developed Kent lab ADCC-ICS assay at our lab. Before using our precious SM cohort samples, we used previously stored patient plasma samples to optimise the assay, as it has not been implemented outside the Kent lab setting, and was the first time being used at Oxford.

We initially screened 13 plasma samples and identified an ADCC responder – J68 – who had strong ADCC responses to Env Pool peptides. We used this patient plasma as a positive control in our experiments screening the SM Cohort to identify ADCC responders. Building up on the data from the Kent lab, our experiments reaffirmed that the ADCC activity is mediated by the antibody(s) present in the patient plasma, and if the patient plasma is substituted, the ADCC response is gone. Also, peptides are necessary for generating the ADCC response, and if peptides are not added to the reaction mix, the ADCC response also vanishes. Hence the assay requires both: a source of antibody (s) – in our experiments, this came from the patient plasma; as well as peptides. Figure 5 on the next page represents this control data which demonstrates the dual necessity of having both plasma and peptides present in order to have an ADCC response.

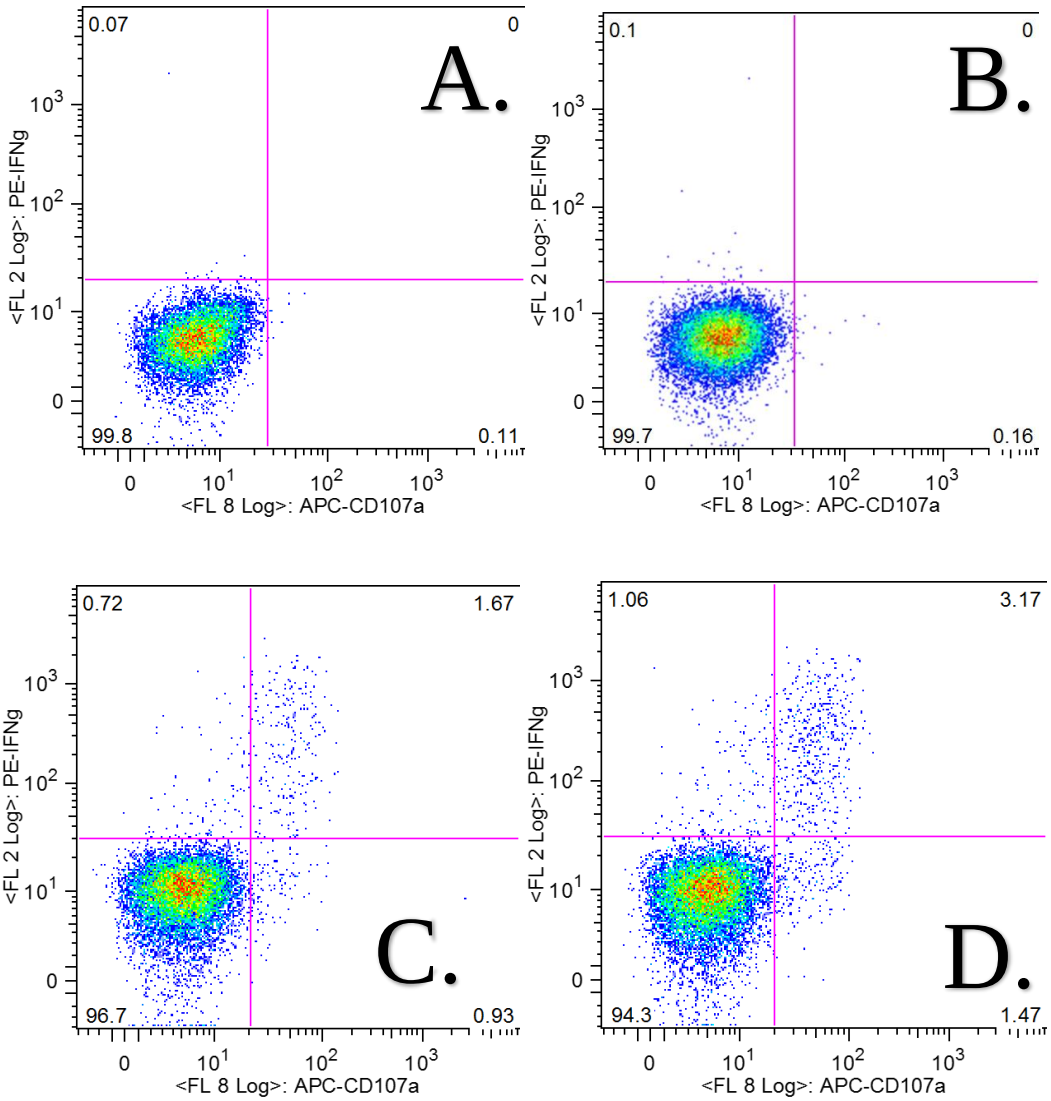


Figure 5:

ADCC responses to Env pooled peptides demonstrating the need for presence of both plasma (containing antibodies) and peptides for documenting a response. Responses are mapped on CD56+ and CD2+ CD3- NK cells expressing IFN-gamma and CD107a. SM 423 plasma was used for these experiments, and Positive Control used was Patient J68.

(A): Plasma from SM 423 not present. No response detected.

(B): Env pooled peptides not present. No response detected.

(C): SM 423 Plasma and Env peptides present. Positive ADCC response

(D): J68 Plasma (Positive Control) ADCC response with Env peptides

5.2.2 ADCC Responses in the SM Cohort

We employed the ADCC-ICS assay on 70 untreated plasma samples from our SM Cohort, which had been collected using convenient sampling in 2008.

We studied ADCC responses to Vpu and Env peptide pools using the ADCC-ICS assay. Patient responses were mapped by gating on the lymphocyte population analysing the expression of IFN-gamma and CD107a from CD56+ and CD2+ CD3- NK cells. Twenty-six (37%) subjects of the seventy assessed showed NK cells activated by the Env peptides, five of whom also showed responses to the pooled Vpu peptides (Figure 5.1 on the next page). We measured the IFN-gamma production here rather than cytotoxicity. No Vpu responses were seen in the absence of Env ADCC responses.

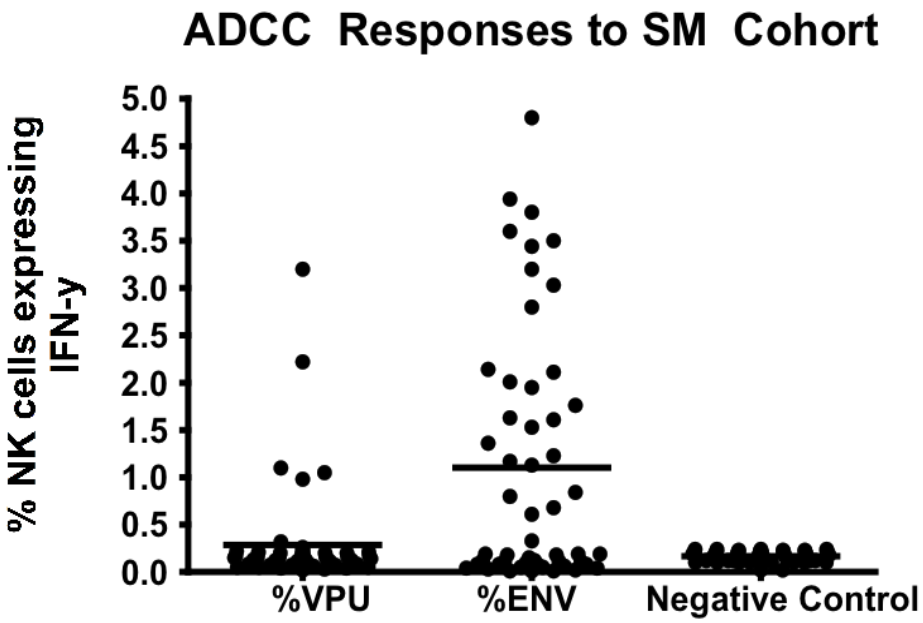


Figure 5-1:

Env and Vpu specific ADCC responses in the SM cohort. 70 subjects were analysed for ADCC responses.

5.2.2 ADCC Responses analysis using Clinical Data of the SM Cohort

We detected Env-specific ADCC responses in just under half of the sampled SM cohort. We next asked whether Env-specific ADCC responses correlated with markers of HIV disease progression. Env-specific ADCC responses, as measured by NK cells expressing IFN-gamma in response to Env peptides, were correlated to viral loads and CD4 T-cell counts were assessed. No correlation was evident between the subject's baseline viral load or CD4 T-cell count and ADCC responses to Env using the ADCC-ICS assay (Figure 5-2 and 5-3 on the next pages). Analysis of correlation between CD4 counts and viral load with percent IFN-gamma expression of NK cells to Env peptides were evaluated using the linear regression test.

We also conducted an analysis stratifying the ADCC responders and non-responders in the SM cohort, and tried to determine if there were any baseline differences in viral loads and CD4 T-cell counts in the two groups. No statistically significant associations were found with viral loads ($p=0.25$) or CD4 T-cell counts ($p=0.85$).

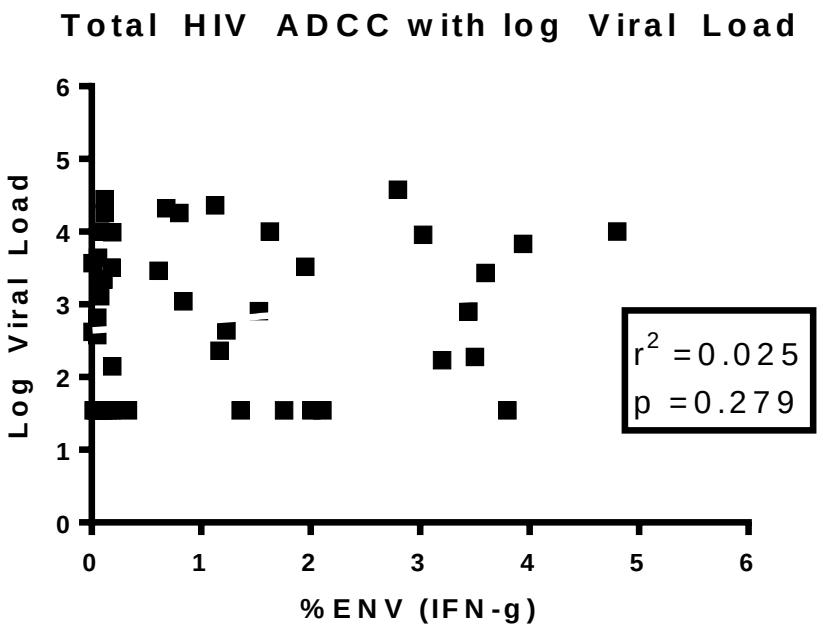


Figure 5-2:

Correlation of Env-specific ADCC responses with viral load data for the SM cohort. No correlation was evident with viral loads against Env pooled peptide ADCC responses

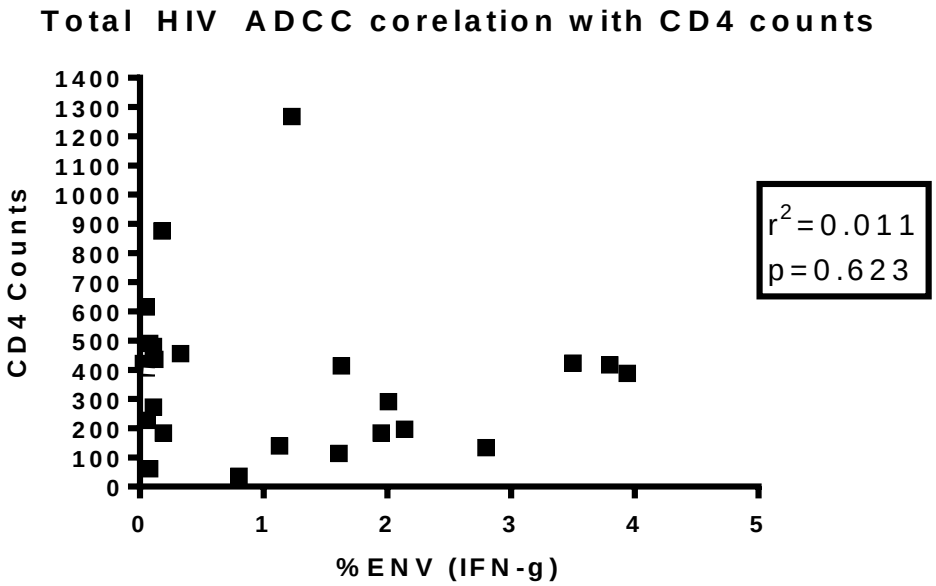


Figure 5-3:

Correlation of Env-specific ADCC responses with CD4 counts for the SM cohort. No correlation was evident with CD4 counts against Env pooled peptide ADCC responses

5.2.3 Delineating Env ADCC responses in the SM Cohort

We had used pooled Env peptides which span all of Env to screen for ADCC responses to Env in the SM cohort. We were interested to see which part of Env is responsible for producing ADCC responses. We formulated two pools, the first one spanning Env peptides 1-105, and the second one spanning Env peptides 106-211, and conducted the ADCC assay on 10 of the Env responders from our SM cohort, who had been chosen randomly (Figure 5-4 on the next page).

In the ten subjects, nine (90%) had responses to the first Env pool, spanning peptides 1- 105. We only had one patient who did not respond to the first pool, and had ADCC responses to the second pool.

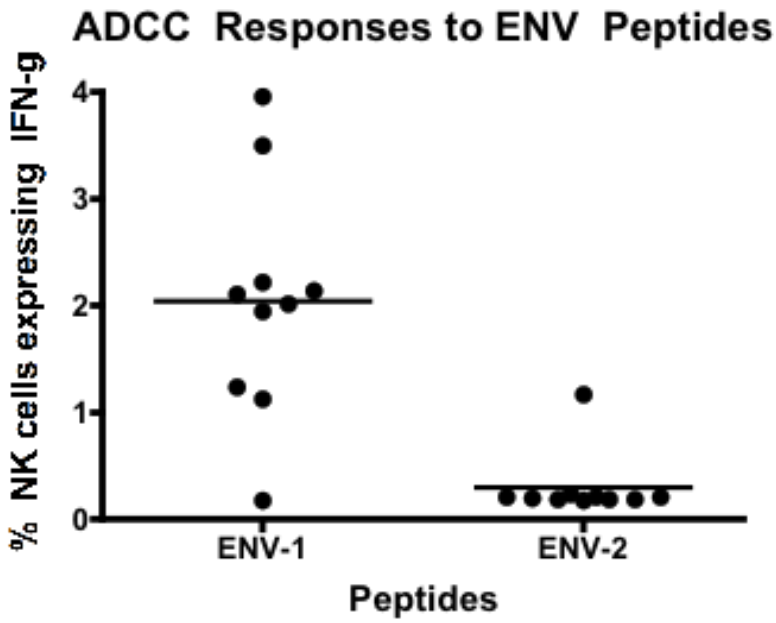


Figure 5-4:

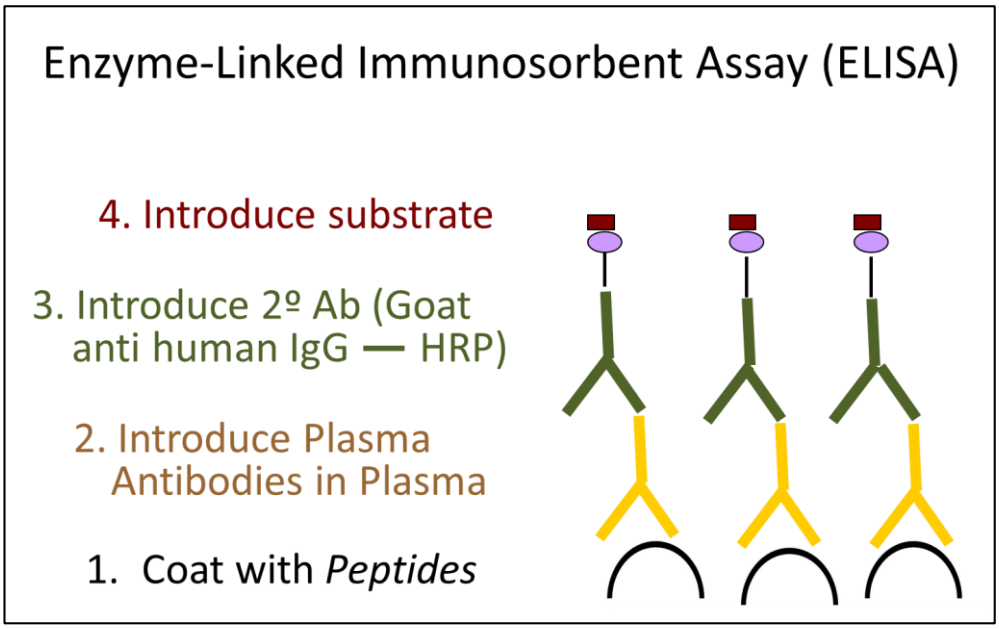
ADCC responses to Env peptides in the SM cohort. Two pools of Env peptides were used to determine ADCC responses to Env peptides – Env-1 spanned Env peptides 1-105 and Env-2 spanned Env peptides 106-211. Ten randomly chosen ADCC responders from the SM cohort were tested and nine had responses to the Env-1.

5.2.4 Delineating ADCC responses in the SM Cohort

As we had very limited plasma, it was not possible to run the ADCC-ICS assay using lone peptides each time – hence we went about developing and optimising an Enzyme-linked Immunosorbent Assay (ELISA) to aid us in identifying peptides that are responsible for the ADCC activity, by identifying those able to bind to antibodies in patient plasma (Table 5-1 below).

Table 5-1:

Principle of the screening Enzyme-linked Immunosorbent assay for identifying Vpu peptides responsible for mediating ADCC.



5.2.5 Delineating Vpu ADCC epitopes in the SM Cohort using an ELISA-based method

After we had optimised our Enzyme-linked Immunosorbent assay, we tested the five Vpu responders and screened them using the 19 Vpu peptides. The ELISA screen revealed Vpu peptides which we then tested in the ADCC-ICS assay.

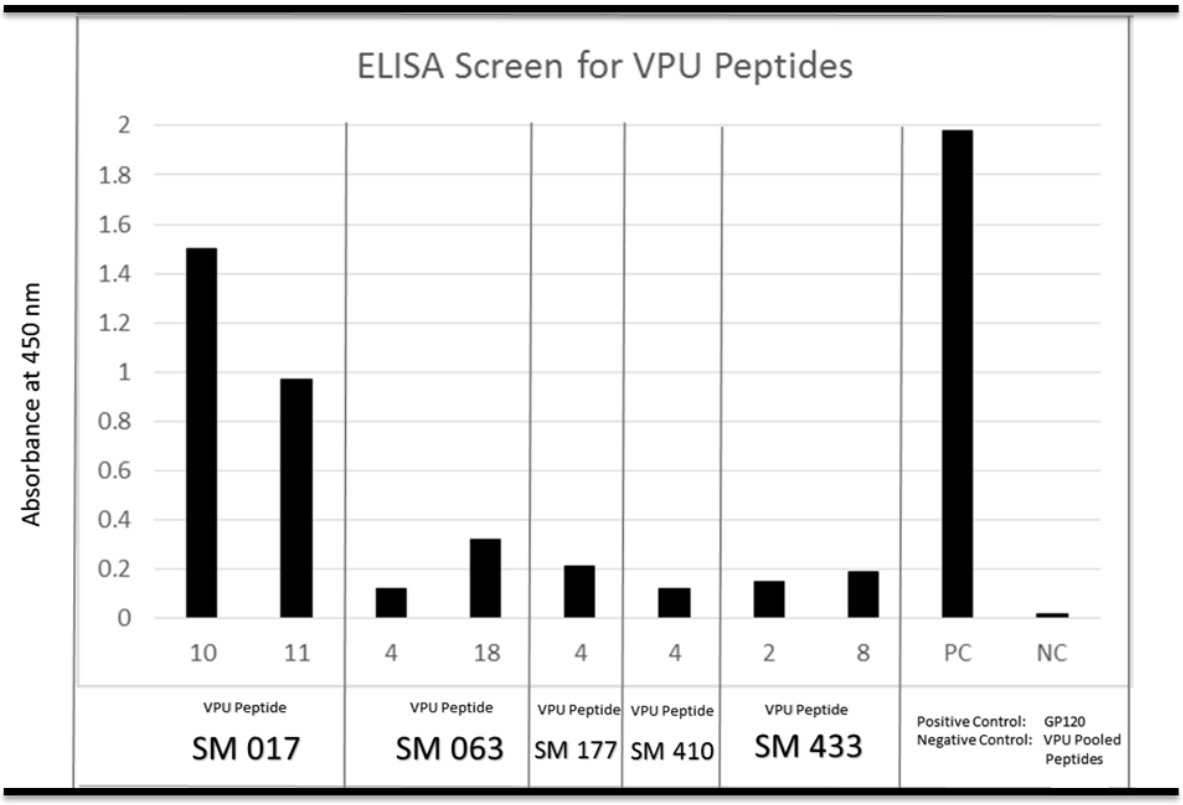


Figure 5-5:

We conducted an ELISA screen using 19 Vpu peptides for each of the 5 Vpu ADCC responders. Peptide 4 was the most commonly identified peptide, with 3 subjects showing ELISA responses.

5.2.6 Delineating Vpu ADCC responses in the SM Cohort using an ELISA-based method

As we had very limited plasma, we pioneered the ELISA-based approach for identifying Vpu epitopes and tested the peptides which were highlighted by the ELISA results. The Vpu peptides identified using the ELISA-method produced responses in the ADCC-ICS assay, helping us to identify which Vpu peptides were responsible for mediating Vpu ADCC activity in the 5 SM cohort Vpu responders.

Figure 5-6 on the next page details the ADCC results.

Table 5-2 details the Vpu epitopes identified.

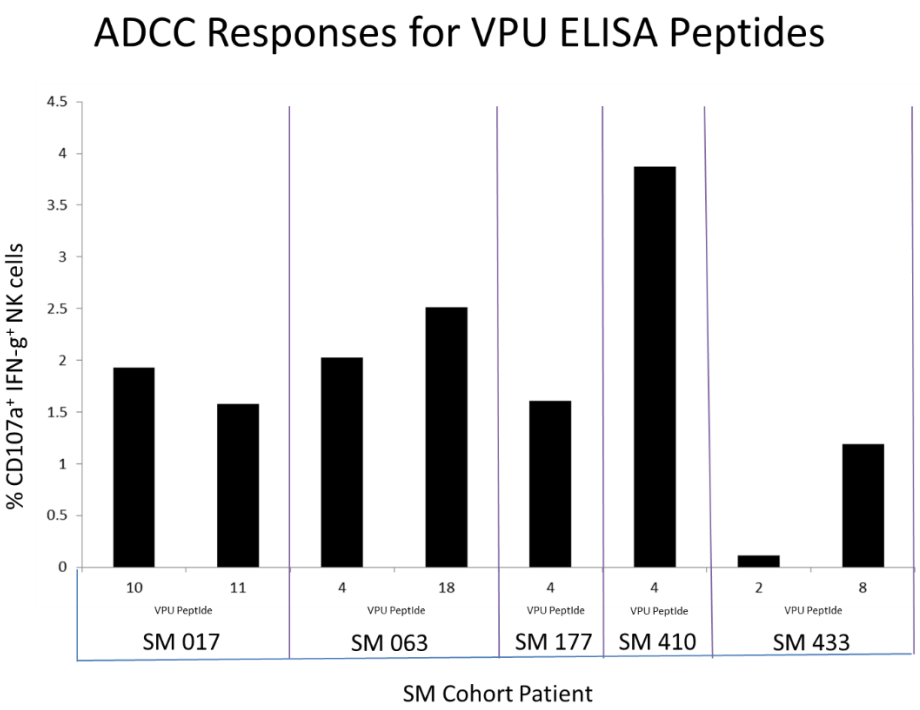


Figure 5-6:

ADCC responses to Vpu peptides in the SM cohort, identified using the ELISA method.

Table 5-2:

Sequences of the Vpu ADCC epitopes in the SM cohort

Vpu Peptide	B Clade Sequence
2 (9-mer)	QILAIVALV
4 (9-mer)	VVAAIIAIV
8 (15-mer)	IVFIEYRKILRQRKI
10 (15-mer)	ILRQRKIDRLIDRIR
11 (15-mer)	RKIDRLIDRIRERAE
18 (15-mer)	SALVEMGHHAPWDVD

5.3 Discussion

We have earlier looked at HLA correlates, and Vpu T cell responses in our SM cohort, which did not reveal any significant associations with clinical characteristics. However, we identified amino acid position 14 that was significantly associated with KIR2DS1. Therefore, we were very interested to look at ADCC responses in the SM cohort, particularly in relation to Vpu.

The ICS-based ADCC assay helps to overcome the variability associated with previous cumbersome ADCC assays which involved radioactivity and significant labour and time-intensive strategies. Before screening our SM cohort for ADCC responses, we employed and undertook extensive analysis of different parameters which could influence the ADCC assay. The assay needs both a source of antibody(s) and peptides, and if any of these two critical parameters is missing, the ADCC response will not happen (Figure 5). The assay is quite robust, and little variability was found when different donors were used for providing fresh whole blood as a source of NK cells. However, the same donor was used as a source of NK cells for our study. Dose titrations were not performed at the initial screening stage for determining ADCC responses in the SM cohort, and neat whole plasma was used. The gating strategy involved mapping NK cells which were CD2+, CD3- and CD56+. It has been shown that CD56 down-regulation happens in chronic HIV infection (Stratov et al., 2008), but the ICS-based ADCC assay uses fresh whole blood plasma from HIV-negative donors, and so this problem of CD56 down-regulation is evaded. The HIV-negative donors we tested were virtually all CD56 positive, and the donor used for the source of NK cells was also CD56+. It has also

been shown from other cohorts that it is the antibody in the plasma of the patients which is mediating the ADCC activity, and if the plasma is removed, the ADCC activity is lost (Chung et al., 2009, Stratov et al., 2008, Johansson et al., 2011). We used primarily peptides for our studies in place of protein, and the main reason for this is the lack of any Vpu proteins present for commercial use.

In this study, we investigated ADCC immune responses amongst a Slow Progressor cohort. We analysed 70 subjects and found that 26 subjects (37%) had ADCC responses to linear Env peptides, five of whom also had responses to Vpu peptides. We then went on to correlate total ADCC responses with viral load and CD4 counts, and no correlation was observed for any of these analysis.

The C1 region of Env has been shown to be a common target of ADCC antibodies (Ferrari et al., 2011). The human monoclonal antibody A32 has been reported to recognize a conformational epitope involving the C1 and C4 gp120 regions following Env binding to CD4 (Moore and Sodroski, 1996). Therefore, it is not surprising that Env is a common target for ADCC antibodies, and quite a big proportion of the SM cohort has ADCC responses against Env pooled peptides. We found that 9 out of 10 subjects (Figure 5-4) analysed had ADCC responses to Env peptide pools 1-105, which contains the C1, C2 and C3 regions. However, the ADCC-ICS assay would have missed responses to conformational epitopes.

Studies from other cohorts corroborate our findings. ADCC responses studied in a cohort of 79 HIV-positive patients revealed the strongest and the commonest ADCC

responses were to the Env peptide pool, with 54 of the 79 responders targeting this antigen (Chung et al., 2011b). More recently, ADCC responses to Env peptides were found in 40 long-term slow progressors from a total of 65 long-term slow progressors (Wren et al., 2013). We found ADCC responses to Env peptides in almost 37% of the cohort, with 9 out of 10 sampled subjects mapping to Env peptides 1-105.

We were most interested in the Vpu ADCC responders. We identified 5 subjects who had responses to the pooled Vpu peptides. Vpu is a multifunctional protein that is expressed partly within the cell membrane and therefore at least part of the Vpu protein may be accessible to ADCC antibodies (Subbramanian and Cohen, 1994, Nomaguchi et al., 2008, Ruiz et al., 2010). Vpu is expressed early in the HIV replication cycle, and ADCC-mediated NK-cell recognition of Vpu-expressing cells may restrict the release of new virions. It is worth postulating that Vpu can be presented in such a manner that elicits functional and effective ADCC responses – thereby Vpu ADCC antibodies might play a crucial role in protection against HIV infection. Interestingly, it has been shown that Vpu-specific antibody responses detected by Western blot in 51 HIV-infected patients were associated with improved prognosis following HIV-1 infection, and seroconversion of anti-Vpu antibody in patients on HAART indicates significant recovery of immunity (Chen et al., 2003).

We were limited by the amount of plasma we had for our SM cohort, and samples were extremely limited, particularly as they were collected back in 2008. Hence, we went about designing an elegant Enzyme-linked Immunosorbent Assay (ELISA)

which allowed us to identify ADCC Vpu peptides without utilising copious amounts of plasma. The assay utilises the principle that antibodies in the patient plasma will bind to peptides coated on the ELISA plate, which are then read by conjugating to a secondary antibody (Goat Anti-human IgG). We tested our ELISA system with Env peptides before applying them to our study to elucidate Vpu ADCC peptide responses.

The ELISA screen revealed in the five Vpu ADCC responders – SM 017, SM 063, SM 177, SM 410 and SM 433 – six Vpu peptides, with Peptide 4 being the most common and occurring in 3 subjects. When the ADCC-ICS assay was done using the peptides identified by the ELISA screen, ADCC responses were documented using the identified Vpu peptides. This validated our ELISA tool, and allowed us to save on precious SM cohort plasma, as otherwise we would have needed to run all the Vpu peptides on each of the subjects, something which our limited plasma stocks did not allow. It was interesting to note that even though the peptides identified by the ELISA screen were the ones which were targeted by the Vpu ADCC responders, there was no quantitative correlation between the ELISA results and the ADCC frequencies. This essentially demonstrates the complex nature of antibody interactions, which are expected to have different avidities and different mechanisms when playing their role in ADCC interactions.

Previously, Vpu peptides 3, 7 and 19 have been identified as epitopes mediating ADCC (Chung et al., 2011a). Another study has shed light on the common 11-amino-acid sequence shared between the 18th and 19th peptides of the Vpu pool,

namely EMGHHAPWDVD as the implicated epitope mediating Vpu ADCC (Stratov et al., 2008). More recently, the three overlapping peptide epitopes (peptides 7–8: VVWTIVFIEYRKILRQRKI, peptides 10–12: ILRQRKIDRLIDRIRERAEDSGN and peptides 18–19: SALVEMGHHAPWDVDDL) in Vpu were shown to be targeted in higher frequencies in a long-term slow progressor cohort (Wren et al., 2013). Therefore, it is interesting that our SM cohort also identified Vpu peptides 10, 11 and 18.

It needs to be kept in mind though that the mapping of ADCC responses using this ADCC-ICS assay is limited to linear epitopes that could be defined with individual peptides. Conformational ADCC epitopes within Vpu recognised by the SM cohort may also be of critical importance, but it is extremely difficult to map these. In addition, the number of SM cohort responders to Vpu peptide-specific ADCC responses is modest (7%) – this is to be expected as multiple other mechanisms might be at play in the SM cohort, which would be responsible for the slow progression of disease. Also, no correlation was seen to exist between the clinical data and the ADCC responses.

We used consensus NIH Vpu peptides for our ADCC assay and this inherently carries its own restriction. It is very much possible that we could have missed epitopes which might have undergone an escape mutation, which has been reported (Chung et al., 2011a). It would be worthwhile to use consensus peptides to chart out the ADCC epitopes, as that would also shed light on any ADCC escape that might be taking place as well.

However, we were greatly limited by the plasma we had, and this was a big limiting factor. When plasma was collected in 2008 for this study, we did not anticipate working on ADCC, and we had to be very careful with the way we used the plasma. Therefore, we were not able to map in detail the Env ADCC epitopes in the SM cohort, and also were limited in elucidating the Vpu epitopes. There are efforts underway to collect more plasma, so that future ADCC studies in the SM cohort do not encounter these restrictions. Having said that, the limited plasma availability also resulted in us pioneering the screening ELISA for identifying potential ADCC epitopes.

This study identifies an immune response associated within our cohort, primarily composed of slow progressors, although the ADCC response is not shown to be linked to the slow progression. However, we have identified patient plasma which has Vpu ADCC antibodies, and it would be extremely interesting to document the antibody-dependent cell-mediated viral inhibition mediated by these Vpu antibodies. The broad ADCC responses encountered in the SM cohort also warrant more detailed studies, to accurately gauge the effect ADCC has in this unique setting.

Chapter 6: ADCVI and Antibody responses from a cohort of Slow Progressors from China

6.1 Introduction

Antibody-dependent cell-mediated virus inhibition (ADCVI) results from an interaction between the target cell, the antibody, and an Fc receptor-bearing effector cell, very much like Antibody-dependent cell-mediated cytotoxicity (ADCC). However, as opposed to ADCC, ADCVI measures the effect of antibody and effector cells on virus output from the infected target cells (Forthal and Landucci, 1998). Therefore, the results from ADCVI assays reveal the percentage of virus inhibition as a consequence of the antibody being tested along with the effector cells, relative to a negative control antibody along with the effector cells.

Most of the antiviral effect of ADCVI is a consequence of target cell killing, and there is a great overlap between ADCVI and ADCC. ADCVI encompasses multiple effector functions, some related to lytic (e.g. ADCC) and some to non-cytolytic (e.g. production of chemokines) mechanisms, dependent greatly on the Fc-gamma receptors that may interfere with HIV infection and replication.

It has been reported that in individuals with acute HIV infection, ADCVI antibodies have been shown to develop as early as the first week after symptom onset or the first month after exposure (Forthal et al., 2001). The ADCVI response occurred with more or less similar timing to the cytotoxic T-cell response, but much earlier than reported for the neutralising antibody response (Gray et al., 2007). In addition, the

ADCVI antibody response was noted to have an inverse relationship with plasma viremia (Forthal et al., 2001). ADCVI antibodies also appear to be more broadly reactive with different strains than other antibodies measured in neutralisation assays.

Animal studies have demonstrated the critical nature of ADCVI antibodies in preventing lentivirus infection. It has been shown that SIV infection or immunisation of rhesus macaques elicits polyclonal and monoclonal antibodies that inhibit SIVmac251 by ADCVI (Forthal et al., 2006). In a series of very elegant experiments in which the broadly neutralising antibody b12 was mutated in the Fc-binding region, it was shown that to confer maximum protection after passive antibody infusion in macaques challenged with SHIV, ADCVI is required (Hessell et al., 2007).

An evaluation of the ADCVI responses in humans following rgp120 vaccination in the Vax004 trial which used a vaccine consisting of rgp120 from two laboratory-passaged strains of HIV-1, HIV-1_{MN}, and HIV-$1_{GNE8,}$ revealed an inverse correlation between HIV infection rate and ADCVI antibody activity measured against a clinical R5 isolate of HIV-1 (Forthal et al., 2007a). It was noted that for every ten percent increase in ADCVI activity, there was a 6.3% decrease in the hazard rate of infection, with a p value of 0.019. Therefore, even though there was no overall efficacy found in the Vax004 trial (Flynn et al., 2005), it is noteworthy that individuals with the most potent vaccine-induced antibody responses had some sort of protection.

Antibodies, which are found at more than 10^5 molecules per millilitre of blood and are secreted in gram amounts every day at mucosal surfaces, play a critical role in the host defences against HIV, and other infections (Klein et al., 2013, Corti and Lanzavecchia, 2013, Schiffner et al., 2013, McCoy and Weiss, 2013). The Y-like antibody structure is basically composed of a two-headed binding region and a constant stalk. The highly variable regions (termed Fab) confers specificity for the recognition of foreign antigens, and the conserved stalk (called Fc) defines the immunoglobulin isotype to which an antibody belongs. Antibody can prevent viral infection through both neutralising and non-neutralising activities. Neutralising antibody is the term used to denote an antibody that prevents infection through its Fab region recognising and blocking functions that mediate virus entry to cells. Protective non-neutralising antibody is the term used to describe an antibody that protects through the action of its Fc region initiating destruction of the offending pathogen by the innate immune response, which may involve the NK cells or other cellular effectors.

Receptors for the Fc segment of immunoglobulin G (IgG) (Fc[gamma] receptors; Fc[gamma]Rs) are expressed on the surface of a number of cells that are involved, or potentially involved, in HIV infection, including natural killer (NK) cells, monocytes, macrophages, dendritic cells, and neutrophils (Takai, 2002, Nimmerjahn and Ravetch, 2007). There have been five major Fc[gamma]Rs identified in humans: Fc[gamma]RI, Fc[gamma]RIIa, Fc[gamma]RIIb, Fc[gamma]RIIIa, and Fc[gamma]RIIIb (Takai, 2002). FcγRIIIa is encoded by a gene that confers either a valine (V) or phenylalanine (F) at amino acid 158 (Ravetch and Perussia, 1989). It has been shown in a study using individuals from

the Multicenter AIDS Cohort Study that the FcγRIIa RR genotype influences the rate of CD4+ cell decline and the risk of acquiring P. jiroveci pneumonia as an AIDS-defining illness (Forthal et al., 2007b), thus reaffirming the role for antibody–Fc[gamma]R interactions in modulating the course of HIV infection.

Historically, a lot of research has gone into identifying broadly neutralising antibodies, and these are considered to be the Holy Grail in tackling HIV infection. The initial group of antibodies against HIV-1 with broad neutralizing activity (bNAbs) defined some of the sites of weakness on the HIV-1 envelope spike: these sites included the third hypervariable domain (V3) loop of gp120 [e.g., the 447-52D antibody (Gorny et al., 1993)], the binding site for CD4, which is the primary receptor for viral entry [b12 (17)]; the CD4-induced site that is the viral binding site for the co-receptor [e.g., 17b (Burton et al., 1994)]; viral glycans [e.g., 2G12 (Trkola et al., 1996)]; and the membrane proximal external region (MPER) of gp41 [4E10 and 2F5 (Buchacher et al., 1994, Muster et al., 1993)]. The second generation of broadly neutralising antibodies have been found to target four major areas on the HIV-1 envelope spike: the CD4-binding site (CD4bs), the N160 glycan-dependent site associated with the V1/V2 loops, the N332 glycan-dependent site at the base of the V3 loop, and the MPER on gp41 (Kwong and Mascola, 2012).

Unfortunately, a broadly neutralising antibody inducing vaccine regimen has not been designed yet. However, there has been a recent surge in interest in antibodies capable of targeting HIV through neutralisation-independent mechanisms, especially antibodies capable of soliciting the help of the cells of the innate arm of

the immune system, particularly through NK cells and ADCC. This surge of interest is a consequence of the interesting results of the recent RV144 HIV vaccine trial in Thailand, which showed modest protection against HIV infection in the absence of strong cytotoxic T-lymphocyte responses or broadly neutralising antibody responses (Rerks-Ngarm et al., 2009). What is most critical and encouraging is that the vaccine regimen was able to induce antibodies capable of activating NK cells for ADCC (Karnasuta et al., 2005, Bonsignori et al., 2012b).

Antibodies capable of mediating ADCC have been found in HIV-1-exposed but uninfected Vietnamese intravascular drug users (Scott-Algara et al., 2003). Higher titres of ADCC antibodies have reported in elite controllers from the Multicenter French trial (Lambotte et al., 2009). These studies and others pertaining to ADCC have been mentioned in detail in the previous chapter.

It has been reported that the human monoclonal antibody A32 (Moore et al., 1993, Wyatt et al., 1995) recognizes a conformational epitope involving the C1 and C4 gp120 regions following Env binding to CD4 (Moore and Sodroski, 1996), and the A32 epitope is expressed on the surface of transmitted/founder (T/F) virus-infected CD4+ T cells beginning at day 3 of *in vitro* infection and can mediate potent ADCC activity with both virus-infected and gp120-coated CD4+ T cells (Ferrari et al., 2011). Equally importantly, MAb A32 Fab blocks the majority of ADCC antibody activity in plasma of subjects chronically infected with HIV-1, indicating that the A32-binding site is highly recognized by the antibodies naturally elicited during

HIV-1 infection and might significantly contribute to the overall ADCC antibody responses (Ferrari et al., 2011).

We have already looked at ADCC responses in our SM cohort, delineating Vpu ADCC specific epitopes in five subjects. We were interested to see whether these subjects' plasma also displays ADCVI activity, and explore further the antibody correlates for SM 017, a subject who has very interesting immune parameters.

6.2 Results

6.2.1 Optimisation of the ADCVI Assay

We employed the Forthal lab ADCVI assay with some slight modifications, finally settling on HIV NL4-3 as our infecting strain of virus, in our lab setting, as this was the first time the assay was being used at Oxford.

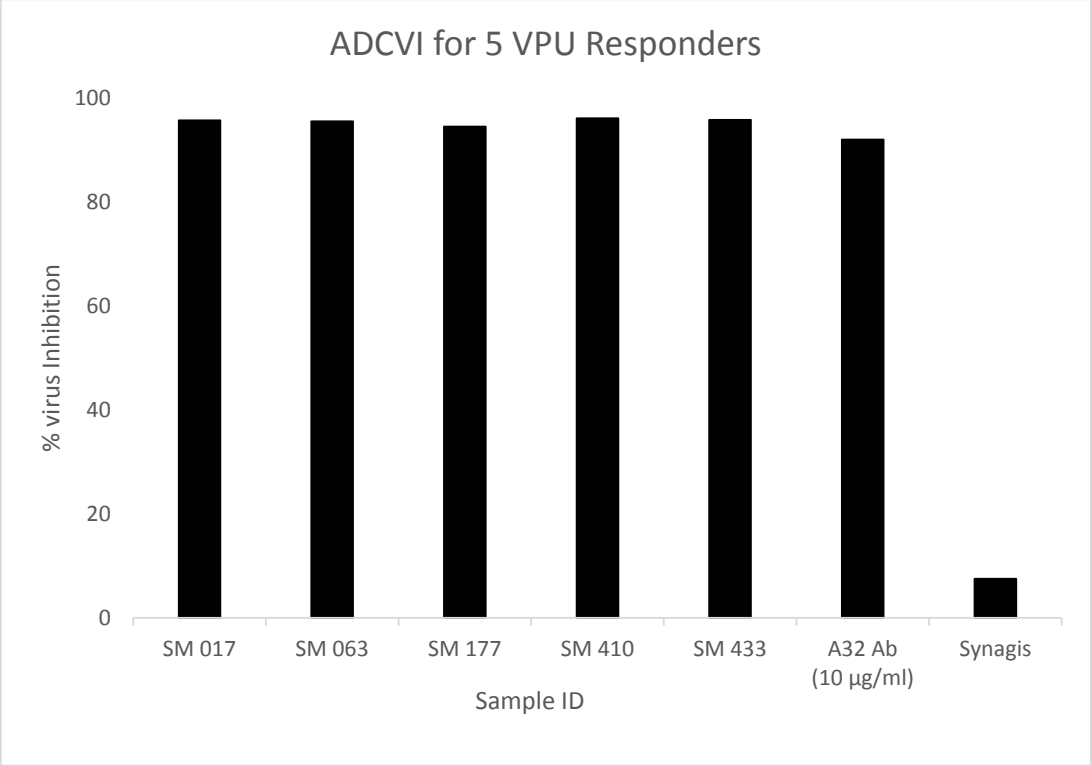
We also were provided with the A32 antibody – courtesy of Prof. Barton Haynes at Duke, which was used as a positive control for Env ADCC responses in optimising our assay. We used Synagis (Palivizumab, a monoclonal antibody) as a negative control. Seven days later after starting the incubation of the plasma with the CEM.NKR-CCR5 cells with a laboratory strain of HIV-1 (HIV NL4-3) at a multiplicity of infection of 0.05, supernatant fluid was collected, and p24 was measured by ELISA (Cambridge BioScience). The percent inhibition due to ADCVI was calculated relative to pooled HIV-negative serum as follows: percent inhibition = 100(1 − ((p24p)/(p24n))), where (p24p) and (p24n) are concentrations of p24 in supernatant fluid from wells containing a source of HIV-positive or -negative Ab, respectively. Plasma dilutions at 1:100 were used in the ADCVI assay.

6.2.2 ADCVI Reponses in the 5 Env/Vpu ADCC Subjects

After optimising the ADCVI in our setting, we were initially interested in the 5 SM cohort subjects who had demonstrated Env and Vpu specific ADCC responses.

We wanted to find out if the plasma from these subjects also mediated ADCVI activity. Therefore, we tested the plasma from these patients which we had also used previously for ADCC experiments, to determine if the antibodies in the plasma of these subjects also inhibit virus replication (Figure 6.1 on the next page).

All 5 subjects – SM 017, SM 063, SM 177, SM 410 and SM 433, showed % inhibition of more than 95%. Interestingly, the magnitude of the ADCC responses did not correlate with the ADCVI responses.



Sample No.	p24 Levels	% Inhibition
SM 017	0.05	95.7
SM 063	0.052	95.5
SM 177	0.056	94.5
SM 410	0.046	96.1
SM 433	0.048	95.8
A32 Ab (Positive Control)	0.044	92
Synagis (Negative Control)	0.95	7.5

Figure 6-1:

ADCVI responses to the 5 Env/Vpu ADCC SM cohort subjects. All 5 displayed strong inhibition. A32 antibody was used as a positive control and Synagis (Palivizumab, a monoclonal antibody) was used as a negative control. The percent inhibition due to ADCVI was calculated relative to pooled HIV-negative serum as follows: percent inhibition = 100(1 − ((p24p)/(p24n))), where (p24p) and (p24n) are concentrations of p24 in supernatant fluid from wells containing a source of HIV-positive or -negative Ab, respectively.

6.2.3 SM 017 ADCC response

We had been limited by the amount of plasma we had for the 5 Env/Vpu ADCC patients, as the plasma had been collected in 2008 and we had not anticipated the need for greater amounts of plasma. Therefore, we tried to trace the 5 subjects to obtain further plasma samples.

Unfortunately, only one subject – SM 017, came in for the repeat bleeding program from the five subjects in 2011, and so we were only able to collect plasma from SM 017.

Therefore, firstly we ran the SM 017 collected in 2011 in the ADCC-ICS assay. To our great surprise, SM 017 from 2011 did not demonstrate any ADCC responses (Figure 6.2 and 6.3 on the next pages).

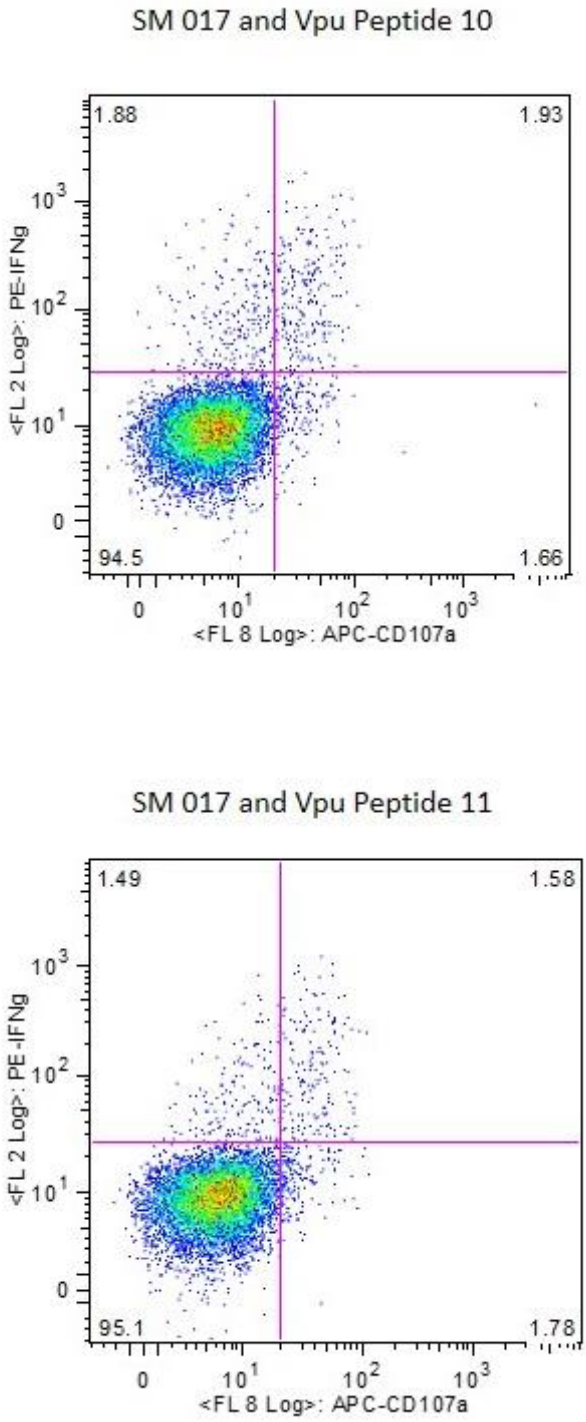


Figure 6-2:

ADCC responses to Vpu pooled peptides to SM 017 in the SM cohort from sampling done in 2008. Responses are mapped on CD56+ and CD2+ CD3- NK cells expressing IFN-gamma and CD107a

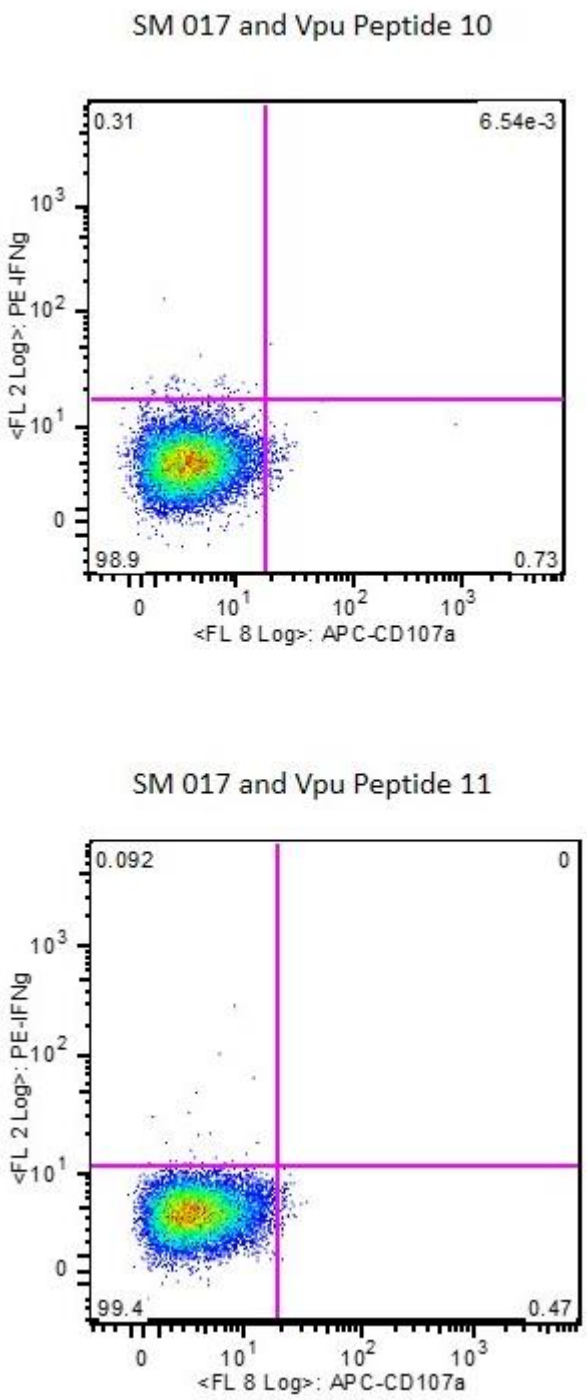


Figure 6-3:

ADCC responses to Vpu pooled peptides to SM 017 in the SM cohort from sampling done in 2011. Responses are mapped on CD56+ and CD2+ CD3- NK cells expressing IFN-gamma and CD107a. No responses were found.

6.2.4 SM 017 ELISA response

SM 017 plasma from 2011 did not display any ADCC responses, contrary to what we expected. To make sure that there was no sample mix-up and to determine if the antibody was still present in the SM 017 plasma from 2011, we ran the ELISA we had designed on SM 017 from both time points using all the Vpu peptides.

Interestingly, the ELISA responses were very similar between the two time points, and stronger responses were revealed to Peptides 10 and 11 from the sample collected in 2011 (Figure 6.4 on the next page).

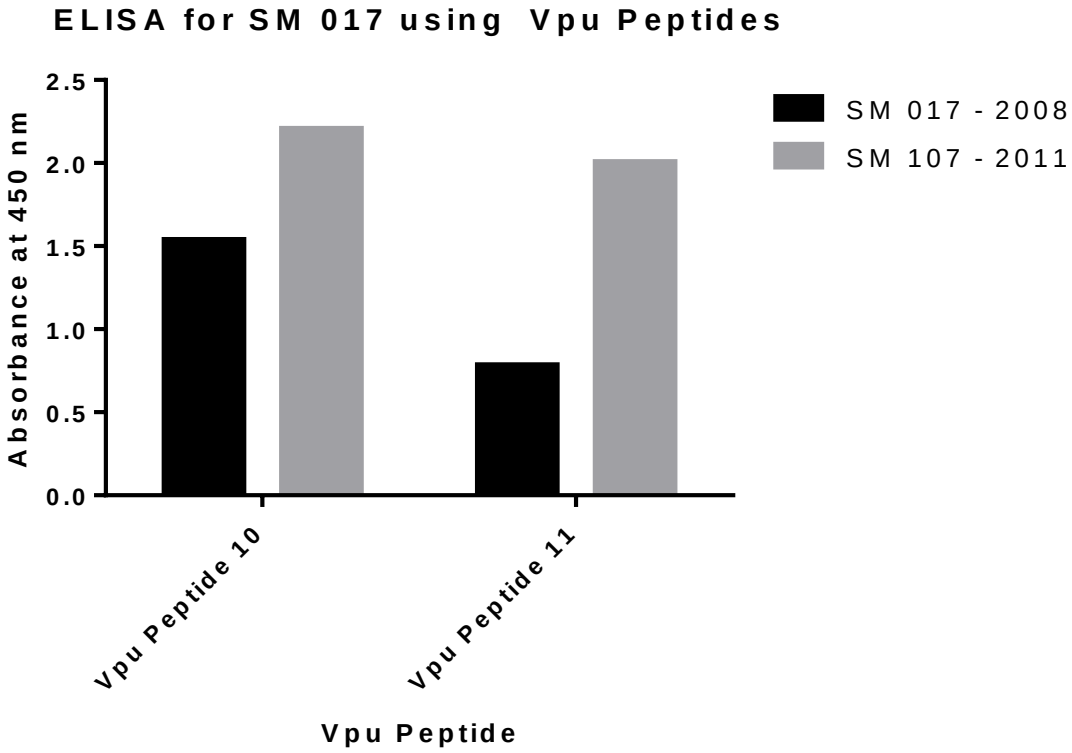


Figure 6-4:

We conducted an ELISA screen for SM 017 from 2008 and 2011. Responses were seen to the same peptides, Peptide 10 and 11, from plasma from both years, although responses from SM 017 – 2011 are stronger compared to the responses seen for SM 017 – 2008.

6.2.5 SM 017 ADCVI response

The ELISA revealed that indeed there was still Vpu-binding antibody (s) present in the plasma collected from 2011. We then used the plasma from SM 017- 2011 and tried to determine if it leads to virus inhibition in the ADCVI assay.

Interestingly, SM 017 from 2011 revealed 96.3% inhibition, compared to 95.1% for SM 017 from 2009 (Figure 6-5 below).

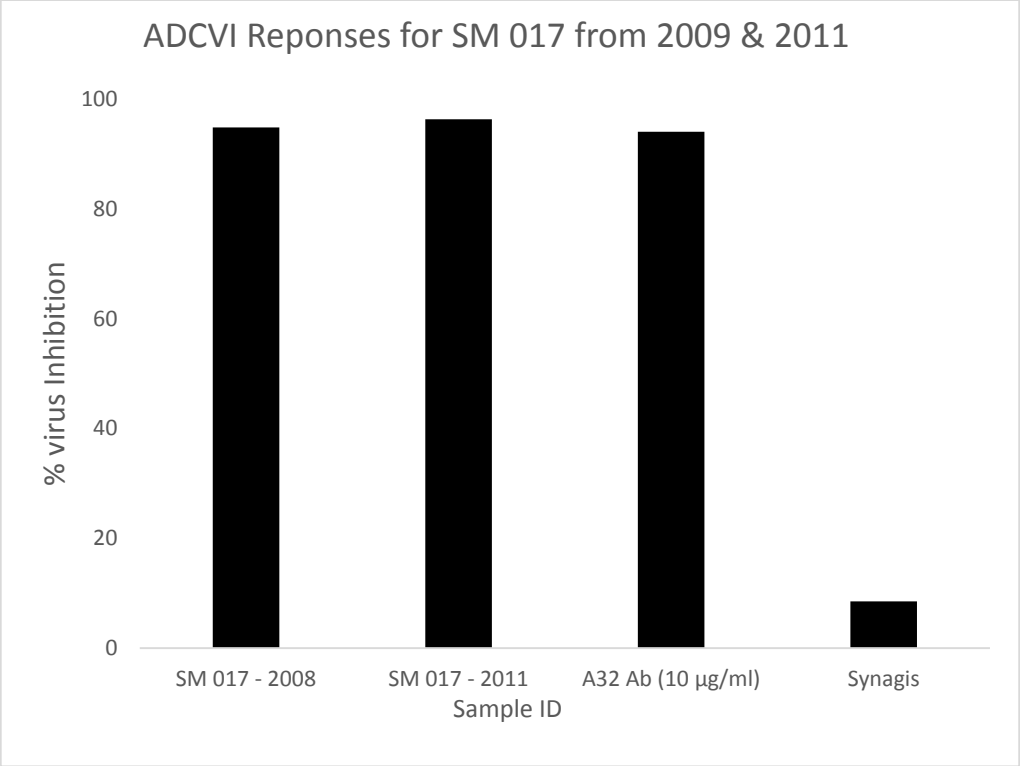


Figure 6-5:

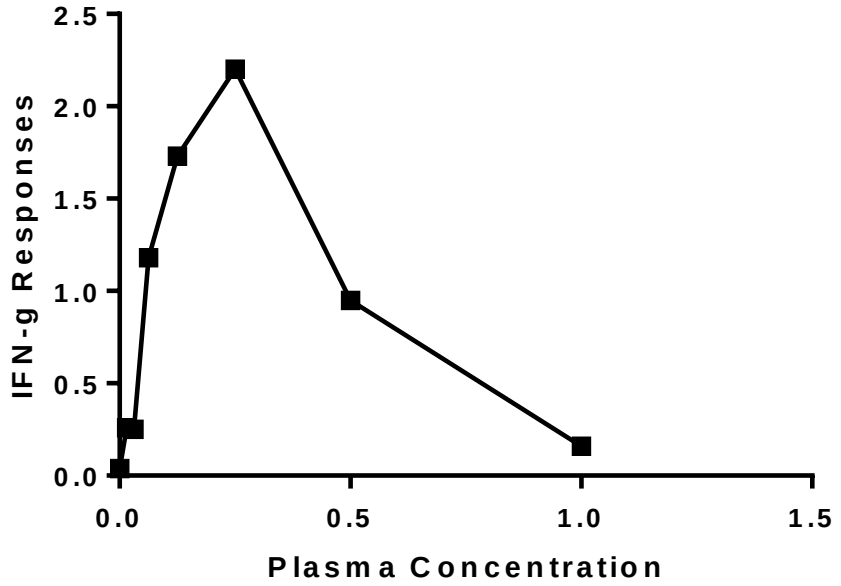
ADCVI responses comparing SM 017 from the years 2011 and 2009. Both displayed strong inhibition. A32 antibody was used as a positive control and Synagis was used as a negative control.

6.2.6 SM 017 Sequencing and Plasma Dilution ADCC

We were puzzled as to why the ADCC response for SM 017 2011 plasma was negative when Vpu binding assays were positive by ELISA and viral suppression occurred when SM017 plasma was incubated with donor NK cells. One possibility was that the Vpu epitopes may have mutated to evade ADCC responses, as has previously been described (Chung et al., 2011a). We sequenced Vpu for SM 017 from 2011, and did not see any sequence variation, compared to SM 017 from 2008 across the whole Vpu protein.

The ELISA and the ADCVI data have demonstrated that SM 017 indeed has antibody (s) present. In addition, the ELISA revealed that the antibody responses are quite strong. Therefore, we conducted the ADCC assay using dilutions of SM 017 – 2011 plasma rather than neat plasma, as was our standard protocol.

Interestingly, the ADCC response became evident at dilutions of plasma of more than 1:2, reaching a peak at 1:4 (Figure 6.6 on the next page).



Plasma Dilution	IFN-g Responses
No Dilution (1)	0.18
1/2 Dilution (0.5)	0.92
1/4 Dilution (0.25)	2.34
1/8 Dilution(0.125)	1.73
1/16 Dilution (0.0625)	1.24
1-32 Dilution ((0.03125)	0.25

Figure 6-6:

ADCC responses to Vpu pooled peptides to SM 017 in the SM cohort from year 2011. Responses are mapped on CD56+ and CD2+ CD3- NK cells expressing IFN-gamma, with serial plasma dilution.

6.3 Discussion

We had earlier screened our SM cohort samples collected in 2008 for ADCC responses and had identified five patients who had ADCC responses to Env and Vpu peptides. We were interested to see whether the plasma from these 5 patients also inhibits virus in the ADCVI assay.

In this study, we looked at ADCVI responses in 5 SM cohort patients who had demonstrated ADCC responses to Env and Vpu peptides in our ADCC assay. We optimised the ADCVI assay in our lab setting, and had used A32 antibody as a positive control. All 5 of the ADCC responders to Env/Vpu had very similar inhibition of virus – nearing 95 %.

We hypothesise that these 5 SM cohort members have antibodies targeting Env and Vpu proteins. HIV-1 Env encodes the only surface-expressed viral protein of HIV, a glycoprotein of 160 kD (gp160), which after translation is cleaved into gp120 and gp41, which remain non-covalently linked to formulate a single subunit of a trimeric 'spike' on the virion surface (Arrildt et al., 2012, Caffrey, 2011, Pinter, 2007). The C1 region of Env has been shown to be a common target of ADCC antibodies (Ferrari et al., 2011). There is otherwise very limited data available apart from this, and antibodies relating to Vpu ADCC responses have not been identified yet.

We were unable to fully map Env-specific ADCC responses in the SM cohort because we were limited by the volumes of plasma available and the large number of overlapping peptides spanning Env. In addition, we were more interested in the Vpu responses, as Vpu has been a focus of intense research efforts since the discovery of tetherin (Perez-Caballero et al., 2009), and we have explored in detail Vpu correlates in the SM cohort. In addition, it is worth mentioning that the large diversity of infecting Env strains, the ability of Env to readily escape antibody responses, and the limited apparent fitness costs of Env variants potentially makes Env a less attractive target than other HIV proteins (Chung et al., 2011a, Peut et al., 2009, Peut and Kent, 2007).

Therefore, we went about trying to follow-up on those 5 ADCC Env/Vpu responders and tried to collect plasma from them for more detailed investigations. Unfortunately, only 1 out of the 5 patients turned up for the follow-up bleed for sample collection in 2011, and we were therefore only able to collect samples from SM 017 in the follow-up sample collection in 2011. This demonstrates the difficulties encountered in getting samples, as the SM cohort is very remote and access requires multiple levels of coordination, sometimes with great effort and limited results.

After getting plasma from SM 017 from the new sampling year, we ran the plasma in the ADCC assay and to our surprise, the ADCC results were negative, with no response whatsoever. We were surprised and thought that perhaps there would be

viral escape happening through mutation, and that might explain the lack of ADCC responses from a later time point for the same SM cohort subject.

Immune escape from HIV was first shown for CTL-based immune responses (Phillips et al., 1991). CTL escape involves complex parameters which normally focus on the effect of the escape mutation on comparative viral fitness, demonstrating the interplay between mutations which cause advantages and which cause deleterious effects on the virus. (Carlson and Brumme, 2008, Stevceva et al., 2007, Goulder and Watkins, 2004). Some CTL immune escape variants have reduced replicative capacity of the virus that slows down the progression of disease (Fernandez et al., 2005, Leslie et al., 2004). Escape from neutralising antibody responses often involves changes in glycosylation patterns and different insertions and deletions (Richman et al., 2003, Wei et al., 2003). There is continuous viral escape and selection by autologous neutralizing antibodies (Mahalanabis et al., 2009, van Gils et al., 2010), and studies have identified multiple escape pathways varying from patient to patient and over the course of infection (Moore et al., 2009, Rong et al., 2009b)

More recently, non-neutralising ADCC antibodies have been shown to force HIV escape mutations (Chung et al., 2011a). By sequencing a large series of linear ADCC epitopes and sequencing autologous HIV RNA across epitopes, the authors were able to find multiple ADCC antibodies that no longer could recognise autologous viral sequences. Viral sequences from very early in infection were shown to be recognised more strongly by recent plasma samples, suggesting evolution of escape

over time. Immune escape was identified initially using ADCC peptide epitopes and an NK cell-activation assay and therefore confirmed using whole Env proteins and a standard killing based assay (Chung et al., 2011a).

Therefore, when we first did not see any ADCC responses, the notion of escape crossed our minds. We sequenced Vpu for our sample collected in 2011 and did not find any mutations, which made us quite perplexed. However, when we ran the ELISA on the sample we were surprised to find responses to Vpu peptides 10 and 11, similar to SM 017 plasma collected in 2008. In fact, the responses were found to be stronger compared to the sample from a couple of years ago, when the ELISA was done using both samples together. In addition, the ADCVI assay showed similar inhibition for SM 017 – 2011, to the sample SM 017 – 2008, both demonstrating >95% inhibition. Therefore, the plasma from SM 017 from 2011 definitely had antibodies, which we were not picking up in the ADCC assay.

Studies have demonstrated that positive outcomes in the context of HIV infection are usually observed in individuals with high titres of antibodies (Lambotte et al., 2009). In addition, ADCC antibody titres in breast milk have been shown to be directly associated with protection against HIV transmission in infants born to HIV-infected mothers (Mabuka et al., 2012). Recently, it has been demonstrated that the magnitude of the ADCC response observed is proportional to the quantity of anti-Env antibody bound to the surface of HIV ADCC target cells (Smalls-Mantey et al., 2012). This leads to the suggestion that it would be beneficial for the high titres of antibody to be composed of a polyclonal antibody population. This would then allow

the clustering of antibodies directed against different Env epitopes, and the vaccine regimen used in the RV 144 trial also induced ADCC-competent antibodies against several Env epitopes (Bonsignori et al., 2012b).

Therefore, working on the hypothesis that SM 017 had higher titres of antibody developing over the years, we re-ran the ADCC experiment with some slight modifications. Instead of using neat plasma, we diluted the plasma sequentially, and then determined whether there was any ADCC activity taking place.

Interestingly, the response in SM 017 – 2011 started to appear with sequential dilutions of the plasma, reaching a peak at a concentration of 1:4 and thereby falling thereafter. This is, to our knowledge, the first instance where ADCC responses have been shown to have a prozone effect using the ADCC-ICS assay. The prozone phenomenon has been observed in ADCC responses in infectious mononucleosis (Hewetson et al., 1979), HTLV-1 (Sinclair et al., 1988) and coronavirus 229E-infected cells (Holmes et al., 1986). We hypothesise that the prozone effect in the ADCC-ICS assay reflects the competition for Fc receptors and/or blocking of cytotoxicity. We therefore hypothesise that the ADCC antibody titres have increased in SM 017 over the years, to augment the ADCC response *in vivo*.

It is worth mentioning here that as we did not dilute the plasma when we were screening the SM cohort for ADCC responses, and we could have potentially missed any ADCC responders. However, we documented ADCC responses to Env peptides to 26 of the cohort members (37%), with only five Vpu responders.

Similar proportions have been reported from ADCC responses from other cohorts, with the commonest responses directed to the Env peptide pools (Chung et al., 2009, Wren et al., 2013, Chung et al., 2011b). However, with the phenomenon of the proozone effect, we would like to advocate using different dilutions of plasma in the ADCC ICS assay for future studies. We might have missed potential ADCC responders in our screening analysis because of the lack of dilution studies. Having said that, the focus of our studies was to identify potential ADCC responses to Vpu, and we were fortunate to identify five Vpu responders, which therefore allowed us to study their immune parameters in detail. For future studies, a strategy which includes using plasma/serum dilutions will be a definite addition to any ICS-based assay experiments.

We present here exciting data which demonstrates the presence of ADCC antibodies in our 5 Env/Vpu ADCC responders from the SM cohort. Most interesting is the prozone effect seen in SM 017 which may reflect increasing titres of ADCC antibodies over time. Plans are now underway to purify the Env/Vpu specific ADCC antibody (s) using peptide-labelled beads, and to determine whether it is the Vpu or Env ADCC antibodies mediating the ADCC/ADCVI response, or whether it is a polyclonal antibody response at work.

Chapter 7: Summary and future directions

UNAIDS estimates in 2011 put the total number of people living with HIV infection at a total of 34.2 million, with about 780,000 people living in China (reviewed in Chapter 1). Multiple host, environmental and virological factors account for the heterogeneity in HIV infection susceptibility and disease progression, giving rise to a complex picture with multiple immune parameters at play.

Our unique SM cohort comprises closely related individuals in rural China who were infected with a very narrow range of clade B HIV-1 strains following plasma donation in the early 90s (Dong, Zhang et al. 2011). Most members of this cohort did not get anti-retroviral therapy (ART) during the first ten years of infection, therefore the confounding effect of ART on the outcome of HIV infection is virtually negligible. However, there is very little information on the events that took place during that 10-year period although there are records of several individuals having died after displaying symptoms compatible with AIDS. This suggests that from the original group of infected subjects, only Slow Progressors would have survived to date, which makes this cohort a unique setting for studies aimed at identifying factors influencing the course of HIV infection.

As the HLA Class I region has been shown to have the strongest genetic association with durable control of HIV (Fellay, Shianna et al. 2007; Dalmasso, Carpentier et al. 2008; Limou, Le Clerc et al. 2009), we set out to investigate the HLA correlates for our unique SM cohort (Chapter 3). We hypothesised that the enrichment of some

HLA alleles in the SM cohort would be the result of either a survival advantage or increased susceptibility to HIV infection. We recruited a neighbouring Han village cohort for comparisons, and employed stringent statistical tests, that utilised clinical data for our SM cohort as well.

Our results (Chapter 3) revealed that there was no enrichment of any specific HLA alleles in the SM cohort. This may be due to the limited study size. However, it is worth mentioning here the lost protective effect for HLA-B*51 which has been described for the Japanese population, because of a major escape mutation that occurred in one of the most dominant HLA-B*51 restricted epitopes (Pol 283-289) in the prevalent HIV-1 populations (Kawashima, Pfafferott et al. 2009). Similar to the Japanese population, our HIV +ve Cohort has also undergone mutations in three dominant HLA-B*51 restricted epitopes: Gag 327-345 (NI9) NANPDCKTI, Pol 743-751 (LI9) LPPVVAKEI and Pol 283-289 (TI8) TAFTIPSI (YongHong Zhang, WeiHua Li et al. 2011). We did however find B*57 to be enriched in the Elite Controllers sub-group of our SM cohort, although this was not statistically significant, which could imply that traditionally protective HLA may not hold true for diverse populations, although this may also be due to small numbers. The clinical data also did not show any correlation with the HLA dataset for the SM cohort (Rai, Zhang et al. 2013).

HIV type 1 infection is characterised by an early peak of viremia that declines coincident with the development of strong Cytotoxic T lymphocytes (CTL) responses (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994; Addo, Yu et al.

2002; McMichael, Borrow et al. 2010). CTLs recognise antigenic peptides displayed by MHC Class I molecules on the infected cell surface and represent a major selective driving force driving HIV evolution. Most studies have focused on the assessment of immune responses directed against the structural HIV-1 proteins Gag, Pol and Env, as well as accessory protein Nef, with very limited data available about Vpu.

Vpu has been the subject of considerable investigation recently due to its role in counteracting the host restriction factor, tetherin. It is known to be a highly variable protein, followed by Tat and Rev, while p24-Gag are the most highly conserved (Yusim, Kesmir et al. 2002; Hasan, Carlson et al. 2012), with a high degree of entropy. The variability of the protein may be explained by its relatively small size, when compared to the other HIV proteins, which allows it to undergo substantial variability without the cost of any significant detriment. The low surface expression of Vpu implies that it is not a substantial target for CTL immune responses, and so other immune parameters, particularly KIR and/or ADCC, might exert a greater selective immune pressure. The role of Vpu in counteracting the host restriction factor, tetherin, also implies that the HIV virus has delegated a critical role to this accessory protein, and so its variability might be a presumptive effort on the part of the virus to make sure that the ability to antagonise tetherin is not lost, and the variability of Vpu serves as a barrier against any potential immunological factor that might successfully be able to counteract the activity of Vpu, and therefore not able to antagonise tetherin, and leading to the inhibition of viral release and propagation. None-the-less, more research effort is needed to definitely ascertain why Vpu remains such a variable protein of HIV.

We investigated the potential selection pressure on Vpu (Chapter 4) in the unique setting of the SM cohort, which was infected with a similar strain of HIV at approximately the same time. We did not find any statistically significant associations in our analysis of HIV-HLA associated polymorphisms in Vpu, and our screening ELISPOTS using overlapping peptides did not reveal any T-cell responses to the protein. This builds up on the already widely-accepted notion that the influence of CTL immune selection on evolution of Vpu at the population level may be reduced compared to other highly variable HIV-1 proteins, quite possibly because Vpu may not be accessible to the Class I processing pathway.

More recently, efforts have been made to determine if NK cells may recognise and place pressure on the virus directly *in vivo*. A recent study has identified 22 positions within the HIV-1 genome in which amino acid polymorphisms were significantly associated with the presence of a specific KIR gene (Alter, Heckerman et al. 2011). Therefore, after looking for CTL pressure, we tried to determine if there were any KIR-associated polymorphisms within the HIV-1 Vpu sequences in the SM Cohort (Chapter 4). We used an approach that utilises stratification analysis by Mantel-Haenszel tests to strengthen the associations inferred between viral polymorphisms at each amino acid residue and the KIR genes. This analysis led to the identification of a single amino acid position in the Vpu sequence which was significantly associated with the presence of a specific KIR gene ($p = 0.0047$): In amino acid position 14 of Vpu, HIV-1 sequences (59%) derived from KIR2DS1+ individuals

encoded for a valine (V) whereas the consensus amino acid alanine (A) at this position is found in the vast majority (76%) of KIR2DS1- individuals.

What is extremely interesting is that amino acid position 14 is known already to be critical for interaction with tetherin (Vigan and Neil 2010). It seems reasonable to propose that this critical amino acid position – Ala14 is under significant immune pressure from the host so it can undergo mutation, and therefore not counteract the activity of tetherin in the host cell. Both Alanine and the other amino acid at Vpu position 14, Valine have non-polar side chains. However, it may represent the immune pressure exerted by the host's NK cells and therefore the virus can evade such NK cell mediated immune pressure by selecting for sequence polymorphisms which do not have a detrimental effect, and can still counteract the activity of tetherin. However, more functional data is needed to delineate this association in greater precision and detail.

After this, we aimed to explore further the role of NK cells in relation to antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cell-mediated virus inhibition (ADCVI), in the context of the SM cohort. There has been a surge of interest in ADCC, as a consequence of the interesting results of the recent RV144 HIV vaccine trial in Thailand, which showed modest protection against HIV infection in the absence of strong cytotoxic T-lymphocyte responses or broadly neutralising antibody responses (Rerks-Ngarm, Pitisuttithum et al. 2009). Notably,

the vaccine regimen was able to induce antibodies capable of activating NK cells for ADCC (Karnasuta, Paris et al. 2005; Bonsignori, Pollara et al. 2012).

Therefore, we first adapted an ICS based ADCC assay in our lab (Stratov, Chung et al. 2008), and then determined the extent of ADCC activity in the SM cohort, focusing on responses to Env and Vpu (Chapter 5). We found ADCC responses to Env in 37% of the SM cohort, with five subjects also having responses to Vpu peptides. The C1 region is known to be a common target of ADCC antibodies (Ferrari, Pollara et al. 2011), and ADCC studies from other cohorts have also revealed strong responses to Env peptides (Chung, Navis et al. 2011). We were most interested in the Vpu responders, and wanted to identify the epitopes responsible for ADCC responses. However, we were limited by the lack of plasma we had. Hence, we devised an Enzyme-linked Immunosorbent Assay (ELISA) which allowed us to identify ADCC Vpu peptides without utilising large amounts of plasma. This therefore allowed us to map the Vpu ADCC epitopes in the SM cohort. We also conducted analysis using clinical data to infer if there were any correlation between the ADCC responses in the SM cohort and slow disease progression, but no correlation was found (Chapter 5).

We wanted to investigate whether the five Env/Vpu ADCC responders also displayed ADCVI (Chapter 6). ADCVI encompasses multiple effector functions, some related to lytic (e.g. ADCC) and some to non-cytolytic (e.g. production of chemokines) mechanisms, dependent greatly on the Fc-gamma receptors that may interfere with HIV infection and replication. We optimised the ADCVI assay in our

lab (Forthal, Gilbert et al. 2007) using HIV NL4-3 as our infecting strain of virus. All 5 Env/Vpu responders had very similar inhibition of virus – nearing 95 %, in the ADCVI assay (Chapter 6).

We followed-up on these 5 Env/Vpu ADCVI responders and tried to get more plasma – unfortunately we were only able to get plasma from one subject – SM 017 as the rest could not be traced. After getting plasma from SM 017 from the new sampling year, we ran the plasma in the ADCC assay and to our surprise, the ADCC results were negative, with no detectable response. Thinking of ADCC escape (Chung et al., 2011a), we performed sequence analysis for the samples from the two time points, however no changes were seen. Even more striking, our ELISA identified that antibodies to Vpu were indeed present (Chapter 6). Therefore, working on the hypothesis that SM 017 had higher titres of antibody developing over the years, we re-ran the ADCC experiment with some slight modifications. Instead of using neat plasma, we diluted the plasma sequentially, and then determined whether there was any ADCC activity taking place. This resolved the puzzle, and to our knowledge, we have shown the prozone effect using the ADCC-ICS assay for the first time.

The antibodies mediating ADCC/ADCVI in SM 017 could be either due to Env or Vpu. We plan now on isolating the antibody from SM 017 using peptide labelled beads, and then use this purified antibody to determine whether it mediates ADCC/ADCVI when it is purified. No ADCC antibody has been identified yet, and

it would be very exciting if the purified Vpu antibody mediates ADCC/ADCVI when it is purified.

To sum up, in this study, we analyse host genetic, virological, and immunologic factors to enrich our understanding of HIV/AIDS parameters in a unique Chinese slow progressors cohort. We build up on the HLA database for slow progressors, and demonstrate a KIR association with Vpu amino acid position 14, something which has not been reported before. We demonstrate ADCC responses in the SM cohort, and also optimised an ELISA-based assay to reduce amount of plasma used for the ADCC-ICS assay. We plan now to isolate the antibodies from a unique patient, and possibly, identify the first Vpu ADCC antibody.

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