Killer Immunoglobulin-like Receptor Polymorphism in A Chinese HIV-1 Infection Cohort

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Contents

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Abstract

Genetic and functional studies have demonstrated that *KIR* gene polymorphism, including different haplotypes, allelic polymorphisms and different expression levels of KIRs, may all play a part in the association with HIV-1 infection outcome. Currently, there are very few studies focusing on the association between *KIR* and HIV in the Chinese population.

In this project, we started to look at the polymorphism of *KIRs* in a unique chronic HIV-1 infected cohort (SM cohort), evaluating the impact of *KIR* and *KIR-HLA* interactions in terms of HIV-1 infection progression. The SM cohort is unique because the major factors such as viral strain, transmission route and timing of infection, which could affect the natural history of HIV-1, have been narrowly controlled. Through comparison with a healthy control population, some genetic associations were identified. The frequency of *KIR2DL3* was lower in the "slow progressors" group; the compound genotype of *KIR3DS1+ Bw4* homozygotes was significantly lower in the "slow progressors" group; additionally, group B genotypes (multiple activating genes) were shown to be likely to mount a greater immune pressure on HIV-1. In terms of KIR footprints, several amino acid positions were identified for which the substitution of an amino acid may be ascribed to the immune response from *KIR*-modulated NK cells rather than from *HLA* restricted CTL immune pressure.

In Chapter 4, we report a novel method to sequence the entire locus of *KIR3DL1/S1*. Two specific pairs of primers have been successfully designed and tested to amplify *KIR3DL1* and *KIR3DS1* exclusively. Using this novel sequencing method, we showed the polymorphism of this locus at a 6-digit level. 8 new *KIR3DS1* alleles, 12 new *KIR3DL1* alleles, 1 new *KIR3DL1* gene and 1 new *KIR3DS1* gene have been identified in this study.

In Chapter 5, we used a valuable acute HIV-1 infection cohort to further study associations between *KIRs* and the clinical outcomes. It was interesting to find that the frequency of *KIR3DS1* was significantly lower in the slow progressors group (31%) than in the acute group (40.7%), which implies that *KIR3DS1* plays a role in HIV-1 disease progression. There are two other trends demonstrated in this study. One trend was that positive *KIR2DL2* and/or *KIR2DS2* (they are in strong linkage disequilibrium with each other) were associated with a higher set point viral load (at 3 month) ($p=0.06$). Another trend was that *KIR3DS1* might have an association with disease progression ($p=0.057$).

Overall, in this study, the role of *KIRs* and *KIR/HLA* interactions were evaluated in acute and chronic HIV-1 infection, which has provided important information for further study.

Acknowledgements

Three years ago, I broke into the wonderland of scientific research and tried to make a transformation from a clinical physician to a medical scientist in the field of translational medicine focusing on infectious diseases. It was an incredible journey, confusing at the beginning, frustrated sometimes in the middle, finally full of hope again at the end. Along the way, there were many people who have helped me. Without them, this thesis would not have been possible.

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List of abbreviations

3TC	Lamivudine
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCVI	Antibody dependent cell mediated virus inhibition
AIDS	Acquired immunodeficiency syndrome
AP	Adaptor protein
APOBEC3G	apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G
ARMS	Amplification refractory mutation system
ART	Antiretroviral therapy
AZT	Zidovudine
CA	Capsid protein
CCP	Clathrin coated pits
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CDS	Coding DNA Sequence
CNV	Copy number variation
COPI	Coat protein for retrograde transport
CTD	C-terminal domain
CTL	Cytotoxic T lymphocytes
CXCR4	C-X-C chemokine receptor type 4
DCs	Dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyriboNucleotide TriphosPhate
EBV	Epstein–Barr virus
Ecs	Elite controllers
ELIspot	Enzyme-linked immunosorbent spot
Env	Envelope
FasL	Fas Ligand
FCS	Fetal calf serum
FDCs	Follicular dendritic cells
Gag	Group-specific antigen
GALT	Gut-associated lymphoid tissue
GWAS	Genome wide association studies
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HICs	HIV controllers
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Inteferon

ILT4	Ig-like transcript 4
IN	Integrase
IPD	Immuno Polymorphism Database
KIR	Killer-cell Immunoglobulin-like Receptors
LAK	Lymphokine-activated killer
LD	Linkage disequilibrium
LRC	Leukocyte Receptor Complex
LTNPs	Long term non-progressors
LTR	Long terminal repeat
MA	Matrix protein
mDCs	myeloid dendritic cells
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MSM	Men having sex with men
NASBA	Nucleic Acid Sequence Based Amplification
NC	Nucleocapsid protein
NCs	Non controllers
Nef	Negative factor
NK	Natural Killer
NKT	Natural killer T
PBMC	Peripheral blood mononucleated cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDCs	plasmacytoid dendritic cells
Pol	Polymerase
PR	Protease
RANTES	Regulated on activation, normal T cell expressed and secreted
Rev	Regulator of expression of virion proteins
RNA	Ribonucleic acid
RPs	Rapid progressors
RRE	Rev response element
RS	Recombination site
RT	Reverse transcriptase
SBT	Sequence-based typing
SDF	Stromal cell-derived factor
SFC	Spot Forming Cells
SIV	Simian immunodeficiency viruse
SM	Shuang Miao village
SNP	Single nucleotide polymorphism
SPs	Slow progressors
SSP	Sequence specific primer
SU	Surface protein
TAR	Trans-activating response
Tat	Transcriptional activators

TGN	Trans-Golgi network
TLR	Toll-like receptor
TM	Transmembrane protein
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
UV	Ultraviolet
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
WHO	World Health Organisation

The code for amino acids and nucleotides

Amino acid codes

Amino Acid	3-Letter	1-Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Nucleotide codes

A	**Adenine**
G	Guanine
C	Cytosine
T	Thymine
U	Uracil
R	Purine (A or G)
Y	Pyrimidine (C or T)
N	Any nucleotide
W	(A or T)
S	(G or C)
M	(A or C)
K	(G or T)
B	Not A (G or C or T)
H	Not G (A or C or T)
D	Not C (A or G or T)
V	Not T (A or G or C)

Chapter 1 Introduction

The first HIV-1 (human immunodeficiency virus type-1) infected case was reported in the United States in 1981 (MMWR, 1981). Two years later, in 1983, HIV-1 was defined as the cause of AIDS (acquired immunodeficiency syndrome) (Barre-Sinoussi, Chermann et al. 1983). Since then, HIV/AIDS has become one of the world's most critical public health challenges.

1. 1 HIV/AIDS epidemiology

1. 1. 1 Global epidemiology

UNAIDS (United Nations program on HIV/AIDS) is the authority that provides the most comprehensive epidemic information on HIV/AIDS, which can be found at the website: http://www.unaids.org. According to the latest report, it is estimated currently there are 34 million people living with HIV and about 30 million people have died of AIDS-related causes since the onset of the epidemic (UNAIDS, 2012 report). In 2011, the global prevalence rate was 0.8%. Although cases have been reported in all regions of the world, 97% of them live in low/middle-income countries, especially in sub-Saharan Africa (**Figure1-1**, **Table1-1**). There were about 2.5 million new infections in 2011 and 1.7 million people died of AIDS. Although the rates of both new infection and death have decreased, HIV is still a leading cause of death worldwide and the number one cause of death in Africa.

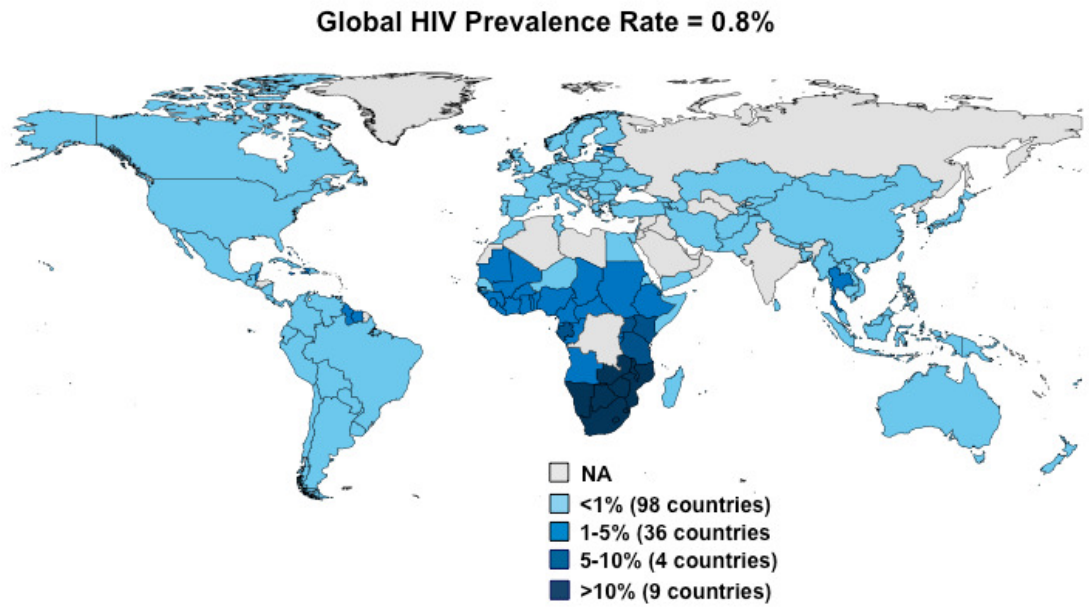


Figure 1-1: Adult HIV prevalence, 2011. Adapted from www.globalhealthfacts.org, the data are estimates; prevalence rates include adults ages 15-49, NA denotes data not available.

Table 1-1: HIV prevalence & incidence by region. Based on data from UNAIDS. Report on the Global AIDS Epidemic; 2012

Region	Total No. (% Living with HIV), 2011	Newly Infected, 2011	Adult Prevalence Rate, 2011
Global Total	34.0 million (100%)	2.5 million	0.80%
Sub-Saharan Africa	23.5 million (69%)	1.8 million	4.90%
South/South-East Asia	4.0 million (12%)	280,000	0.30%
Eastern Europe/Central Asia	1.4 million (4%)	140,000	1.00%
Latin America	1.4 million (4%)	86,000	0.40%
North America	1.4 million (4%)	51,000	0.60%
Western/Central Europe	900,000 (3%)	30,000	0.20%
East Asia	830,000 (2%)	89,000	0.10%
Middle East/North Africa	300,000 (1%)	37,000	0.20%
Caribbean	230,000 (0.7%)	13,000	1.00%
Oceania	53,000 (0.2%)	2,900	0.30%

There are many prevention strategies to deal with the epidemic including behaviour change programs, condoms, HIV testing, blood supply safety measures, and male circumcision etc. HAART (highly active antiretroviral therapy), the use of potent drug combinations that can inhibit the plasma HIV-RNA to undetectable levels, was introduced in 1996 and began to be widely used. Moreover recently, both the treatment of the discordant couples and the use of antiretroviral-based microbicide gels have been shown to be beneficial in reducing HIV transmission (Williams, Abdool Karim et al. 2011; Anglemyer, Rutherford et al. 2013).

Although HIV/AIDS has changed from what was initially a uniformly fatal disease to what is now seen a treatable chronic disease, access to treatment still remains a big problem in resource-poor countries. Meanwhile patients on HAART commonly suffer from side effects or acquire drug-resistant virus, necessitating a change in anti-viral regimen. There is still a desperate need for an HIV vaccine to decrease the morbidity and mortality of the epidemic. Before a successful vaccine can be achieved, better understanding of disease pathogenesis and human immune responses to HIV infection is needed.

1. 1. 2 HIV epidemic in China

In China, the first HIV/AIDS case, in a foreigner, was reported in 1985 (Yu, Xie et al. 1996). The first local patients were four haemophilia patients who were found to be HIV positive after transfusion of imported blood products in 1986 (Zeng, Fan et al. 1986). Epidemic estimates show that there were about 780,000 people living with HIV in China at the end of 2011, accounting for 0.058% of the total population. There were 154,000 cases of AIDS among them. The estimated numbers of new infection and death in 2011 were 48,000 and 28,000 respectively. Among all the infected people, 46.5% were thought to be infected through heterosexual transmission, 17.4% were men having sex with men (MSM), injecting drug use accounted for 28.4%, 6.6% were former blood donors, and 1.1% were infected

through mother-to-child transmission (2012 China AIDS response progress report, from Ministry of Health of the PRC). In 2009, China reported that AIDS had become the country's leading cause of death among infectious diseases for the first time ever, surpassing both tuberculosis and rabies (Chinese CDC report, 2009).

Although the national prevalence remains low, the epidemic in some areas is severe. At the end of 2011, there were 5 provinces (Yunnan, Sichuan, Guangxi, Guangdong, and Xinjiang) in which the number of HIV/AIDS cases reported surpassed 50,000. These accounted for 60% of the total reported cases (**Figure 1-2**).

Figure1-2: Geographic distribution of the estimated 780,000 people living with HIV/AIDS in China in 2011 (Adapted from UNAIDS/WHO 2011 Estimates for the HIV/AIDS Epidemic in China)

According to the case reporting system, HIV/AIDS has been reported in all provinces in China. As for the cumulative reported cases, there were 6 provinces (Yunnan, Guangxi, Henan, Sichuan, Xinjiang, and Guangdong) reporting the highest numbers which represented 75.8% of the total cumulative cases (**Figure 1-3**).

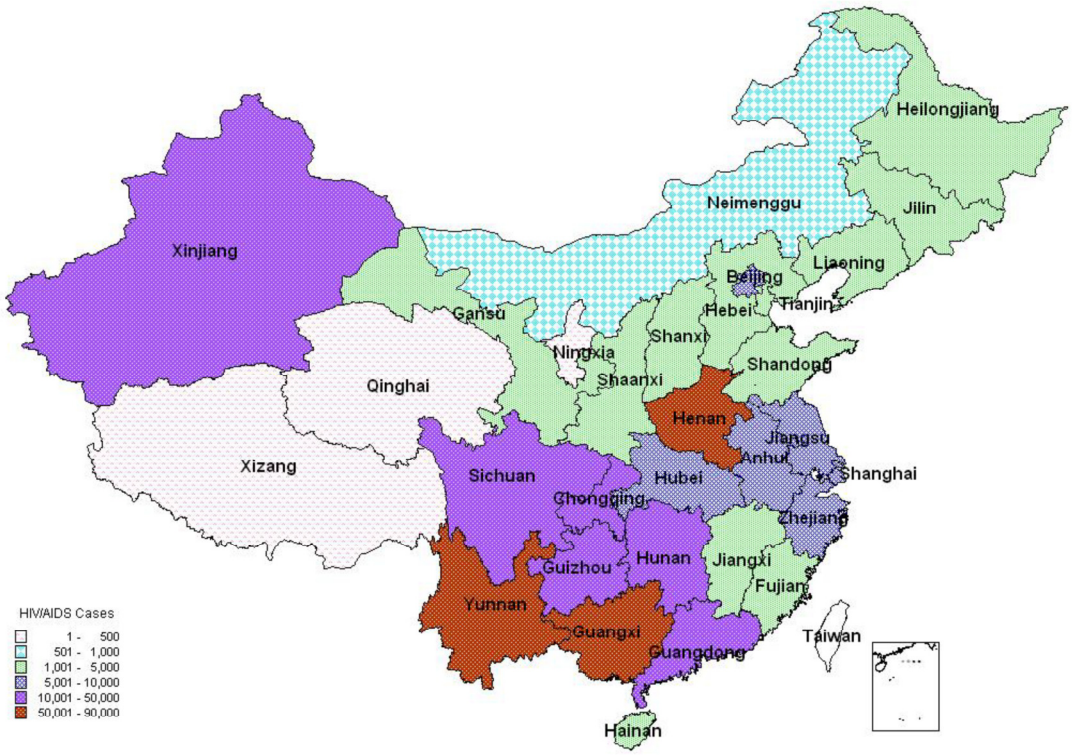


Figure 1-3: Geographic distribution of cumulative cases of HIV/AIDS in China (by the end of 2011) (Adapted from UNAIDS/WHO 2011 Estimates for the HIV/AIDS Epidemic in China)

1. 2 HIV-1 infection

1. 2. 1 Viral structure

HIV-1 is a human retrovirus that belongs to the genus Lentivirus. It is roughly spherical in shape with a diameter of about 120nm (McGovern, Caselli et al. 2002).

1. 2. 1. 1 The viral envelope

HIV-1 viral particles are surrounded by a lipoprotein membrane taken from the host cell when the newly formed virus particles bud. There are about 14 glycoprotein complexes (Zhu, Liu et al. 2006) integrated into the lipid membrane known as Env (Envelop protein) as well as some host proteins captured from the cell membrane such as HLA molecules embedded throughout the viral surface. Each Envelope protein consist of trimers of an external

glycoprotein gp120 (spiking through the surface) and a transmembrane spanning protein gp41, anchoring the viral envelope (Barre-Sinoussi 1996). The binding between gp120 and gp41 is loose and therefore gp120 could be shed spontaneously and may be detected in the serum of HIV-infected patients.

1.2.1.2 The viral core

Inside the viral envelope is the matrix protein called P17 which lies between the viral envelope and the viral core to ensure the integrity of the virion particle (Wu, Alexandratos et al. 2004). Within the matrix protein is a cone-shaped core or capsid, consisting of about 2,000 copies of viral protein, P24. The capsid contains two copies of HIV-RNA, each of which has a complete copy of the virus's genes. The HIV-RNA is bound to the nucleocapsid proteins, p6 and p7 which protect the RNA from digestion by nucleases. Inside the capsid all the enzymes which are necessary for replication are present: a reverse transcriptase p66 (RT),an integrase p32 and a protease p11 (Barre-Sinoussi 1996)(**Figure 1-4**).

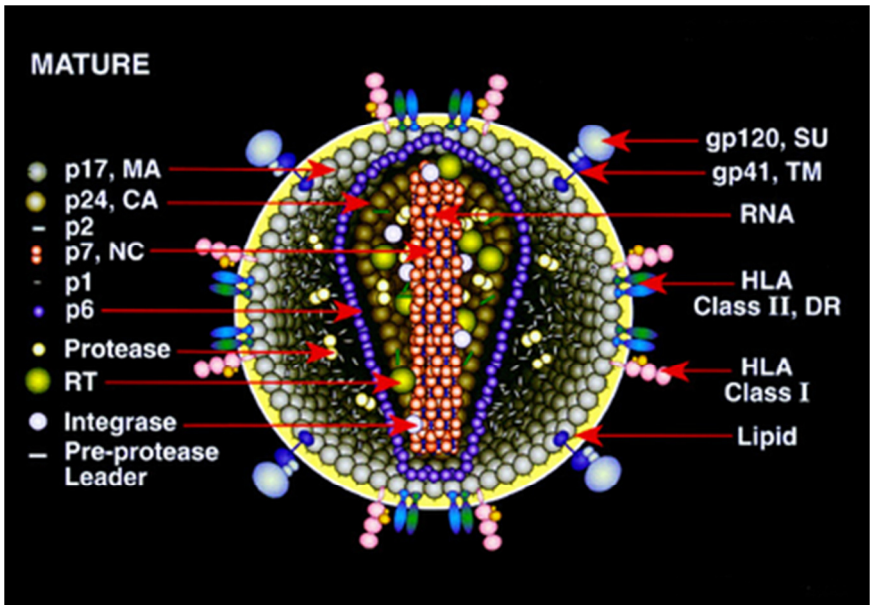


Figure 1-4: HIV-1 virion model showing the structure (Adapted from http://www.mcb.uct.ac.za/cann/335/HIVmature.jpg). MA: matrix protein; CA: capsid protein; NC: nucleocapsid protein; RT: reverse transcriptase; SU: surface protein; TM: transmembrane protein.

1. 2. 2 The organisation of the HIV-1 genome and its proteins

The entire HIV-1 genome is about 9.7Kb long (Ratner, Haseltine et al. 1985). Being a retrovirus, the classical structural scheme of the genome is: 5'-LTR-*gag*-*pol*-*env*-LTR-3'. The LTR (long terminal repeat) region consists of the two ends of the viral genome that are connected to the host cellular DNA after integration. They do not encode for any viral proteins but act as the triggering switches to control the production of new viruses. *Gag* represents group-specific antigen, *pol* is polymerase, *env* means envelope. These three major genes encode major structural proteins as well as essential enzymes. In addition to these, there are 6 other genes, 2 of them are important regulatory genes, *Tat* and *Rev*. The other 4 genes are accessory genes: *Nef*, *Vpr*, *Vif* and *Vpu*, which are not essential for virus replication *in vitro* (Frankel and Young 1998). Generally speaking, the gag proteins provide the infrastructure of the virus, the *pol* gene encodes the proteins responsible for replication, and the other genes help HIV enter the host cell and act to enhance viral replication (**Figure 1-5**).

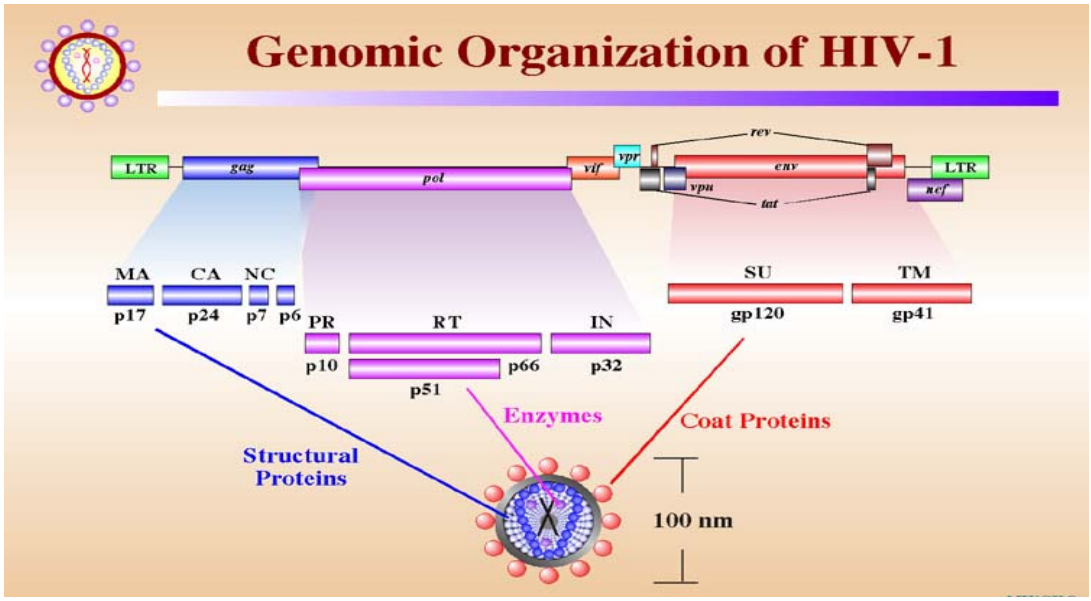


Figure 1-5: The genomic organization of HIV-1 (Adapted from http://www.stanford.edu/group/virus/retro/2005gongishmail/HIV.html). MA: matrix protein; CA: capsid protein; NC: nucleocapsid protein; RT: reverse transcriptase; SU: surface protein; TM: transmembrane protein.

Gag

Group-specific antigen genes encode the capsid proteins. The precursor is a 55-KDa protein (p55) called assemblin, indicating its role in viral assembly, which is further processed to p17 matrix protein (MA), p24 capsid protein (CA), p7 nucleocapsid protein (NC) and p6 protein by the viral protease. The roles and functions of the 4 proteins are summarised in the following table (**Table 1-2**).

Table 1-2: The roles and functions of *gag* proteins

protein	Roles and functions
MA (p17)	Ensures the integrity of the virion particle; Targets *gag* precursor polyproteins to the plasma membrane prior to viral assembly; Lines the inner surface of the virion membrane; Helps incorporate *Env* glycoproteins with long cytoplasmic tails into viral particles (Freed, Englund et al. 1995; Mammano, Kondo et al. 1995).
CA (p24)	Forms the core of the virus particle; Functions primarily in assembly and is also important for infectivity, participating in viral uncoating (Luban 1996; Gamble, Yoo et al. 1997).
NC (p7)	Protects the viral RNA from digestion by nucleases; Delivers the full-length viral RNAs into the assembling virion (Berkowitz, Ohagen et al. 1995).
p6	Binds *Vpr* during viral assembly (Lu, Bennett et al. 1995; Kondo and Gottlinger 1996); Helps mediate efficient particle release (Huang, Orenstein et al. 1995);

Pol

The polymerase gene encodes 3 proteins: the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN). All of them are crucial enzymes in the HIV-1 replication cycle.

Protease (PR) is responsible for cleaving at several polyprotein sites to produce the final mature proteins such as: the final p17, p24, p7 and p6 proteins from *Gag* and PR, RT and IN from Pol. Meanwhile conformational rearrangements must occur within the particle to

produce mature infectious viruses (Kaplan, Manchester et al. 1994). Protease is a major target for antiviral drug design and several PR inhibitors are in wide use clinically, although mutants resistant to multiple inhibitors have been observed (Condra, Schleif et al. 1995; Ridky and Leis 1995). These mutations responsible for resistance can be located both in the inhibitor binding pocket and at distant sites, and some of them even lead to proteins with increased catalytic activities (Schock, Garsky et al. 1996).

Reverse transcriptase (RT) has three activities: RNA-dependent DNA polymerase, ribonuclease H and DNA-dependent DNA polymerase. RT catalyses DNA polymerisation and cleaves the RNA portion of RNA-DNA hybrids generated during the reaction. Finally, double-stranded cDNA is integrated into the host genome generating a long-term infection. RT has also been a major target for antiviral drug design. Currently two classes of RT inhibitors are in use: nucleotide analogues such as AZT (zidovudine) and 3TC (lamivudine), which are designed to bind to the polymerase active site, and non-nucleoside inhibitors such as nevirapine. Drug-resistance mutations have also been observed and mapped to different parts of RT, including the active site, DNA –binding cleft and the flanking regions, suggesting there are both direct and indirect alteration effects (Tantillo, Ding et al. 1994; Ren, Esnouf et al. 1995).

Integrase (IN) catalyses sequential reactions to integrate the viral genome into a host chromosome. Integration can happen at many target sites within the genome. There appears to be a preference for integration at sites of kinked or distorted DNA in *in vitro* studies (Miller, Bor et al. 1995). There are at least 4 steps for the whole integration, of which strand transfer is usually targeted by integrase inhibitors such as raltegravir and elvitegravir. These new drugs are very potent, but resistance, mutations have also begun to be reported (Geretti, Armenia et al. 2012).

Env

The *Env* gene codes for gp160. This precursor protein is later cleaved by protease into two parts: gp120, the surface protein (SU) and gp41, the transmembrane protein (TM). The mature gp120-gp41 proteins are bound by non-covalent interactions and are associated as a trimer on the cell surface. Gp120 binds to the major receptor for HIV-1, CD4 molecules, to initiate the entry of the virus. The binding causes a conformational change in the envelope, to expose the chemokine-receptor binding site. Co-receptor binding triggers Env to undergo full conformational changes that expose the hydrophobic gp41 fusion peptide, which induces therefore, the fusion of HIV and host cell membranes. However the gp120-CD4 interaction is not sufficient for HIV entry: entry also depends on members of a group of chemokine receptors that serve as essential viral co-receptors. In terms of viral tropism, there are two classes of co-receptor. M (Macrophage)-tropic HIV uses CCR5; T-tropic strains use CXCR4. Other molecules, including CCR3 and CCR2b can serve as co-receptors for some HIV-1 isolates (Clapham and Weiss 1997). The physiological ligands for CXCR4, CCR5, and CCR3 (SDF-1, RANTES/MIP-1α/MIP-1β, and eotaxin, respectively) are each able to inhibit viral entry by competing with the virus for binding to the cognate co-receptor (Bleul, Farzan et al. 1996; Choe, Farzan et al. 1996; Clapham 1997).

Tat

Tat, as an important regulatory gene, encodes polypeptides, which are strong transcriptional activators through binding to the TAR (trans-activating response) elements. Although the integrated virus could use host cellular transcriptional factors to initiate replication, the elongation of viral transcripts is inefficient in the absence of Tat protein. Tat enhances the activity of transcribing polymerases and may also enhance the rate of transcription initiation. Under some circumstances, Tat increases the production of viral mRNA 100-fold and consequently is regarded as essential for viral replication. The reason for its importance is not

entirely clear but it was suggested that tat might assemble into transcription complexes and recruit or activate factors that phosphorylate the RNA polymerase II C-terminal domain (CTD) to convert it from an initiating to an elongating enzyme (Parada and Roeder 1996; Yang, Herrmann et al. 1996; Zhou and Sharp 1996).

Rev

Rev (regulator of expression of virion proteins) is considered the most functionally conserved regulatory protein of lentiviruses. The final viral proteins are translated from various lengths of mRNA. For example: The gag and gag-pol proteins are translated from unspliced mRNA; *Vif*, *Vpr*, *Vpu*, and *Env* are translated from singly spliced viral mRNA; *Tat*, *Rev*, and *Nef* are translated from multiply spliced mRNA. Host cells (eukaryotic cells) have mechanisms to prevent the export from the cell nucleus of incompletely spliced mRNA transcripts, which poses a problem for HIV-1. The Rev protein provides the virus' solution to this problem. In the early stages of replication, the three proteins translated from the fully spliced mRNA are exported from the nucleus by means of the normal host cellular mechanisms, then the expressed Rev protein enters the nucleus and binds to the Rev response element (Fischer, Huber et al. 1995). Rev contains a leucine-rich nuclear export signal that allows it to shuttle between the nucleus and cytoplasm (Meyer and Malim 1994): it interacts with a nucleoporin-like protein, hRip/Rab, located at the nuclear pore (Bogerd, Fridell et al. 1995; Fritz, Zapp et al. 1995). The interaction with hRip/Rab may be bridged by CRM1, a nuclear export receptor that is important for Rev export (Ullman, Powers et al. 1997). Thus, Rev binding to the RRE is believed to target the attached mRNA to the nuclear export machinery.

Nef

The *Nef* (negative factor) gene encodes a multifunctional 27-kd myristoylated protein produced by an ORF located at the 3' end of the virus genome (Niederman, Hastings et al.

1993), which is one of the first proteins to be produced in infected cells and the most immunogenic of the accessory proteins. The *Nef* gene is dispensable *in vitro*, but is essential for efficient viral spread and disease progression *in vivo*.

Nef has been shown to have a number of functions: the down-regulation of CD4 and MHC class I, the stimulation of virion infectivity, and the capacity to alter the activation state of cells.

Nef down-regulates CD4 at three levels: to redirect some CD4 from the trans-Golgi network (TGN) to the endosomal compartment; to trigger the accelerated internalization of CD4 molecules that have reached the cell surface; and to target CD4 from the endosome to the lysosome (Piguet, Chen et al. 1998; Kim, Chang et al. 1999). These effects are achieved because Nef acts as a bridge between CD4 and the cell protein trafficking machinery. Nef binds to CD4 through the recognition of a dileucine-based signal in the proximal region of the receptor cytoplasmic tail; meanwhile Nef recruits downstream partners including adaptor protein complex of clathrin coated pits (CCP), or AP, which recruit clathrin to the cytoplasmic tail of receptors containing endocytosis signals (Le Gall, Erdtmann et al. 1998; Piguet, Chen et al. 1998) and the β subunit of COPI (coat protein for retrograde transport) (Daro, Sheff et al. 1997) which plays a crucial role in ER-Golgi transport. The down-regulation of CD4 on the surface of HIV-1 infected cells avoids blocking the release of new virions through inadvertent capture of viral envelope on host cell CD4 molecules (Ross, Oran et al. 1999). CD4 downregulation also reduces the possibility of lethal superinfection events: moreover this activity promotes T cell activation, which is favourable for viral gene expression. It is noteworthy that these effects are not mutually exclusive, therefore it might explain why Nef-induced CD4 down-regulation is so well conserved amongst primate lentiviruses.

Nef-mediated down-regulation of MHC class I molecules is less efficient than the modulation of CD4. A tyrosine found in the cytoplasmic tails of HLA-A and B, but not HLA-C, is crucial for Nef binding. In the presence of Nef, the tyrosine residue associates with the binding pocket of the clathrin adaptor protein (AP-1) complex to promote the internalisation and degradation of the HLA-A and B molecules (Roeth, Williams et al. 2004; Wonderlich, Williams et al. 2008). Significantly, HLA-C cytoplasmic tails lack two amino acids necessary for this interaction. Residues in Nef essential for MHC class I down-regulation differ from those necessary for CD4 down-regulation (Greenberg, DeTulleo et al. 1998; Mangasarian, Piguet et al. 1999). MHC class I molecules present foreign antigenic peptides to cytotoxic T lymphocytes to induce the killing of infected cells. HIV evades the killing by CTL through down-regulating the HLA-A and B molecules : meanwhile HLA-C molecules, as the most important ligands for inhibitory KIRs (HLA-C1 molecules are the ligands for KIR2DL2/2DL3; HLA-C2 molecules are the ligands for KIR2DL1), are not down-regulated which enables HIV to evade killing by Natural killer cells (Collins, Chen et al. 1998; McMichael 1998).

In addition, Nef can recruit several protein kinases involved in cell signalling to interfere with T-cell activation. Nef is also able to promote the production of virions and boost their infectivity (Piguet and Trono 1999).

Vpr

Vpr (viral protein R) is a 96-amino acid protein which becomes localised to the nucleus (Cohen, Subbramanian et al. 1996). The main functions for Vpr include importing of the proviral DNA as a component of the pre-integration complex to the nucleus (Miller, Farnet et al. 1997), inducing G2 cell cycle arrest (Emerman 1996), and influencing mutation rates

during viral DNA synthesis (Mansky 1996). In HIV-2 and SIV, the *Vpx* gene is apparently the result of a *Vpr* gene duplication event, possibly by recombination.

Vpu

Vpu (virus protein U): Vpu is an 81-amino acid protein, which is specifically found in HIV-1 but not HIV-2. Sometimes the newly synthesized Env proteins (gp160) could be trapped in the endoplasmic reticulum by newly synthesized CD4. Vpu promotes degradation of CD4 in the ER to allow Env transport to the surface for viral assembly (Cohen, Subbramanian et al. 1996). Vpu can also down-regulate cell surface expression of MHC class I proteins (Kerkau, Bacik et al. 1997) and stimulate the release of virions by degrading tetherin, which is a host cell transmembrane protein binding the virus to the inner plasma membrane (Neil, Zang et al. 2008; Douglas, Viswanathan et al. 2009).

Vif

Vif (viral infectivity factor): *Vif* is important for the production of highly infectious mature virions. *Vif*- deficient HIV isolates cannot replicate in $CD4^+$ T cells, some T cell lines (non-permissive cells) or in macrophages. Although they can enter target cells and initiate Reverse transcription, the level of viral DNA synthesis is markedly reduced (Cohen, Subbramanian et al. 1996; Simon and Malim 1996). It is interesting that *Vif* mutants do not show defects in permissive cells implying that expression of *Vif* in permissive cells blocks an inhibitor of viral infectivity. This inhibiting factor was identified as APOBEC3G (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G) (Sheehy, Gaddis et al. 2002). APOBEC3G specifically deaminates cytosine to uracil in mRNA or DNA, resulting in an accumulation of G-to-A mutations that leads to degradation of viral DNA. Vif blocks this effect by forming a complex with APOBEC3G.

1. 2. 3 HIV-1 lifecycle

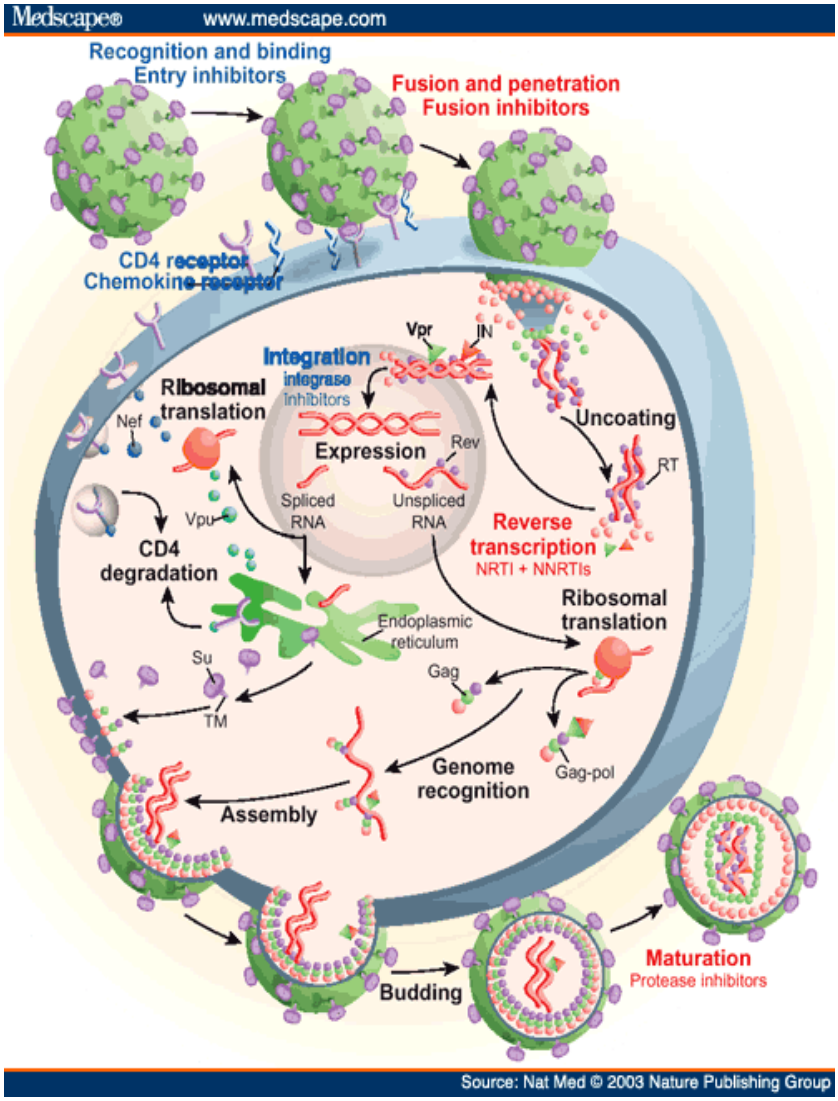


Figure 1-6: HIV-1 life cycle. Adapted from nature medicine (Pomerantz and Horn 2003). The texts in red and blue are targets for drug therapy.

The life cycle of HIV is shown in **Figure 1-6**, which is composed of 5 basic steps:

1. 2. 3. 1 Entry and Fusion

HIV uses gp120 to bind to the receptor CD4 and a co-receptor (CC or CXC chemokine receptors, predominantly HIV isolates use either CCR5 or CXCR4 as co-receptors) on the surface of the target cells (T-lymphocytes, T-cell precursors within the bone marrow and thymus, monocytes, macrophages, eosinophils, dendritic cells and microglial cells of the central nervous system)(Moore and Ho 1993). The binding causes a conformational change to expose the gp41 fusion peptide, which promotes the virus-cell membrane fusion and releases the viral core into cytoplasm (Gallo, Finnegan et al. 2003).

1. 2. 3. 2 Reverse Transcription

The viral core is then uncoated and the single-stranded HIV-RNA is Reverse transcribed by RT into a partially duplex linear DNA that enters the host cell's nucleus (Freed 2001).

1. 2. 3. 3 Integration

Integrase then catalyses integration of the viral DNA into the host genome (Miller, Bor et al. 1995). Unpaired dinucleotides at the provirus 5' ends are removed and gaps between host and viral DNA are repaired by host DNA enzymes, which completes the integration process (Van Maele, Busschots et al. 2006).

1. 2. 3. 4 Transcription

The viral transcripts are generated from the promoter located in the 5'LTR upon activation of host cells. During the course of viral transcription, Tat greatly enhances the rate of transcription. A set of unspliced, partially spliced, and small spliced mRNAs are transported from the nucleus to the cytoplasm with the help of Rev, where they encode Gag and Pol polyprotein precursors or are packed into progeny virions as genomic RNA; encode the

proteins of Env,Vif,Vpu, and Vpr; and are translated into Rev, Tat, and Nef respectively (Emerman and Malim 1998; Frankel and Young 1998).

1. 2. 3. 5 Assembly and budding

Proteolytic processing of the Gag and Gag-Pol polyproteins is catalysed by protease to generate MA, CA, NC, p6, PR, RT, and IN to assemble a new virus particle. Together with Vif, Vpr, Nef, and the genomic RNA, an immature virion begins to bud from the cell surface. For maturation, the Env polyprotein must first be released from complexes with CD4 on the surface of the host cells. Vpu assists this process by promoting CD4 degradation and Nef promotes endocytosis and degradation of surface CD4 as well. Finally the virus particle buds and is released from the cell surface coated with Env proteins to complete the viral life cycle (Frankel and Young 1998).

1. 3 The host immune response to HIV infection

1. 3. 1 Natural HIV-1 infection history

Infection with HIV-1 results in progressive loss of immune function characterised by depletion of CD4+ T-lymphocytes, leading to opportunistic infections and malignancies. The median time from initial infection to the development of AIDS among untreated patients ranges from 8-12 years (Lyles, Munoz et al. 2000). The natural history of HIV-1 infection is defined as the duration from acquisition of the infection to death without treatment (Bartlett 2004). The classical course of infection is characterized by three phases: acute or primary infection phase, the asymptomatic phase (clinical latency), and AIDS. The schematic figure is shown in **Figure 1-7**.

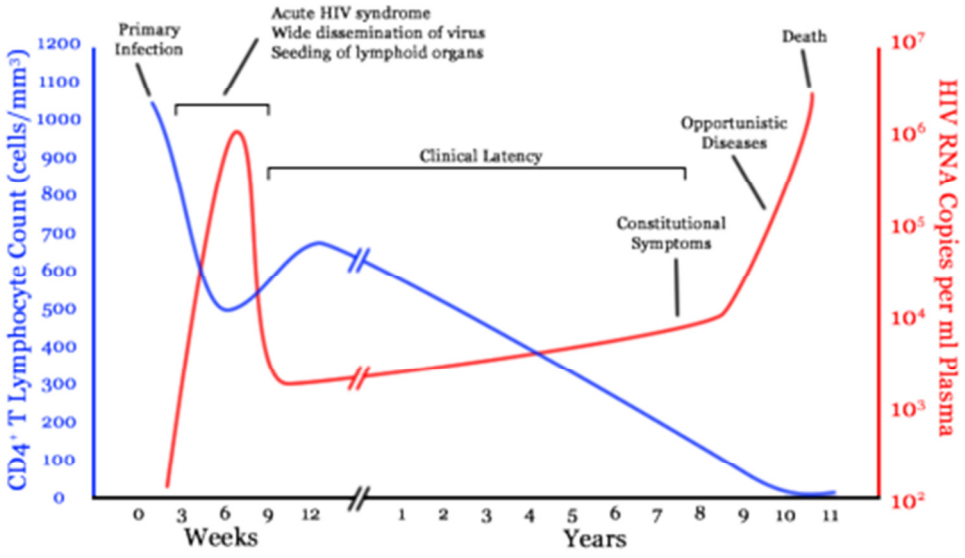


Figure 1-7: Nature history of HIV-1 infection (Adapted from http://www.pipelinedrugs.com/biotechnology_encyclopedia/500px-Hiv-timecourse.png)

Primary HIV-1 infection refers to the period when HIV-1 first spreads throughout the body and induces immune responses. The diagnosis of acute HIV-1 infection requires an understanding of populations most at-risk, the clinical manifestations associated with primary infection and the appropriate laboratory tests. Our knowledge of events occurring in the earliest stages of acute HIV-1 infection is mostly inferred from either culture of the infected mucosal tissue *in vitro* (Hu, Frank et al. 2004; Veazey, Klasse et al. 2005; Margolis and Shattock 2006) or from experimental infections of rhesus macaques with SIV (Miller, Li et al. 2005; Li, Skinner et al. 2009). It is assumed there will be similarities to human infection, as SIV shares a very similar genetic structure to HIV-1 and uses CD4 as its primary receptor (Soogoor and Daar 2005).

Following transmission of the virus, there is a period of 0-10 days, known as the eclipse phase, before viral RNA becomes detectable in the plasma. Initially, the primary targets for HIV-1 are the memory $CD4^+$ T cells in the mucosa. When the innate immune system is activated and recruits macrophages and lymphocytes, these cells can also be targets of HIV-1

(Mogensen, Melchjorsen et al. 2010). The virus begins to replicate in the target cells as described above (in "HIV-1 life cycle" part). When the virus and viral-infected cells reach the lymph nodes, the infection is amplified by encounter with large numbers of $CD4^+$ T cells. Destruction of infected (as well as bystander) $CD4^+$ T cells results in the massive depletion of CD4 memory T cells.

21-28 days later, the virus enters the blood again and infects more lymphocytes, leading to peak plasma viraemia along with decreased numbers of circulating CD4 T cells (Mogensen, Melchjorsen et al. 2010). During this phase, gut-associated lymphoid tissue (GALT) is particularly severely affected, where activated $CD4^+CCR5^+$ memory T cells are present in high numbers (Brenchley, Schacker et al. 2004; Brenchley, Paiardini et al. 2008). Based on data from monkey models, it is estimated that 20% of $CD4^+$ T cells in the GALT are infected, whist 60% of uninfected $CD4^+$ T cells at this site become activated and die by apoptosis; therefore, ~80% of $CD4^+$ T cells in the GALT can be depleted in the first 3 weeks of HIV-1 infection (Brenchley, Schacker et al. 2004). $CD4^+$ T cell numbers are low at the time of peak viraemia but later return to near normal levels in the blood but not in the GALT. There are at least three mechanisms that result in the loss of $CD4^+$ T cells in HIV-1 infection. Firstly, infected cells can be killed directly by the virus; Secondly, infected cells are more likely to undergo apoptosis; Thirdly, infected $CD4^+$ T cells are killed by $CD8^+$ cytotoxic lymphocytes that recognize viral peptides (Murphy, Travers et al. 2008).

Around the time of the peak viral load, the infected patients begin to manifest typical clinical symptoms (flu-like syndrome: complaints of fever, rash, malaise, sore throat, and myalgia of a week or two in duration, occasionally with neurological manifestations) when the CD4 count has decreased to 200-500 cells/mm^3. The period between transmission and peak viraemia is a crucial phase known as the "window of opportunity" in which an HIV-1 vaccine

could control viral replication, prevent widespread $CD4^+$ T cell depletion and suppress generalized immune activation.

Following the acute phase, the host immune responds robustly to decrease the viral load and increase the circulating CD4 T cells; however these efforts are not sufficient to clear the infection. The presence of antibodies in the peripheral blood, known as seroconversion, occurs in the first 3-6 weeks. Over 12-20 weeks, the viral load eventually decreases to a stable status called "set point" , the level of which is usually the best indicator of future disease progression (Mellors, Rinaldo et al. 1996; Blattner, Oursler et al. 2004; Goujard, Bonarek et al. 2006; Mellors, Margolick et al. 2007). In the absence of HAART, the set point is maintained by a balance between virus turnover and the immune responses.

The asymptomatic phase is generally of long duration but the length varies between different people. There is persistent replication of the virus and a gradual decline in the function and numbers of CD4 T cells during the clinically "silent" period.

A number of terms have been used to describe individuals in terms of variable rates of disease progression and viral control, including "long term non-progressors" (LTNPs) (Canducci, Marinozzi et al. 2009; Limou, Le Clerc et al. 2009; Okulicz, Marconi et al. 2009), "elite controllers" (ECs) (Deeks and Walker 2007) , "slow progressors" (SPs) (Gillespie, Kaul et al. 2002; Zhang, Jiang et al. 2010; Kamya, Boulet et al. 2011) , "HIV controllers" (HICs) (Lambotte, Boufassa et al. 2005) , "non-controllers" (NCs) (Shaw, Hunt et al. 2011) and "rapid progressors" (RPs) (Fontaine, Coutlee et al. 2009; Lajoie, Fontaine et al. 2009; Casado, Colombo et al. 2010). The most common definitions for each group of patients are presented below (**Table 1-3**) (unpublished data from The UK HIV Genomics Consortium).

Table 1-3: The definition for different terms used to describe HIV-1 disease progression

Term	General definition
LTNPs	Asymptomatic without ART for 10 years or more, no $CD4^+$ T cell counts < 500 cells/mm^3 during this period
ECs	Spontaneously maintain viral loads < 50 copies/ml without ART
SPs	Asymptomatic individuals infected for 8 or more years with a $CD4^+$ T cell counts > 500 cells/mm^3 without ART
RPs	Individuals with $CD4^+$ T-cell counts <300 cells/mm^3 within 3 years
HICs	HIV infected individuals with at least 3 measurements of plasma HIV-RNA < 2000 copies/ml over at least a 12-month period in the absence of ART
NCs	HIV infected individuals with plasma HIV RNA > 10,000 copies/ml without ART

AIDS When an individual's CD4 T cell counts falls below 200 cells/mm^3, the clinical syndrome is known as AIDS. This indicates profound immunosuppression and the individual is susceptible to a range of opportunistic infections and tumours.

Many factors such as geographic characteristics, viral subtypes, and host factors (age, gender, exposure group, immunologic and genetic background) may influence the natural history of HIV-1 infection at both an individual and at a population level. A sophisticated understanding of these factors may lead to the ability to predict individual HIV susceptibility, disease progression risk and to select targets for future drug and vaccine development.

1. 3. 2 Innate immune response

The innate immune system is the body's first line of defence, comprising the cells and mechanisms that protect the host from infection in a non-specific manner.

Sexual transmission is the most frequent mode of transmission and accounts for more than 80% of HIV infections worldwide (Joint United Nations Programme on HIV/AIDS. 2010). Dendritic cells (DCs) are located in the mucosae and the lymphoid tissues and are proposed to be among the first cells that encounter HIV-1.

Dendritic cells exhibit a versatile response when encountering HIV-1, mediating effects that may be beneficial or detrimental or a combination of the two. DCs can be classified into several subsets in terms of their different distribution, immunological function, and cell surface markers. Examples of some DC subsets include myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in the blood and Langerhans cells in the tissue (Banchereau, Briere et al. 2000; Liu 2001). DCs are present at low frequencies in blood, together constituting 0.5-2% of total PBMCs (Rissoan, Soumelis et al. 1999; Liu 2001). Langerhans cells exist mostly in the skin and the stratified squamous epithelia, constituting 2-3% of epidermal cells (Zaitseva, Blauvelt et al. 1997; Banchereau, Briere et al. 2000). mDCs, pDCs, and Langerhans cells can be infected by HIV-1 (Soto-Ramirez, Renjifo et al. 1996; Blauvelt, Asada et al. 1997; Kawamura, Gulden et al. 2003; Smed-Sorensen, Lore et al. 2005). All of them can express modest amounts of CD4 and HIV co-receptors including CCR5, CXCR4, CCR3, CCR8 etc (Granelli-Piperno, Moser et al. 1996; Rubbert, Combadiere et al. 1998). In general, replication of HIV in DCs is less productive than in CD4 T cells (Granelli-Piperno, Delgado et al. 1998; Granelli-Piperno, Finkel et al. 1999; Kawamura, Gulden et al. 2003; Steinman, Granelli-Piperno et al. 2003). The reasons for this might be low levels of expression of the HIV receptors and co-receptors by DCs, rapid degradation of internalized

HIV in intracellular DC compartments (Moris, Nobile et al. 2004; Turville, Santos et al. 2004; Nobile, Petit et al. 2005), and expression of host factors that inhibit HIV replication, such as *APOBEC3G*.

In addition, follicular DCs (FDCs), which are found only in the B-cell follicles and germinal centres of the peripheral lymphoid tissues, can act as reservoirs of HIV. FDCs are not productively infected by HIV-1 but they can trap and retain large amounts of HIV at their surface and promote the migration of resting CD4 T cells to facilitate their infection (Spiegel, Herbst et al. 1992; Heath, Tew et al. 1995; Smith, Gartner et al. 2001; Burton, Keele et al. 2002). For this reason, FDCs are thought to be detrimental in their contribution to HIV pathogenesis.

DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) is a C-type lectin receptor also known as CD209, on the surface of both macrophages and dendritic cells (Geijtenbeek, Torensma et al. 2000; Engering, Van Vliet et al. 2002; Soilleux, Morris et al. 2002). DC-SIGN plays a crucial role in the dissemination of HIV-1 by DCs (Geijtenbeek, Kwon et al. 2000). DC-SIGN represents a novel class of HIV-1 receptor; it captures and binds the virus at sites of entry but does not allow viral infection, instead it transmits the virus efficiently to T cells located in lymphoid tissues (Geijtenbeek and van Kooyk 2003). Nef can upregulate the expression of DC-SIGN and increase the clustering of DCs with T cells, thereby promoting HIV infection (Sol-Foulon, Moris et al. 2002). In addition to its adhesion function, DC-SIGN also functions as an antigen receptor that captures and internalises antigens for presentation by DC.

HIV-pulsed DCs promote viral infection of co-cultured T cells (Pope, Betjes et al. 1994). Recent studies have demonstrated some possible mechanisms underlying this DC-mediated transmission. The pathways could be across the “infectious synapse” (McDonald, Wu et al.

2003; Arrighi, Pion et al. 2004) or trans-infection mediated by DC-derived exosomes (Wiley and Gummuluru 2006).

Cytokine storm

HIV triggers innate immune responses through toll-like receptors, including TLR7 and TLR8 (Diebold, Kaisho et al. 2004; Heil, Hemmi et al. 2004), leading to the potent activation of dendritic cells and the release of huge amounts of type I IFNs and tumour necrosis factor α. In addition to TLR7/8 recognition, TLR2, 4 and 9 have also been implicated in recognition and modulation of HIV viral replication (Thibault, Fromentin et al. 2009). Primary HIV infection induces a robust cytokine storm (Stacey, Norris et al. 2009) , which is characterised by the rapid induction of IFN-α, IL-15, and inducible protein-10 (IP-10) and a slower increase in proinflammatory factors. The magnitude of the storm is even greater than that observed in acute hepatitis B and C virus infection, which implies that this cytokine cascade actually contributes to immunopathological consequences instead of helping resolve the acute infection (de Jong, Simmons et al. 2006; Cameron, Ran et al. 2007).

NK cells in HIV infection will be discussed below in **1. 4**.

Activation of other innate cell subsets

In addition to dendritic cells and NK cells, other innate cells subsets are also involved in the acute HIV-1 infection. For example, monocytes may become activated and contribute to the control of infection through producing cytokines and chemokines (Kuwata, Kodama et al. 2007). Meanwhile they may also promote viral replication and contribute to the spread of virus, including transport of virus into the brain (Clay, Rodrigues et al. 2007). $CD3^+CD56^+$ natural killer T cells and CD1d-restricted natural killer T (NKT) cells are also considered to be activated in acute HIV-1 infection. They may mediate immunoregulatory function through

secreting Th1 and Th2 cytokines or exert their effector functions via cytolysis (Borrow and Bhardwaj 2008).

1. 3. 3 Adaptive immune response

1. 3. 3. 1 Cellular immune response to HIV-1

The $CD8^+$ T cell response

The cellular immune response is crucial to combat viral infection. $CD8^+$ cytotoxic T lymphocytes (CTLs), which eliminate infected cells, play a key role in this process. Their role has been clearly shown in studies in SIV macaque models. When $CD8^+$ CTLs were depleted using specific monoclonal antibodies, the high levels of acute viraemia showed no decrease (Matano, Shibata et al. 1998; Jin, Bauer et al. 1999; Schmitz, Kuroda et al. 1999). Similar to SIV infection in macaques, the first T cell response to HIV-1 infection appears when viraemia reaches its peak, and the T cell response approaches its peak 1-2 weeks later, as viraemia declines (Goulder and Watkins 2004). The immune response timeline could be seen below in **Figure 1-8**.

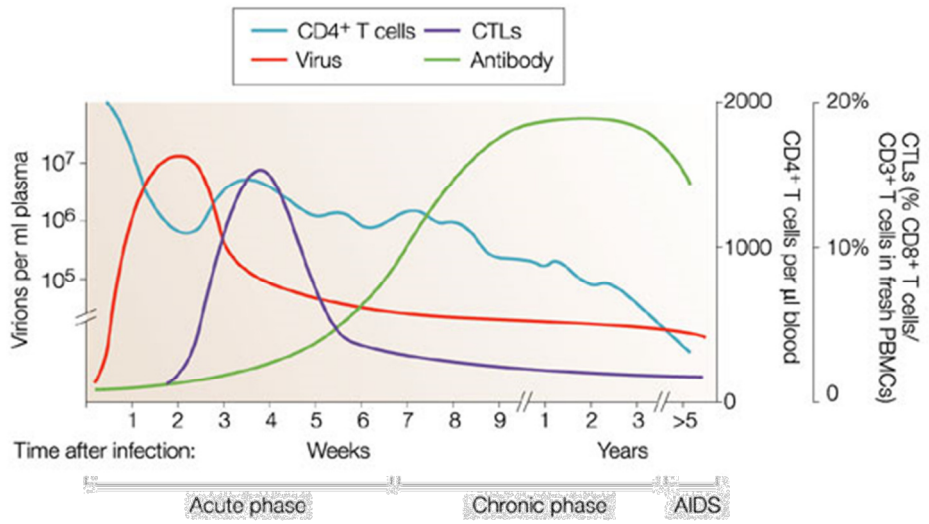


Figure 1-8: The immune response timeline in SIV infection. Adapted from (Goulder and Watkins 2004)

Effector CTLs can directly kill infected cells via production of perforin and granzymes or induce apoptosis of the infected cells through interaction of Fas and FasL (McMichael and Rowland-Jones 2001). CD8 T cells also display a non-cytotoxic antiviral activity involving several cytokines and chemokines including IFN-γ, TNF-α, MIP-1α/β and RANTES etc (Saksena, Wu et al. 2008).

Following the peak of CTLs, rapid selection of mutations occurs in the virus genome (Bernardin, Kong et al. 2005). Most of the amino acid changes in the virus are within epitopes expressed by the founder virus instead of escape mutant virus (Goonetilleke, Liu et al. 2009). Compared to CTL escape mutations, mutations in the viral envelope protein selected by neutralising antibodies emerge later, at about 12 weeks. Some early mutations should be explained with caution. A few mutations could be reversions from the transmitted strain, which are no longer under selection from the partner's immune system; a few mutations could be selected by ADCVI (antibody dependent cell mediated virus inhibition) or

NK cells. The selection of mutations usually involves multiple amino acids changes rather than a single amino acid change. Under such circumstances, various mutants are tested until the fittest is selected. The selection could be in a sequential manner throughout the course of HIV infection, and the affected amino acids could also be in the flanking regions of the T cell epitope in residues that are important for antigen processing (Draenert, Le Gall et al. 2004; Tenzer, Wee et al. 2009).

The first T cell responses target at Env and Nef epitopes (Turnbull, Wong et al. 2009). Responses to other viral proteins such as Gag and Pol tend to arise later but may be more important for controlling the viraemia and maintaining the set point. The more highly conserved epitopes that are targeted by CTLs, the greater the fitness cost paid by the virus through escape mutation. So those immunodominant responses targeting conserved epitopes are more likely to result in a low level of viraemia at the set point (Streeck, Jolin et al. 2009). Patients with HLA-B*27, HLA-B*5701 , HLA-B*5703 and HLA-B*58 do well clinically in terms of disease progression because their CD8 T cell responses target more conserved viral regions, particularly in Gag, and mutations in these epitopes have a high fitness cost. The range of epitopes recognised by CTLs at the early stages is narrow, but later on the response becomes broad, often targeting more than 10 epitopes (Addo, Yu et al. 2003).

In chronic HIV infection, the expanded HIV-1 specific T cells persist at high frequencies, often 1-2% of all circulating CD8 T cells. Without treatment, the high number can persist into late infection and they can still be detected when AIDS develops (McMichael and Rowland-Jones 2001).

The $CD4^+$ T cell response

As activated $CD4^+$ T cells are specific target cells for HIV-1 infection, virus-specific $CD4^+$ T cells are particularly susceptible to HIV-1 infection and are depleted in the early stage of the

infection (Douek, Brenchley et al. 2002; Li, Duan et al. 2005; Mattapallil, Douek et al. 2005); therefore it is always difficult to show $CD4^+$ T cell responses to HIV proteins. Nevertheless, several epitopes for $CD4^+$ T cells have been mapped, particularly in Gag (Kaufmann, Bailey et al. 2004), but such responses often decrease rapidly (Oxenius, Fidler et al. 2001; Gloster, Newton et al. 2004). The early intervention using ART, which prevents the killing of $CD4^+$ T cells, can rescue strong HIV-1 $CD4^+$ T cell responses (Rosenberg, Billingsley et al. 1997; Oxenius, Price et al. 2000).

1. 3. 3. 2 Humoral immune response to HIV-1

Neutralising antibodies

The neutralising antibodies for autologous virus develop slowly, usually emerging 12 weeks or longer after HIV-1 transmission (Richman, Wrin et al. 2003; Wei, Decker et al. 2003; Gray, Moore et al. 2007). As for those antibodies that show some degree of neutralisation of heterologous viruses, they arise in no more than 20% of patients, usually several years after infection (Richman, Wrin et al. 2003; Gray, Madiga et al. 2009; Stamatatos, Morris et al. 2009). The earliest humoral immune response occurs 8 days after the eclipse phase as a form of immune complex; however the free specific antibodies for gp41 and gp120 arise 23 and 38 days respectively after transmission (Derdeyn, Decker et al. 2004; Alam, McAdams et al. 2007; Gray, Moore et al. 2007; Tomaras, Yates et al. 2008). The first HIV-1-specific IgA responses in mucosal secretions are also specific for gp41 during acute HIV-1 infection (Yates, Stacey et al. 2013). These early antibodies do not select escape mutations, which implies that they are not very effective against HIV-1; it may because these antibodies target denatured or non-functional Env forms (Crooks, Moore et al. 2007; Tobin, Trujillo et al. 2008). The first antibodies which can induce escape mutants arise 12 or more weeks after transmission. The range of epitopes targeted by the first neutralising antibodies is narrow

(Moore, Gray et al. 2008), thus permitting rapid viral escape (Richman, Wrin et al. 2003; Wei, Decker et al. 2003).

Most of the neutralising activity elicited by HIV-1 is strain and subtype-specific. Those broad-specificity, neutralising antibodies to conserved Env regions recognise domains such as carbohydrate epitopes, the CD4 binding site, and the CD4-induced co-receptor binding domain, are rarely generated during HIV-1 infection; they can develop only after 20-30 months of infection (Gray, Madiga et al. 2009; Shen, Parks et al. 2009; Stamatatos, Morris et al. 2009). The time delay is partly due to the impaired CD4 help. There are genetic factors affecting the maturation of the antibody response to HIV-1 which determine the generation of this rare, late, broad-specificity, neutralising antibody response.

Non-neutralizing antibodies

Only a part of the envelope-specific responses generated against HIV-1 bears neutralising activity; the other parts fail to impair HIV-1 infectivity directly and hence lack neutralising ability. Currently, there is mounting interest in the potential role of these non-neutralising antibodies in the control of HIV-1 infection. These antibodies can bind to infected cells and recruit activated effector cells, which in turn induce cytolysis or apoptosis. These antibodies utilise the Fc portion binding to the Fc receptors (FcγRs) on effector cells, which could be NK cells, macrophages, dendritic cells, and neutrophils. This binding can induce both cytolysis (Kagi, Vignaux et al. 1994) and the release of antiviral cytokines (Berke ; Russell and Ley 2002) depending on the nature of the receptors. The inhibition effects caused by this mechanism are called ADCC (antibody-dependent cell-mediated cytotoxicity) and ADCVI (Antibody-dependent cell-mediated viral inhibition). Antibodies capable of ADCC/ADCVI have been detected early in infection, and a higher magnitude of Fc-mediated NK cell

responses early after infection correlated with lower set point (Connick, Marr et al. 1996; Forthal, Landucci et al. 2001), nevertheless, the longitudinal association of these responses with clinical outcome is not clear.

To summarise, the sequential immune responses in acute HIV-1 infection can be shown in **Figure 1-9**.

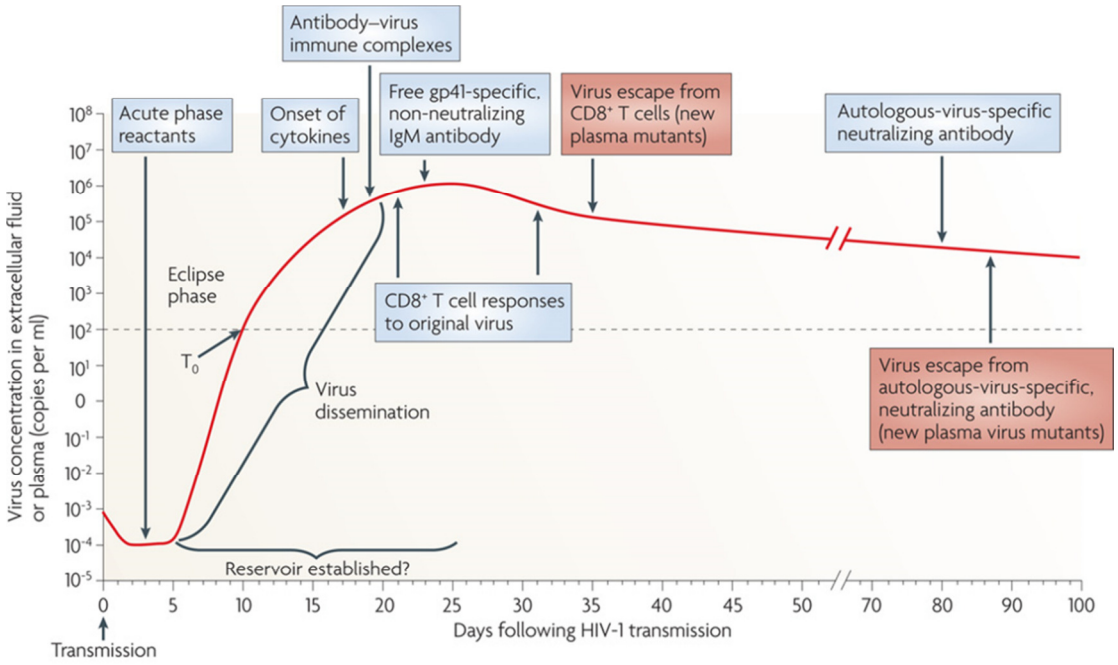


Figure 1-9: The alignment of innate and adaptive immune responses during acute HIV-1 infection. Adapted from (McMichael, Borrow et al. 2010).

1. 3. 4 Host genetic determinants for HIV acquisition and viral control

People show distinct variation in susceptibility to infection by HIV-1 and especially in the clinical outcome after infection. Understanding why some people can establish and maintain effective control of HIV-1 and others do not is extremely crucial in the effort to develop new treatments for HIV/AIDS. Currently the era of studying human genetics has transitioned from candidate-gene studies to genome-wide association studies and more recently to comprehensive exome and genome sequencing studies.

CCR5 is a CC-chemokine receptor which functions as a co-receptor for binding and entry of M-tropic HIV strains into CD4 T cells (Deng, Liu et al. 1996; Dragic, Litwin et al. 1996). The natural chemokine ligands that bind to this receptor are RANTES (Regulated on Activation, Normal T cell Expressed and Secreted protein, also known as CCL5) (Proudfoot, Fritchley et al. 2001; Struyf, Menten et al. 2001; Slimani, Charnaux et al. 2003) and macrophage inflammatory protein (MIP) 1α and 1β (also known as CCL3 and CCL4, respectively). It also interacts with CCL3L1 (Struyf, Menten et al. 2001; Miyakawa, Obaru et al. 2002). For CCR5, there is a mutation consisting of a 32-base-pair deletion which is common in Caucasians (Liu, Paxton et al. 1996; Samson, Libert et al. 1996) but absent in most non-European populations (Martinson, Chapman et al. 1997; Zimmerman, Buckler-White et al. 1997). The deletion leads to a non-functioning protein which fails to localize to the cell surface (Rana, Besson et al. 1997). Homozygous individuals with CCR5Δ32 alleles are highly resistant to M-tropic viruses (Liu, Paxton et al. 1996; Samson, Libert et al. 1996), however, heterozygous individuals with CCR5Δ32 alleles don't show the protective effect (Dean, Carrington et al. 1996).

CCR5 ligands including RANTES, MIP1α and MIP1β have been considered to be potential candidate genes for HIV resistance. The polymorphism of the genes encoding these ligands could be associated with HIV acquisition. For example: variation in the promoter of the *CCL5* gene has been associated with an increased risk of HIV-1 acquisition and disease progression among European-Americans (McDermott, Beecroft et al. 2000; Gonzalez, Dhanda et al. 2001), but this effect is not seen in Japanese (Liu, Chao et al. 1999). Another association between *CCL3* gene variation and a reduced risk of HIV infection was only seen in populations of African ancestry and didn't exist in European-American or Japanese cohorts (Modi, Lautenberger et al. 2006; Hu, Song et al. 2012). Taken together, these observations

suggest that variation in the genes encoding CCR5 ligands may affect acquisition risk in specific populations.

Human genetic variation can also influence the progression of HIV infection to AIDS. As mentioned above, although heterozygous individuals with CCR5Δ32 alleles didn't show resistance to HIV acquisition, they did show a two-year delay to AIDS (Dean, Carrington et al. 1996). CCR5 promoter variation has also shown associations with different rates of HIV progression (Martin, Dean et al. 1998; McDermott, Zimmerman et al. 1998).

APOBEC3G (apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G) is expressed in cells that are non-permissive to HIV-1 replication and is an important inhibitory factor for HIV-1 replication and infectivity. HIV has evolved to counteract this effect using *Vif*, as mentioned before. It was reported that the H186R mutation in exon 4 of the *APOBEC3G* gene was associated with more rapid HIV progression in African-Americans (An, Bleiber et al. 2004).

HLA role shown by GWAS

Many HLA alleles have been associated with different outcomes of HIV infection, and several effects are consistently maintained across different studies, including *HLA-B*57*, *B*27*, *B*58* and *B*35*. The first whole-genome association study of major determinants for host control of HIV-1 (Fellay, Shianna et al. 2007) identified two independently acting groups of polymorphisms which explained 9.6% and 6.5% of the total variation in HIV-1 set point, respectively. The first one, located in the HLA complex, the P5(*HCP5*) gene, actually is a proxy for *HLA-B*5701*, which is well known to be associated with a better outcome in HIV infection (Migueles, Sabbaghian et al. 2000). Although due to the perfect linkage disequilibrium with *HLA-B*5701*, the protective effect of *HCP5* could be ascribed in this way, actually *HCP5* itself is also a good candidate to interact with HIV-1, possibly through

an antisense mechanism (Vernet, Ribouchon et al. 1993). The second most significant polymorphism is a variant located 35kb upstream of the *HLA-C* gene: the -35C variant is associated with protection and -35T is associated with susceptibility. The *-35kb C/T* genotypes correlated with levels of *HLA-C* mRNA transcripts (Stranger, Forrest et al. 2005) and cell surface expression (Thomas, Apps et al. 2009), which implies that high levels of *HLA-C* expression might confer an advantage against the virus. Another GWAS in HIV controllers in an international cohort (Pereyra, Jia et al. 2010) further demonstrated that all the positive SNP signals concentrated around the HLA class I region on chromosome 6, which again highlights the central role of HLA class I in protection against HIV disease progression.

Further analysis from the two GWAS showed that the major genetic associations, after correction for multiple comparisons, were due to polymorphisms in amino acids in the HLA-B peptide-binding groove. The most significant amino acid positions associated with control are positions 67, 70, and 97 in HLA-B, which are all involved in binding peptides within the peptide-binding groove. Positions 62 and 63 in the HLA-B peptide-binding groove were also found to be independently significant (Carrington and Walker 2012).

HLA heterozygote and rare allele advantage

Based on HIV genetic epidemiological data, it is plausible that both *HLA* heterozygotes (Carrington, Nelson et al. 1999; Tang, Costello et al. 1999) and rare *HLA* alleles (Trachtenberg, Korber et al. 2003) have shown advantages to HIV-infected individuals. The mechanism underlying this observation could be that *HLA* heterozygous individuals will present a broader repertoire of viral peptides to T cells than will homozygotes. As for the rare allele advantage, it could be that the most frequent HIV strains are more likely to have

adapted to HLA alleles common in a particular population, leaving the less frequent allotypes advantageous for the host.

HLA class I loci

Although the *HLA-B*57* allele tops all others in terms of its protective effect on HIV, the basis for this protection is not yet very clear and probably involves an array of mechanisms (Martinez-Picado, Prado et al. 2006; Kiepiela, Ngumbela et al. 2007; Crawford, Lumm et al. 2009; Kloverpris, Stryhn et al. 2012; Mendoza, Royce et al. 2012). *HLA-B*57* restricts three or four immunodominant epitopes located in conserved regions of Gag, especially the epitope TW10, and escape mutations within these targeted epitopes are commonly observed in viruses isolated from *HLA-B*57* positive patients at a cost of reduced viral fitness. The reduced fitness of HLA-B*57-associated mutations may in part explain the HLA-B*57 protective role. Although *HLA-B*57* is highly enriched among the most extreme controllers, many patients processing this gene can progress to AIDS as rapidly as those without this allele. In this sense, the influence of HLA on HIV pathogenesis is complicated and involves multiple factors including genetic, viral and environmental factors, which could modify the final outcome.

HLA-B*27 is also protective in terms of viral control and AIDS progression (Hendel, Caillat-Zucman et al. 1999; Gao, Bashirova et al. 2005; Pereyra, Addo et al. 2008). In spite of the similarities to B*57, B*27 protection has its own distinct features. The HLA-B*57-restricted CD8 response targets up to four Gag epitopes, however B*27 restriction is focused on a single gag epitope, KK10. Under B*27-restricted pressure, the virus experiences a complex pattern of mutations that result in a completely escaped virus with wild-type levels of replication ability (Schneidewind, Brockman et al. 2007). Development of this escape pattern leads to rapid disease progression (Goulder, Phillips et al. 1997; Kelleher, Long et al. 2001;

Feeney, Tang et al. 2004): however, the complexity of the mutations and the required compensatory mutations means that it takes quite a while for the escape pattern to emerge.

*HLA-B*35* alleles associate with susceptibility to AIDS across multiple studies (Itescu, Mathur-Wagh et al. 1992; Sahmoud, Laurian et al. 1993; Carrington, Nelson et al. 1999). HLA-B*35-restricted CD8 T cells rarely recognize Gag (Streeck, Lichterfeld et al. 2007). Subsets of *HLA-B*35* alleles were identified (Gao, Nelson et al. 2001): one is termed HLA-B*35-Px allotypes (B*3502, B*3503, B*3504) which bind peptides with hydrophobic residues at position 9 (Steinle, Falk et al. 1996), the other one consists of the HLA-B*35-Py allotypes (B*3501 and B*3508), which prefer binding peptides with tyrosine at the same position (Hill, Elvin et al. 1992; Smith, Reid et al. 1996). It was shown that B*35-Px allotypes associate most strongly with the susceptible phenotype (Gao, Nelson et al. 2001), which implicates differential peptide binding and presentation in the underlying mechanism. Other possible mechanisms might involve different binding affinity to Ig-like transcript 4 (ILT4), an inhibitory receptor expressed on DCs, which may lead to more profound DC dysfunction in HLA-B*35 Px-positive individuals, thereby accelerating disease progression (Huang, Goedert et al. 2009).

HLA class II loci

There are no consistent genetic data indicating that polymorphisms at the HLA class II loci associate with different HIV/AIDS outcomes.

KIR polymorphism (discussed below).

1. 4 Natural killer cells

1. 4. 1 NK cell introduction: biology and receptors

Natural killer (NK) cells originate from the common lymphoid progenitor cell in the bone marrow. NK cells account for 5–15% of peripheral blood mononuclear cells and are defined phenotypically by the expression of CD56 and lack of expression of CD3 (Robertson and Ritz 1990). Based on the cell-surface density of CD56, NK cells are further divided into two distinct populations (Lanier, Le et al. 1986). The majority (about 90%) of human NK cells in peripheral blood are $CD56^{dim}$ $CD16^{high}$, whereas about 10% of peripheral blood NK cells are $CD56^{bright}CD16^{dim}$ or $CD16^{-}$.

$CD56^{dim}$ NK cells produce low levels of cytokines (Cooper, Fehniger et al. 2001) but are potent mediators of ADCC (antibody-dependent cell-mediated cytotoxicity), LAK (lymphokine-activated killer) activity and natural cytotoxicity. The $CD56^{dim}$ NK-cell subset has high-level expression of KIRs whereas $CD56^{bright}$ NK cells have high expression of CD94/NKG2A (Voss, Daley et al. 1998; André, Spertini et al. 2000). $CD56^{bright}$ NK cells produce high levels of cytokines following stimulation (Cooper, Fehniger et al. 2001). This subset has low-density expression of CD16 and exhibits low natural cytotoxicity and ADCC, but potent LAK activity. The schematic figure could be seen below (**Figure 1-10**).

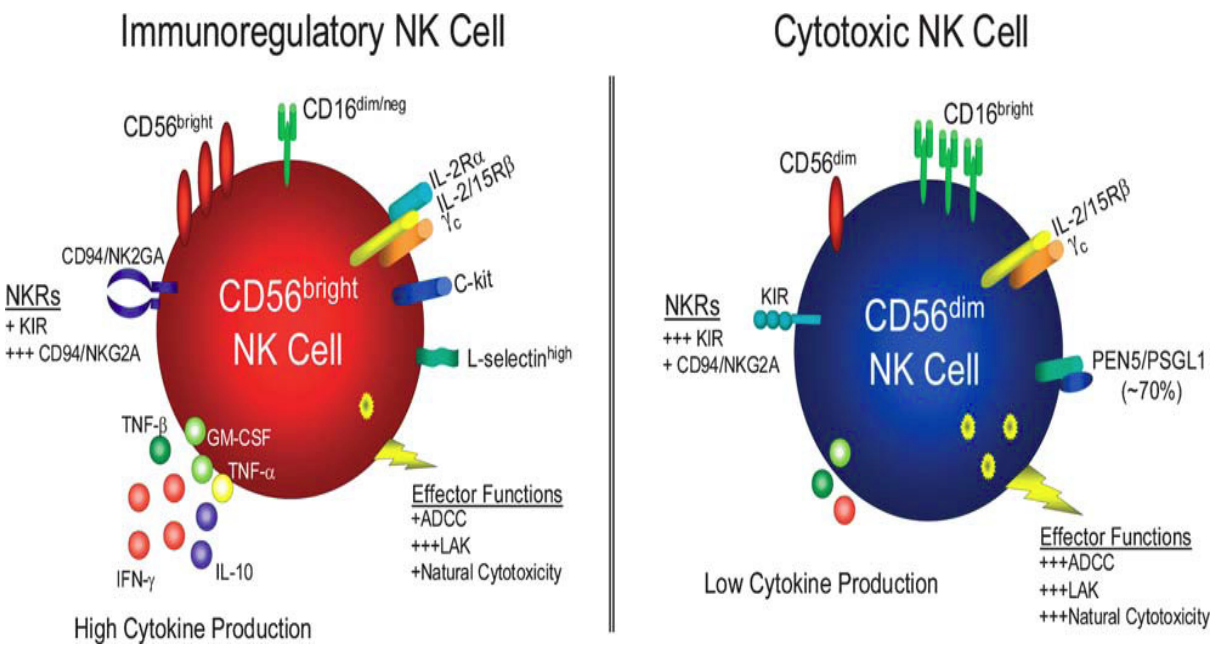


Figure 1-10: CD56^{bright} and CD56^{dim} NK cell subsets exhibit differential receptor profiles and immune functions. Adapted from (Cooper, Fehniger et al. 2001)

NK cell receptors

NK cell can be rapidly activated in the absence of specific immunisation. Whether or not a NK cell is activated is determined by the balance of an array of activating signals and inhibitory signals transduced from the surface receptors. Activating receptors include: the C-type lectin-like receptors NKG2D and CD94:NKG2C/E, natural cytotoxicity receptors NKp44, NKp30, NKp46, and CD16 (Fc-γ-RIII), which is the low-affinity Fc receptor and mediates antibody-dependent cytotoxicity; the main inhibitory receptors are KIRs and the CD94:NKG2A heterodimer.

NK cell function: secretion and killing mechanism

NK cells participate in the early control of virus infection, especially herpesvirus infection (Lee, Miyagi et al. 2007) and in tumour immunosurveillance (Smyth, Hayakawa et al. 2002).

Although named "natural" effector cells, NK cells still need priming by various factors such as IL-15 presented by dendritic cells (Lucas, Schachterle et al. 2007) or macrophages

(Mortier, Advincula et al. 2009), IL-12 (Guia, Cognet et al. 2008) or IL-18 (Chaix, Tessmer et al. 2008) to achieve their full function. NK cells also impact DC, macrophages and neutrophils through cytokine production (Moretta, Marcenaro et al. 2005) and regulate downstream antigen-specific T and B cell responses. In this sense, NK cells are not simply "innate immunity" cells, they actually play a crucial "bridge" role between innate immunity and adaptive immunity.

NK cells can secret Th1 cytokines such as IFNγ and tumour necrosis factor α (TNF α) and exert cytotoxic effects on their target cells. IFNγ activates macrophages for phagocytosis and helps to shape T cell responses; TNFα acts to promote direct NK killing of tumour cells. The killing could be mediated by degranulation of cytotoxic granules or by surface expression of ligands such as Fas ligand (FasL) and TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) that activate death receptors on target cells. The granules in the cytoplasm of NK cells contain perforin and granzymes. Upon release towards the target cells, perforin forms pores and helps granzymes to enter, inducing either apoptosis or osmotic cell lysis.

The mechanism underlying NK cell activation involves a hypothesis named "missing self" (Karre, Ljunggren et al. 1986). When cells are infected by viruses or undergo cellular transformation, the MHC class I molecules on the cells surface decrease; NK cells can detect the lack of surface class I and then exert their cytotoxic function. The recognition is mediated by MHC class I specific receptors including KIRs and CD94/NKG2A heterodimers (Moretta, Bottino et al. 1996). NK cells circulate through the blood, lymphatics and tissues, on patrol for the presence of transformed or pathogen-infected cells. Thus, NK cells spare normal cells which express self-MHC class I molecules, whereas they selectively kill target cells "in distress" that down-regulate MHC class I molecules and/or up-regulate stress-induced self molecules such as NKG2D ligands (Raulet and Guerra 2009).

NK cell education, licencing, arming and tuning

Many models have been proposed to explain NK cell development.

The first two models are ‘licensing’ or ‘education’ models (Kim, Poursine-Laurent et al. 2005; Anfossi, Andre et al. 2006). Bone marrow-derived NK cells show evidence of enhanced proliferation if they express an inhibitory receptor for a self-MHC class I molecule. To some extent, ‘licensing’ is similar to the process of positive selection which occurs in the thymus for T cells with appropriate self-HLA-reactive T-cell antigen receptors (Starr, Jameson et al. 2003). Licensing results in two types of self-tolerant NK cells-licensed NK cells with self-MHC class I inhibitory receptors, and unlicensed NK cells. In these models the interaction between inhibitory NK cell receptors and self HLA during development confers a signal that renders an NK cell functionally competent, whilst NK cells that lack an inhibitory receptor to self HLA remain inert. Yokoyama and colleagues refer to this education process as 'licensing' (Yokoyama and Kim 2006).

The third and fourth model are ‘arming’ or ‘tuning’ models. In these models, the presence of a dominant inhibitory signal during development results in the ‘arming’ of an NK cell, whereas the presence of no inhibition, and/or too much activation results in the disarming (Fernandez, Treiner et al. 2005) or tuning (Salcedo, Andersson et al. 1998) of NK cells, which then circulate in the peripheral circulation as a hyporesponsive subset of cells.

The inhibitory HLA class I binding receptors including inhibitory KIRs and NKG2A/CD94 mediated education and activating KIRs mediated education can to some extent be compared to the positive and negative selection of T cells; education by inhibitory binding receptors would be similar to positive selection, and education by activating KIRs would be the analog of negative selection. T cell selection leads to deletion or survival, however NK cell

education results in different level of activation without affecting survival (Kim, Poursine-Laurent et al. 2005; Anfossi, Andre et al. 2006).

1. 4. 2 NK cells in HIV-1 infection

Phenotypic changes

NK cell populations are significantly expanded during acute infection before HIV-1 seroconversion (Alter, Teigen et al. 2007). Although NK cells can be activated and KIR expression can be increased after HIV infection, NK cell cytotoxicity is reduced during acute infection (Naranbhai, Altfeld et al. 2013).

There are significant changes amongst the subsets of NK cells during HIV-1 infection. NK cell numbers are elevated during acute HIV-1 infection, with an expansion of $CD56^{dim}CD16^{pos}$ NK cells and an early depletion of $CD56^{bright}CD16^{neg}$ NK cells (Alter, Teigen et al. 2005). During chronic infection, it is observed there is a decline in the proportion of $CD56^{pos}$ cells as well as an expansion of $CD56^{neg}CD16^{pos}$ NK cells (Hu, Hultin et al. 1995; Azzoni, Papasavvas et al. 2002; Mavilio, Benjamin et al. 2003; Alter, Malenfant et al. 2004). $CD56^{neg}CD16^{pos}$ NK cells lack the majority of NK cell effector functions, including killing, cytokine secretion and ADCC, and also exhibit aberrant DC editing activity (Ahmad, Morisset et al. 1994; Fehniger, Herbein et al. 1998; Ziegner, Campbell et al. 1999; Scott-Algara and Paul 2002; De Maria, Fogli et al. 2003). Taken together, these studies have shown that ongoing viral replication has caused depletion of $CD56^{dim}CD16^{pos}$ NK cells with a parallel increase in functionally anergic $CD56^{neg}CD16^{pos}$ NK cells resulting in a sequential impairment of NK cell function during HIV-1 infection (Alter, Teigen et al. 2005).

Whether these two NK cell subsets in humans share a precursor–product relationship or are the result of independent pathways of development is not known. Some evidence suggests that $CD16^{-}CD56^{hi}$ NK cells are precursors of $CD16^{+}CD56^{low}$ NK cells. First, the proportion of $CD16^{-}CD56^{hi}$ NK cells in the blood is increased in patients shortly after bone-marrow transplantation and declines over time in these patients, when the $CD16^{+}CD56^{low}$ NK cell population becomes more prominent (Gottschalk, Bray et al. 1990; Jacobs, Stoll et al. 1992). Second, telomere length, which progressively diminishes with cell division and age, is significantly shorter in $CD16^{+}CD56^{low}$ NK cells compared with $CD16^{-}CD56^{hi}$ NK cells from the same donor (Ouyang, Baerlocher et al. 2007).

Genetic studies suggesting a role for KIR^{+} NK cells in HIV-1 outcome

GWAS provided evidence that NK cells might play a role in the control of HIV-1 replication and disease outcome (Fellay, Shianna et al. 2007; Pereyra, Jia et al. 2010). Earlier genetic studies (Flores-Villanueva, Yunis et al. 2001) showed that HIV-1-infected individuals with homozygosity for the HLA-Bw4 motif experience significantly better control of HIV-1 viraemia and slower HIV-1 disease progression. In addition to their role in presenting viral peptides, the HLA-Bw4 group of molecules also function as ligands for KIR. In 2002, a study of AIDS progression in an ART naïve cohort (Martin, Gao et al. 2002) demonstrated that the compound genotype *KIR3DS1+ HLA-B Bw4 80I* was protective in terms of HIV-1 progression. Surprisingly, in the same cohort, the combination of *KIR3DL1*h/y + HLA-B Bw4-80I* was also shown to be protective against HIV progression (Martin, Qi et al. 2007). The copy number variation in the *KIR3DL1* and *KIR3DS1* was also shown to play a role against HIV-1 infection (Pelak, Need et al. 2011): an increase in *KIR3DS1* count associates with a lower viral set point in the presence of its putative ligand; an increase of *KIR3DL1* count shows the same effect when both *KIR3DS1* and the appropriate ligands are present. All

these genetic studies suggest that KIR^+ NK cells and their interactions with their HLA ligands, might play a critical role in the outcome of HIV-1 infection.

Functional studies of NK cells in HIV-1 infection

NK cells expressing KIR3DS1 and, to a lesser extent, the inhibitory receptor KIR3DL1 specifically expand in acute HIV-1 infection in the presence of *HLA-B Bw4 80I* (Alter, Rihn et al. 2009). *KIR3DS1*$^+$ NK cells have stronger activity in HIV-1-infected individuals encoding for *HLA-Bw4* alleles (Long, Ndhlovu et al. 2008) and can inhibit HIV-1 replication *in vitro* more potently (Alter, Martin et al. 2007). Furthermore, the *KIR3DL1* (+) NK cell subset also showed increased functional potential (Boulet, Song et al. 2010). Current models of NK cell development suggest that NK cells need to receive an inhibitory signal through an inhibitory KIR during maturation to become functionally competent (Kim, Poursine-Laurent et al. 2005; Elliott, Wahle et al. 2010; Elliott and Yokoyama 2011). Although there is accumulated evidence to show the interaction between KIR3D molecules and HLA-Bw4, many puzzles still remain: no direct binding between HLA-Bw4 and KIR3DS1 has been shown to date (Carr, Rosen et al. 2007; O'Connor, Yamada et al. 2011; Vivian, Duncan et al. 2011); since inhibitory and activating KIR exert different effects on NK cell licencing, when an individual expresses both KIR3DL1 and KIR3DS1 receptors, it is difficult to evaluate how they really influence NK cell function. Moreover, due to the close location of different *KIR* genes, linkage disequilibrium is also an issue worth considering when analysing single *KIR* gene effects.

The sequence of the HLA class I –presented epitopes are thought to play a crucial role in the interaction of KIR and HLA (Altfeld and Goulder ; Boyington, Motyka et al. 2000), especially the C-terminal part of the epitope. The positions 7 and 8 (in a nonamer peptide) have been shown to be critical for the epitope to modulate KIR/HLA binding (Brackenridge,

Evans et al. 2011). During HIV-1 infection, naturally occurring amino acid changes in HIV-1- encoded peptides can significantly modulate the binding of the associated inhibitory KIRs (Thananchai, Gillespie et al. 2007; Fadda, Korner et al. 2012).

HIV evasion from NK cell mediated immune pressure

Viruses have developed several ways to evade the host's immune pressure, and the evolution of these evasion mechanisms reflect directly the immune selection pressure exerted by the host (Lodoen and Lanier 2005).

Using *Nef*, HIV-1 has evolved to escape from strong CTL response restricted by *HLA-A* and *HLA-B* alleles, while maintaining the HLA-C ligand for the broadly expressed and inhibitory KIR2DL molecules on the surface of the infected cells to provide an inhibitory signals to NK cells (Cohen, Gandhi et al. 1999). Also Nef can induce down-regulation of NKG2D ligands including MIC A, ULBP1, 2, and 3 on surfaces of the infected cells (Cerboni, Neri et al. 2007), which suggests that NKG2D may also be involved in the recognition and control of HIV-1 infection.

In summary, multiple NK cell receptors have been involved in HIV-1 disease progression and transmission, with the implication that NK cells might contribute significantly to the control of HIV-1 infection. On the other hand, HIV-1 may have also have evolved mechanisms to escape from NK cell recognition. Nevertheless, to what extent these viral evasion strategies modulate HIV-1 pathogenesis still needs greater understanding.

1. 5 Killer immunoglobulin-like receptors

1. 5. 1 *KIR* genes

The *KIR* gene family is encoded within a 100-200 Kb region of the Leukocyte Receptor Complex (LRC) located on chromosome 19 (19q13.4), currently consisting of 15 gene loci

(*KIR2DL1, KIR2DL2/L3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1/S1, KIR3DL2, KIR3DL3*) and 2 pseudogenes, *KIR2DP1* and *KIR3DP1* (Trowsdale 2001). The *KIR* genes are arranged in a "head-to-tail" cluster and encode molecules that belong to the immunoglobulin superfamily of receptors (Wilson, Torkar et al. 2000). The lengths of KIR genes vary from 4 to 16 Kb (full genomic sequence).

KIR genes can be classified according to the number of extra-cellular domains which are encoded to form either KIR2D or KIR3D molecules. *KIR* genes can also be divided into L or S representing long or short respectively in terms of the length of the cytoplasmic domain. *KIR2DL* and *KIR3DL* genes encode inhibitory molecules (*KIR2DL4* is an exception, which has both inhibitory and activation potential), whereas *KIR2DS* and *KIR3DS* encode activating receptors.

KIR genes can be divided into two groups of haplotypes, named A and B. Haplotype A comprises nine genes and encodes predominantly inhibitory receptors, there is only one activating gene *KIR2DS4* in haplotype A. Conversely the B haplotypes represent several groups of haplotypes and contain more activating *KIRs* (**Figure 1-11**).

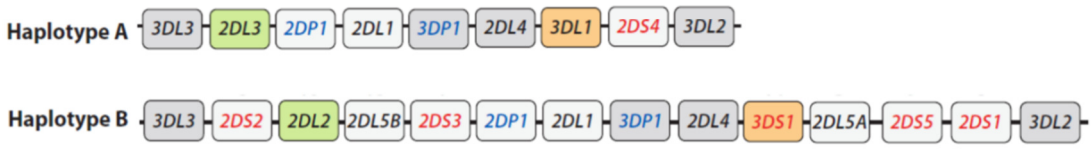


Figure 1-11: Haplotypes of KIR genes**.** Gene pairs *KIR2DL2/L3* and *KIR3DL1/S1* are alleles of the same loci (shown in green and orange respectively). The four framework genes are shown as grey boxes. Genes encoding activating KIR with short tails and pseudogenes are denoted by red and blue letters, respectively. Adapted from (Bashirova, Thomas et al. 2011).

The number of alleles for each *KIR* gene is shown below (**Figure 1-12**).

KIR Alleles								
Gene	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3
Alleles	43	28	34	46	41	15	22	14
Proteins	24	11	17	22	17	7	8	5
Nulls	1	0	1	0	0	0	0	1
Gene	2DS4	2DS5	3DL1	3DS1	3DL2	3DL3	2DP1	3DP1
Alleles	30	16	73	16	84	107	22	23
Proteins	13	11	58	12	61	55	0	0
Nulls	0	0	1	1	1	0	0	0

Figure 1-12: The number of alleles for each KIR gene**.** Data are from the IPD-KIR database released on April 2011 (http://www.ebi.ac.uk/ipd/).

Conserved genes are located at the centromeric (*KIR3DL3*) and telomeric (*KIR3DL2*) ends of the haplotype, as well as in the central part (*KIR3DP1* and *KIR2DL4*) of the locus (Pyo, Guethlein et al. 2010). These framework genes define two regions of gene-content variation, one in the centromeric part of the locus, the other in the telomeric part. The combination of *Cen-A* and *Tel-A* forms the group A KIR haplotype, a relatively short haplotype with a predominance of inhibitory receptors. The group B KIR haplotypes are characterized by more activating *KIRs* and *KIRs* that have reduced or lost recognition of HLA class I. *Cen-A1* and *Tel-A1* are the centromeric and telomeric motifs, respectively, of the canonical A *KIR* haplotype; *Cen-B1* and *Cen-B2* are alternative centromeric motifs of common B haplotypes, and *Tel-B1* is the common telomeric motif of B haplotypes (Cooley, Weisdorf et al. 2010).

The gene content of the common motifs is shown in (**Figure 1-13**).

		Centromeric part									RS	Telomeric part								
Haplotype	Cen motif	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5	2DP1	2DL1	3DP1		2DL4	3DL1	3DS1	2DL5A	2DS3/5	2DS1	2DS4	3DL2	Tel motif
A	Cen-A1																			Tel-A1
B	Cen-A1																			Tel-B1
	Cen-B1																			Tel-A1
	Cen-B2																			Tel-A1
	Cen-B1																			Tel-B1
	Cen-B2																			Tel-B1

Figure 1-13: The centromeric and telomeric regions. They are separated by a unique recombination site (RS) sequence functioning to reassort the centromeric and telomeric gene motifs. The conserved framework genes are shaded grey, haplotype B genes are blue, and haplotype A genes are red. Adapted from (Cooley, Weisdorf et al. 2010).

1. 5. 2 KIR-HLA interaction

HLA class I molecules provide ligands for KIR receptors. The interaction of the inhibitory KIRs with their associated ligands is much better established, compared to their activating counterparts. The inhibitory receptors KIR2DL1, 2DL2, and 2DL3 are specific for HLA-C. KIR2DL1 binds HLA-C2 (lysine at position 80); whereas KIR2DL2/L3 bind HLA-C1 (asparagine at position 80) (Colonna, Borsellino et al. 1993; Wagtmann, Rajagopalan et al. 1995). The affinities of these interactions are different and it is thought that KIR2DL1/HLA-C2 and KIR2DL2/HLA-C1 are relatively stronger whilst KIR2DL3/HLA-C1 is relatively weaker (Winter, Gumperz et al. 1998; Moesta, Norman et al. 2008).

As for the 3D KIRs, KIR3DL1 recognises *HLA-B* alleles with the Bw4 serological motif (HLA-Bw4) and also some *HLA-A* alleles which contain the Bw4 motif. KIR3DL2 recognises HLA-A3 and HLA-A11 (Gumperz, Litwin et al. 1995; Pende, Biassoni et al. 1996). However for the activating receptors, the specific interaction with HLA class I molecules is less defined, although they share high similarities with their inhibitory counterparts. Binding of KIR2DS1 to HLA-C is very similar to that of KIR2DL1 but occurs

at a much lower affinity (Stewart, Laugier-Anfossi et al. 2005). Furthermore, albeit KIR2DS2 and KIR3DS1 share considerable sequence homology with KIR2DL2 and KIR3DL1 respectively, binding to the relevant ligands has not been convincingly established.

The summary of the interaction between KIRs and HLA class I molecules can be seen in **Table 1-4**.

Table 1-4: KIR molecules and their HLA ligands

Receptor	ligand
KIR2DL1	HLA-C2: C*02, C*04, C*05, C*06
KIR2DL2/L3	HLA-C1: C*01, C*03, C*07, C*08 Some HLA-C2: C*0501, C*0202 Some HLA-B: B*4601, B*7301
KIR3DL1	Some HLA-A and HLA-B expressing the Bw4 epitope HLA B*08, B*27, B*57, B*58
KIR3DL2	HLA-A: A*03, A*11
KIR2DS1	HLA-C2: C*02, C*04, C*05, C*06
KIR2DS2	unknown
KIR2DS3	unknown
KIR2DS5	unknown
KIR3DS1	unknown
KIR3DL3	unknown
KIR2DS4	HLA-C: C*0501, C*1601, C*0202 HLA- A*1102
KIR2DL4	HLA-G

KIR2D and HLA-C

The KIR-HLA interaction is sensitive to single amino acid substitutions in the KIR, which can vary its epitope specificity (Winter and Long 1997) or abrogate recognition of HLA (Biassoni, Pessino et al. 1997; Winter and Long 1997; Winter, Gumperz et al. 1998). It was demonstrated that for KIR2DL1 and KIR2DL3, mutagenesis at position 44 was enough to "swap" the C1 and C2 specificities. This means that the KIR2DL1 mutant with lysine 44 acquires C1 specificity, whereas the KIR2DL3 mutant with methionine 44 acquires C2 specificity. The strengths of the KIR/HLA-C interactions are variable： KIR2DL1-HLA C2 is

considered to be the strongest inhibitory combination, with KIR2DL2- HLA C1 conferring intermediate inhibition, and KIR2DL3-HLA C1 having the weakest inhibitory effect (Winter, Gumperz et al. 1998; Moesta, Norman et al. 2008; Moesta and Parham 2012).

These different binding abilities could explain some clinical associations. In regulating the resolution of HCV infection, the weaker inhibition conferred by KIR2DL3-HLA C1 was found to be protective, possibly because it enables stronger NK cell responses than KIR2DL2-C1 or KIR2DL1-C2 (Khakoo, Thio et al. 2004). Conversely, the stronger inhibitory interaction of maternal KIR2DL1 with foetal C2 in pregnancy appears to render uterine NK cells hypofunctional, thereby tending to cause several types of disorders: recurrent miscarriage, preeclampsia, and foetal growth restriction (Hiby, Walker et al. 2004; Hiby, Apps et al. 2010).

KIR2DL1 appears exclusively specific for C2, demonstrating no detectable cross-reactivity with C1 (Moesta, Norman et al. 2008).By contrast, KIR2DL2/3, cross-reacts with C2 and may use it as a functional ligand. This interaction, KIR2DL2-C2 is weaker than that between KIR2DL1 and C2, but significantly stronger than KIR2DL3 -C2 (Moesta and Parham 2012).

KIR2DL2/3 is located in the centromeric region: *KIR2DL3* being characteristic for *Cen-A*, while *KIR2DL2* is characteristic for *Cen-B*. It is noteworthy that *KIR2DL2* and *KIR2DS2* are in almost complete LD with each other (Moesta and Parham 2012). This is important when we interpret the role of *KIR2DL2* in terms of its association with many diseases.

KIR not only interact with the α1 and α2 domains of HLA class I, but also directly contact the HLA-bound peptide (Malnati, Peruzzi et al. 1995; Rajagopalan and Long 1997; Zappacosta, Borrego et al. 1997). KIR binding and function are particularly sensitive to the peptide residues at position 7 and 8 (Rajagopalan and Long 1997; Boyington, Motyka et al.

2000). Such sensitivity to peptide interactions could render NK cells able to detect subtle changes in the peptide repertoire, as occurs during viral infections. In principle this would be a more sensitive sensory mechanism than the down-regulation of HLA expression on target cells which results in the absence of self-HLA class I (Rajagopalan and Long, 2010).

1. 5. 3 KIR polymorphisms

The *KIRs* are a multigene family and its members are highly polymorphic. The KIR molecules also have complex, clonal expression patterns on NK cells. In terms of KIR polymorphisms, there are several different levels (Gardiner 2008).

1. *KIR* gene content For one individual, the *KIR* gene numbers can vary from 9 to 17 genes. There are only 3 *KIR* genes present on almost every haplotype: *3DL3, 3DL2*, *3DP1* and *2DL4*. *KIR3DL3* , *KIR3DP1* and *3DL2* are framework genes, *KIR2DL4* is the only *KIR* gene which is expressed by all NK cells (Rajagopalan and Long 1999) and its alleles are codominantly expressed (Chan, Kurago et al. 2003).

2. *KIR* gene alleles and haplotypes For each *KIR* gene locus, the numbers of alleles are variable (Robinson, Halliwell et al. 2013). For example, in the latest database (Robinson, Halliwell et al. 2013), *KIR3DL1* has 92 alleles, *KIR3DS1* has 16 alleles; Haplotype A contains 9 genes and only one activating gene *KIR2DS4*; B haplotypes thus contain more activating KIRs.

3. KIR expression patterns Except *KIR2DL4*, which is expressed ubiquitously on NK cells, KIRs are expressed in a stochastic way leading to a situation where in one individual, different subsets of NK cells can express different combination of KIRs. For any given *KIR* gene, donors also vary in the frequency of NK cells expressing the receptor. For example, one donor may express *3DL1* on 50% of their NK cells and the next person could express it on

only 5% of their NK cells (Gumperz, Paterson et al. 1996). There is also variation in the expression levels of a given KIR. For example, particular alleles of *3DL1 (*002, *01502*) are expressed at higher levels on NK cells and they are also found on a higher percentage of NK cells (Gardiner, Guethlein et al. 2001; Yawata, Yawata et al. 2006). There is also redundancy within allelic polymorphism: for example, the *3DL1*004* allele is poorly expressed at the cell surface (Pando, Gardiner et al. 2003); *2DS4*003* is truncated and not expressed at the cell surface (Parham 2005). Once the KIR expression pattern has emerged during development, it appears to be stable within one individual. For this given KIR pattern, both the percentage and level of expression remain unchanged in this individual over time (Gumperz, Paterson et al. 1996; Mingari, Vitale et al. 1997; Shilling, Young et al. 2002).

4. Copy number variations If one person possesses two copies of a given *KIR* gene, they will express it at a higher frequency compared to those having only one copy (Yawata, Yawata et al. 2006; O'Connor, Guinan et al. 2007).

5. HLA polymorphism HLA class I provides ligands for the KIRs, so that in order for a functional interaction to occur, both receptor and ligand pairs should be expressed within the same individual. However, *HLA* and *KIR* segregate independently on different chromosomes (Vilches and Parham 2002), and *HLA* is the most polymorphic gene system which extremely increases the complexity of the polymorphism.

1. 5. 4 How to evaluate the role of KIR in a disease?

Most of the evidence for a role for KIR in disease comes from disease-association studies. Since the *KIR* genes are genetically complicated (polygenic and polymorphic) and have sophisticated interactions with HLA, it is speculated that the association might be subtle. It is considered that examination of KIR alone will not be as informative as examination of combinations of KIR and HLA in association with particular disease outcomes (Carrington

and Martin 2006). Given the polymorphic nature of both *KIR* and *HLA* genes, these studies require large sample numbers and need to be controlled with caution. Generally phenotypic and functional studies are required in tandem to validate the associations found with genetic studies.

1. 5. 5 The role of KIRs in different human diseases

Given the critical role of KIRs in the regulation of NK cell function, their associations with protection or susceptibility in different human diseases is not surprising. Both the presence and absence, as well as allelic variation at some of the KIR loci have been associated with increased risk of several human diseases and disease outcomes (Kulkarni, Martin et al. 2008), such as viral infections, autoimmunity, reproductive failure, and cancer etc.

Viral infections: HCV, HBV, HIV

In a study of HCV resolution (Khakoo, Thio et al. 2004), the weak *KIR2DL3+HLA-C1* interactions were shown to be protective in Caucasians and African Americans with expected low infectious doses of HCV. This might suggest that the differences in the ability of distinct KIR-HLA genotypes to modulate NK cell activity cannot overcome the high-dose infection but should be enough to alter the outcome when faced with low-dose infection.

KIR2DL1 in combination with *HLA-C2* confers susceptibility to chronic hepatitis B , whereas homozygotes for the inhibitory receptor *KIR2DL3* or *KIR2DL3* in the presence of the HLA-C1 C1 genotype shows protection against Chronic hepatitis B (Gao, Jiao et al. 2010).

The main KIRs involved in HIV infection are *KIR3DL1/3DS1* and KIR2D which we will talk about in detail later.

Autoimmune disease: Rheumatoid arthritis, psoriatic arthritis, multiple sclerosis, Crohn's disease etc.

The *KIR2DS2* gene was significantly enriched among patients with rheumatoid vasculitis compared with normal individuals (odds ratio 5.56, P = 0.001) and in patients with RA but no vasculitis (odds ratio 7.96, P = 0.001). The *KIR2DS2* gene significantly increases the risk of patients with Rheumatoid arthritis to develop vasculitic complications (Yen, Moore et al. 2001).

The absence of *KIR2DS1* and *KIR2DS2* was associated with protection against psoriatic arthritis, whereas these receptors conferred susceptibility to disease when the HLA ligands for the corresponding inhibitory KIRs were missing. The presence of *KIR2DS1* and/or *KIR2DS2* along with HLA alleles encoding ligands for *KIR2DL1* and *KIR2DL2/3*, genes that were present in nearly all individuals sampled, was neutral (Martin, Nelson et al. 2002).These data suggest that, in the presence of their HLA ligands, corresponding inhibitory KIR may neutralise the effect of the activating KIR. *KIR2DS2* has been implicated in the pathogenesis of vascular damage among individuals with rheumatoid arthritis (Yen, Moore et al. 2001), and it would be of interest to determine whether this effect could be attributed to *KIR2DS2* in the absence of alleles encoding HLA ligands for *KIR2DL2/3*.

Absence of the inhibitory *KIR2DL3* gene is associated with an increased risk of developing multiple sclerosis in individuals carrying *HLA-C1* alleles (Jelčić, Hsu et al. 2012).

There is a protective effect for *KIR2DL2* in the absence of its HLA ligand C1 (Hollenbach, Ladner et al. 2009) for Crohn's disease.

Reproductive disease: preeclampsia, foetal growth restriction and recurrent miscarriage, etc.

The combination of maternal *KIR AA* genotype with a foetal *HLA-C2* is associated with an increased risk of pre-eclampsia (Hiby, Walker et al. 2004).

Pregnancy disorders were less frequent in mothers that possessed the telomeric end of the KIR B haplotype, which contains activating *KIR2DS1* (Hiby, Apps et al. 2010).

Cancer: cervical neoplasia etc.

Specific inhibitory *KIR/HLA* ligand pairs decrease the risk of developing neoplasia, whereas the presence of the activating receptor *KIR3DS1* results in an increased risk of disease, particularly when the protective inhibitory combinations are missing (Carrington, Wang et al. 2005). This led the authors to suggest that NK cell activation may promote cancer development. All the disease associations of KIR and HLA could be summarized in **Table 1-5**.

Table 1-5: The disease associations of KIR and HLA

Disease	KIR-HLA ligand	Effect	Reference
Infectious diseases			
HIV	*KIR3DS1+HLA-Bw4 80I*	Slower progression	(Martin, Gao et al. 2002)
		Protection from opportunistic infection	(Altfeld, Kalife et al. 2006)
	*KIR3DL1*h/y + HLA-B Bw4-80I*	Slower progression	(Martin, Qi et al. 2007)
HCV	*KIR2DL3+HLA-C1*	Resolution of HCV infection	(Khakoo, Thio et al. 2004)
HBV	*KIR2DL3+HLA-C1*	Protection against chronic hepatitis B	(Gao, Jiao et al. 2010)
P.falciparum	Homozygosity for KIR genotype A	Protection from infection	(Yindom, Forbes et al. 2012)
Autoimmune and inflammatory conditions			

Rheumatoid arthritis	*KIR2DS2*	Susceptibility	(Yen, Moore et al. 2001)
Psoriatic arthritis	*KIR2DS1/2DS2*	Susceptibility	(Martin, Nelson et al. 2002)
Multiple sclerosis	Absence of *KIR2DL3*	Susceptibility	(Jelčić, Hsu et al. 2012)
Primary sclerosing cholangitis	*KIR3DL1/Bw4*; *KIR2DL1/HLA-C2*	Protection	(Karlsen, Boberg et al. 2007)
Idiopathic bronchiectasis	*HLA-C1/C1* and *2DS1/2DS2*	Susceptibility	(Boyton, Smith et al. 2006)
Reproduction			
Preeclampsia	Maternal KIR AA genotype with a foetal *HLA-C2*	Susceptibility	(Hiby, Walker et al. 2004)
Recurrent miscarriages	Increased *KIR2DS2* and decreased *HLA-C2* frequency, overall increased frequency of activating *KIR*.	Susceptibility	(Wang, Zhao et al. 2007)
Cancer			
Malignant melanoma	*KIR2DL2/L3*; *HLA-C1*	Susceptibility	(Naumova, Mihaylova et al. 2005)
Leukaemia	*KIR2DL2/L3*	Susceptibility	(Verheyden, Bernier et al. 2004)
Hodgkin's lymphoma	*KIR2DS1*;*KIR3DS1*	Protection	(Besson, Roetynck et al. 2007)

Taken together, it is now appreciated that KIR play a critical role in determining the function of NK cells, but this role must be examined in the context of KIR ligands.

1. 5. 6 KIRs in HIV-1 infection

KIR3DL1/S1 and HLA-B Bw4 Synergism in AIDS Outcomes

In 2002, a study of AIDS progression in an ART naïve cohort (Martin, Gao et al. 2002) demonstrated that the compound genotype *KIR3DS1+ HLA-B Bw4 80I* was protective in terms of HIV-1 progression; it was intriguing that without *KIR3DS1*, *HLA-B Bw4 80I* was not associated with any of the AIDS outcomes measured, moreover in the absence of *HLA-B Bw4 80I* alleles, *KIR3DS1* was significantly associated with more rapid progression to AIDS. This study clearly showed the epistatic interaction between *KIR* and *HLA* in HIV/AIDS progression.

This combination was further associated with lower viral load and protection from opportunistic infections (Altfeld, Kalife et al. 2006). But this effect should be interpreted with caution because the difference in viral replication (*Bw4 80I/KIR3DS1* carriers average HIV-1 RNA level of 4.75 $\log_{10}$ copies/ml *versus* viral load of 4.89 $\log_{10}$ copies/ml for those who did not carry both genes) is modest, and may not be sufficient to explain the benefit in long-term clinical outcome.

Later on a functional study (Alter, Martin et al. 2007) showed that NK cells expressing *KIR3DS1* could strongly be activated by autologous HIV-1–infected *Bw4-80I*$^+$ CD4$^+$ T cells and could significantly inhibit HIV-1 replication in these cells *in vitro* compared with NK cells expressing the inhibitory receptor KIR3DL1, or neither of these receptors, which provided a plausible mechanistic basis for the epidemiological studies above.

However the synergistic protective effect of the compound genotype *KIR3DS1+HLA-B Bw4 80I* was not seen in two other studies (Gaudieri, DeSantis et al. 2005; Barbour, Sriram et al. 2007) . Moreover no direct binding of *KIR3DS1* to *HLA-B Bw4-80I* has so far been

demonstrated (Carr, Rosen et al. 2007; O'Connor, Guinan et al. 2007). So this protective effect has not yet been unequivocally demonstrated.

The *KIR3DL1* allotypes can be divided into two groups: KIR3DL1*l/x (lower expression and lower inhibitory activity) including 3DL1*005, *007, *009 and KIR3DL1*h/y (high expression and high inhibitory capacity) including 3DL1*001, *002, *008, *015,*020 (Martin, Qi et al. 2007). Surprisingly, in the same cohort (Martin, Gao et al. 2002) in which *KIR3DS1+ HLA-B Bw4 80I* was protective, the combination of *KIR3DL1*h/y + HLA-B Bw4-80I* was also shown to be protective against HIV (Martin, Qi et al. 2007). These results imply that efficient engagement of both activating and inhibitory *KIR* is beneficial for the host to counter the viral infection. The protection conferred by activating receptors is relatively straightforward to explain; the protection by some KIR3DL1 allotypes which have high expression and high inhibitory ability is harder to explain, but might be attributed to more potent tuning of the NK cells during their maturation process, eventually leading to more powerful activation of those NK cells (Bashirova, Thomas et al. 2011).

Currently, there are two mechanisms by which the inhibitory KIRs could act: one is that NK cell killing of virus-infected cells could be altered by inhibitory KIR expression; the other one is that the virus-specific $CD8^+$ T cell response could be modified by corresponding KIR expression (on NK cells or T cells). KIRs on both NK cells and CD8+ T cells have been demonstrated to shape adaptive immunity (Gerosa, Baldani-Guerra et al. 2002; Piccioli, Sbrana et al. 2002; Raulet 2004). Inhibitory KIRs on CD8+ T cells have been shown to promote the survival of a subset of memory phenotype $CD8^+$ αβ T cells with enhanced cytolytic potential by reducing activation-induced cell death (Ugolini, Arpin et al. 2001; Young, Uhrberg et al. 2001; Young and Uhrberg 2002; Gati, Guerra et al. 2003).

HIV-specific $CD8^+$ T cells can also express KIR (Alter, Rihn et al. 2008). As discussed previously, the combination of KIR3DL1+HLA-Bw4 80I has been associated with slow progression to AIDS (Martin, Qi et al. 2007): in this study, a model based on NK cell development was used to explain the protective effect, but an alternative explanation was that KIR3DL1 might enhance the protective effect of HLA-B- restricted responses to HIV-1.

KIR2D in control of HIV

GWAS of HIV-1 infection (Fellay, Shianna et al. 2007; Pereyra, Jia et al. 2010) identified one SNP, which is a variant located 35kb upstream of *HLA-C* gene: -35C is the allele associated with protection and -35T is associated with susceptibility. The protective variant is associated with increased *HLA-C* expression on the surface of T cells (Thomas, Apps et al. 2009). Intriguingly, the protective effect of this SNP could not be assigned to a specific *HLA-C* allele or phylogenetically related subgroup (Thomas, Apps et al. 2009), which could be regarded as suggesting a potential NK-mediated rather than CD8-dependent mechanism.

Further study showed increasing HLA-C expression was associated with protection against multiple outcomes independently of individual *HLA* allelic effects in both African and European Americans, in spite of their different *HLA-C* frequencies and linkage relationships with *HLA-B* and *HLA-A* (Apps, Qi et al. 2013). Additional evidence that the levels of HLA-C expression may be relevant to disease outcome comes from a study in the SM cohort, in which the -35 SNP association with clinical status was confirmed in a Han Chinese population, and the high-expressing HLA-C (-35 C/C) alleles were shown to lead to greater selection pressure on viral evolution (Blais, Zhang et al. 2012).

The association of the -35 SNP with control of HIV-1 infection is extremely unlikely to be a direct result of the SNP itself. Corrah et al re-evaluated the relationship between this SNP and HLA-C expression level and proposed that it was the particularly low expression of the 35T-

associated HLA-Cw*07 allele that contributes substantially to the low HLA-C expression associated with the -35 T allele, and possibly to the relatively high risk of disease progression (Corrah, Goonetilleke et al. 2011).

Since *HLA-C* alleles serve as ligands for KIR2D receptors, there is a possibility that this protective effect is NK-cell-dependent through the interaction with its ligand. This possibility currently remains to be adequately addressed.

KIR2DL2 has been found to enhance both beneficial and detrimental HLA class I-mediated immunity to HCV and HTLV-1 (Seich Al Basatena, Macnamara et al. 2011). The underlying mechanism could be that, in the face of chronic antigen stimulation, the antigen-specific T cells, whether protective or detrimental T cells survive longer if they carry KIR2DL2 and therefore exert stronger protective or detrimental effects.

KIR footprints in HIV-1 evolution

HLA class I imposes a characteristic change (footprints) on HIV-1 sequences at a population level over time. These kinds of amino acid substitutions in the viral proteome have been regarded as markers of $CD8^+$ T cell pressure (McMichael and Klenerman 2002). Likewise, KIR-associated amino-acid polymorphisms in the HIV-1 sequence of chronically infected individuals have also recently been shown on a population level (Alter, Heckerman et al. 2011). This suggests that, like T cells, NK cells may also recognize specific regions of the HIV proteome and place selection pressure on the virus.

Crystal structures of KIR/HLA complexes show that KIR interacts with the α1 and α2 helix of HLA class I and makes direct contact with the carboxy-terminal portion of the bound peptide (Boyington, Motyka et al. 2000; Fan, Long et al. 2001). Particular amino acid changes in the bound peptide, especially at position 7 and 8, could abrogate the inhibition of

NK cells mediated through KIR and lead to target cell lysis (Correa and Raulet 1995; Malnati, Peruzzi et al. 1995; Fadda, Borhis et al. 2010).

Compared to the binding of self-peptides that confers a strong inhibitory signal to NK cells through KIRs, the HIV peptides may bind differently resulting in a weaker binding to an inhibitory KIR, such that NK cells are activated, or mediate a stronger binding to an activating KIR which also activates NK cell cytotoxicity. This direct KIR-mediated immune pressure may drive HIV-1 to evolve and generate "escape mutations" to evade this form of innate recognition. However the extent of KIR-associated NK cell pressure on HIV evolution has yet to be defined.

1. 5. 7 *KIR3DL1* and *KIR3DS1*

Among all the KIR genes, *KIR3DL1* and *KIR3DS1* attracted more attention than other KIRs in HIV-1 infection, and this has been demonstrated in many studies (Martin, Gao et al. 2002; Qi, Martin et al. 2006; Alter, Martin et al. 2007; Martin, Qi et al. 2007; Boulet, Sharafi et al. 2008; Boulet, Song et al. 2010). There are 92 alleles for *KIR3DL1* and 16 alleles for *KIR3DS1* in the latest KIR allele database (Robinson, Halliwell et al. 2013). In order to determine the independent role that each of these genes plays in HIV-1 disease progression in our cohort, we begin to develop a sequence–based method to generate nucleotide sequences for the entire *KIR3DL1/S1* locus.

KIR3DL1 and *KIR3DS1* share the same locus, which expands to 14kb within the LRC (lymphocytes related complex) region, and they share 97% similarities of extracellular domains to one another. *KIR3DL1/S1* has 9 exons and 8 introns. The main difference between *KIR3DL1* and *KIR3DS1* is that there is a 2bp deletion in exon 8 of *KIR3DS1* that leads to a

premature stop codon in exon 9, making exon 9 of *KIR3DS1* much shorter than that of *KIR3DL1* (**Figure 1-14**).

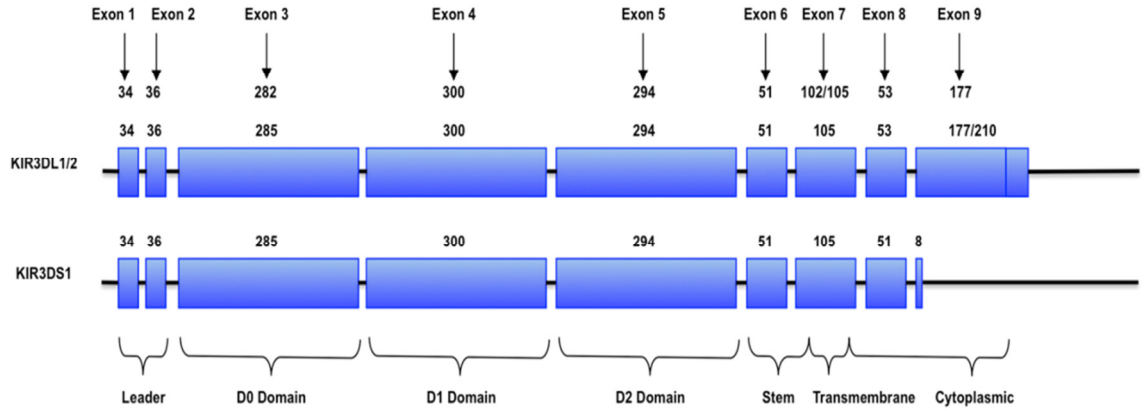


Figure 1-14: *KIR3DL1/2* and *KIR3DS1* gene organisation. Adapted from http://www.ebi.ac.uk/ipd/kir/introduction.html

1. 6 Main objectives

1. To study the frequencies of *KIR* gene distribution in the SM cohort and compare them to those *KIR* gene frequencies in a healthy control group to show the potential impact of *KIR* in terms of HIV disease progression.

2. To study the combination role of *KIR/HLA* between the two groups.

3. Are there any KIR footprints on HIV-1 evolution in this cohort? In other words, is there any HIV-1 virus sequence mutation associated with the presence or absence of particular KIRs?

4. We utilise *KIR3DL1* and/or *KIR3DS1* positive samples to test and optimise a novel sequencing method, which can sequence the locus of *KIR3DL1/S1* exclusively. In this manner, we could further show the polymorphism of these two genes at a more detailed level.

5. Another valuable acute HIV-1 infection cohort, which was established prospectively in a MSM population, was used to study the potential role of KIRs in acute HIV-1 infection.

Chapter 2 Materials and methods

Materials

Plasticware and other lab supplies

Product	Cat No.	Supplier
15ml Falcon tubes	352096	BD Biosciences, UK
50ml Falcon tubes	352070	BD Biosciences, UK
Leucosep tubes	227289	Greiner Bio-One, UK
PCR plates	PCR-96-FS-C	Bioline Labs Ltd
384-WELL PLATE	THER-384	Elkay Laboratory Products (UK) Ltd
Agencourt SPRIPlate 96R - Ring Magnet Plate	A29164	Beckman Coulter UK Ltd
MicroAmp® Optical 96-Well Reaction Plate	N8010560	Applied Biosystems
96-Well G-50 Gel Filtration Plate	CGP04/10	Geneaid

Kits

Product	Cat No.	Supplier
BigDye(R) Terminator v3.1 Cycle Sequencing Kit	4337455	Applied Biosystems
MultiTest IMK kit	340503	BD Bioscience
NucliSens HIV-1 QT assay	30443	BioMerieux

Chemical and biological reagents

Product	Cat No.	Supplier
Agarose	BIO-41025	Bioline Labs Ltd
Sephadex G-50 superfine	G5050-100G	Sigma Aldrich Company Ltd
Cell lysis solution	2300720	5 prime
DNA Hydration solution	158914	Puregen, Gentra systems
Isopropanol	59080	Sigma Aldrich Company Ltd
Phosphate-buffered saline	14190-094	Invitrogen
Protein precipitation solution	2300720	5 prime
RBC lysis solution	2301300	5 prime

PCR reagents

Product	Cat No.	Supplier
dNTP mix	BIO-39028	Bioline Labs Ltd
PCR water	BIO-37080	Bioline Labs Ltd
Biotaq polymerase	BIO-21060	Bioline Labs Ltd
Platinum® Taq DNA Polymerase	10966034	Gibco- Use Invitrogen
Quick-Load® 1 kb DNA Ladder	N0468L	New England Biolabs (UK) Ltd
Gel Loading Dye, Blue (6X)	B7021S	New England Biolabs (UK) Ltd

Sequencing reagents

Product	Cat No.	Supplier
BigDye® Terminator v1.1 & v3.1 5X Sequencing Buffer	4336697	Applied Biosystems
Hi-Di Formamide	4311320	Applied Biosystems
Expand Long Range dNTPack	4829034001	Roche Diagnostics Ltd
ExoSAP-IT For PCR Product Clean-Up	78201 1 ML	Affymetrix UK Ltd
Expand Long Range dNTPack	4829034001	Roche Diagnostics Ltd

Methods

2. 1 Introduction to the SM cohort

SM denotes Shuang Miao village, which is an isolated rural community in Henan province in central China, where most residents have been living for decades. Between 1993 and 1995, many villagers in this community participated in a scheme for paid plasma donation which was common in rural China at that time. Most of the donors were not aware of their HIV-1 infection until 2004 when a large-scale HIV screening programme was carried out in China. HIV-1 transmission among paid plasma donors in China is thought to be due to either contamination of blood collection equipment or pooled red cells being returned to donors (Kaufman and Jing 2002). A previous study has shown that the paid plasma donation associated HIV-1 subtype B′ epidemic in China is monophyletic (Zhang, Chen et al. 2004). The SM cohort was set up in 2004 when 261 HIV-infected adults were recruited into this study: none of them had been treated with ART before 2004. Meanwhile 252 healthy donors from a geographically nearby village, who were thought to have a similar genetic background to the HIV-1 infected individuals in SM village but did not participate in the plasma donation scheme, were recruited into this study as controls. Informed consent was obtained from each participant prior to sample collection. Ethical approval was obtained from Beijing You'an Hospital and the University of Oxford Tropical Ethics Committee.

The SM cohort provides us with an unique opportunity to study the host genetic factors that could affect the clinical outcome, because in this cohort the major factors such as viral strain, transmission route and timing of infection that could affect the natural history of HIV-1 have been narrowly controlled (Dong, Zhang et al. 2011).

2. 2 Extraction of genomic DNA

Genomic DNA was isolated from 3ml whole blood using the Puregene DNA Isolation kit. 9ml RBC Lysis Solution was dispensed into a 15ml Falcon tube and 3ml whole blood was added. They were mixed by inverting the tube 10 times, and then incubated at room temperature for 10 minutes. Then, cells were centrifuged at 2000 rpm for 10 minutes, therefore, the colour of the solution turned red. The supernatant was discarded and the remaining white blood cells were washed twice with PBS then resuspended in 600ul Cell Lysis Solution (Sometimes when needed the whole workflow can stop here and samples are stable in Cell Lysis Solution for at least 2 years at room temperature). Samples within Cell Lysis Solution were mixed with 200ul of Protein Precipitation Solution, vortexed vigorously and centrifuged at 13,000g for 15 minutes. The supernatant was decanted into a clean 2ml vial containing 600ul isopropanol. After the tube was gently inverted 50 times, genomic DNA became visible as threads or a clump floating in the solution and then the tube was centrifuged at 13,000g for 3 minutes to pellet the genomic DNA. The supernatant was discarded and DNA was washed with 70% ethanol. After discarding the ethanol, centrifuging the tube, and removing all the solution in the tube, the genomic DNA was dissolved in 50ul hydration solution. Samples were stored at -20℃ for the genotyping and viral sequence analysis.

2. 3 KIR genotyping

KIR genes are similar to each other and KIR genotyping was performed by using PCR amplification with two pairs of primers specific for each locus. PCR-SSP (sequence specific primer) is also variably known as allele-specific PCR or amplification refractory mutation system (ARMS).Two pairs of primers annealing at 2 different exonic positions of the same gene in 2 independent reactions were used to detect the presence of the gene of interest. A panel of 60 KIR specific primers were then used in 30 PCR reactions to detect 15 *KIR* genes.

A pair of control primers was included in each reaction to amplify a 796 bp product from the third intron of the human *HLA-DRB1* (*DRB1* is present in all individuals) gene as a positive control to check for PCR efficiency. The list of the primers could be seen in **Figure 2-1**.

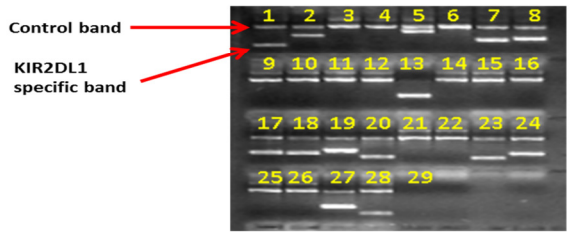
Number	KIR Gene	Primer F	Primer R	Lane	size
1	**2DL1**	5'- GTT GGT CAG ATG TCA TGT TTG AA	5'- GGT CCC TGC CAG GTC TTG CG	1	146
		5'- TGG ACC AAG AGT CTG CAG GA	5'- TGT TGT CTC CCT AGA AGA CG	2	330
2	**2DL2**	5'- CTG GCC CAC CCA GGT CG	5'- GGA CCG ATG GAG AAG TTG GCT	3	173
		5'- GAG GGG GAG GCC CAT GAA T	5'- TCG AGT TTG ACC ACT CGT AT	4	151
3	**2DL3**	5'- CTT CAT CGC TGG TGC TG	5'- AGG CTC TTG GTC CAT TAC AA	5	550
		5'- TCC TTC ATC GCT GGT GCT G	5'- GGC AGG AGA CAA CTT TGG ATC A	6	800
4	**2DL4**	5'- CAG GAC AAG CCC TTC TGC	5'- CTG GGT GCC GAC CAC T	7	254
		5'- ACC TTC GCT TAC AGC CCG	5'- CCT CAC CTG TGA CAG AAA CAG	8	288
5	**2DS2**	5'- TTC TGC ACA GAG AGG GGA AGT A	5'- GGG TCA CTG GGA GCT GAC AA	9	175
		5'- CGG GCC CCA CGG TTT	5'- GGT CAC TCG AGT TTG ACC ACT CA	10	240
6	**2DS3**	5'- TGG CCC ACC CAG GTC G	5'- TGA AAA CTG ATA GGG GGA GTG AGG	11	242
		5'- CTA TGA CAT GTA CCA TCT ATC CAC	5'- AAG CAG TGG GTC ACT TGA C	12	190
7	**2DS4**	5'- CTG GCC CTC CCA GGT CA	5'- TCT GTA GGT TCC TGC AAG GAC AG	13	204
		5'- GTT CAG GCA GGA GAG AAT	5'- GTT TGA CCA CTC GTA GGG AGC	14	197/219
8	**2DS5**	5'- TGA TGG GGT CTC CAA GGG	5'- TCC AGA GGG TCA CTG GGC	15	126
		5'- ACA GAG AGG GGA CGT TTA ACC	5'- ATG TCC AGA GGG TCA CTG GG	16	178
9	**3DL1**	5'- CGC TGT GGT GCC TCG A	5'- GGT GTG AAC CCC GAC ATG	17	191
		5'- CCC TGG TGA AAT CAG GAG AGA G	5'- TGT AGG TCC CTG CAA GGG CAA	18	186
10	**3DL2**	5'- CAA ACC CTT CCT GTC TGC CC	5'- GTG CCG ACC ACC CAG TGA	19	211
		5'- CCC ATG AAC GTA GGC TCC G	5'- CAC ACG CAG GGC AGG G	20	130
11	**3DS1**	5'- AGC CTG CAG GGA ACA GAA G	5'- GCC TGA CTG TGG TGC TCG	21	300
		5'- CCT GGT GAA ATC AGG AGA GAG	5'- GTC CCT GCA AGG GCA C	22	180
12	**3DL3**	5'- GTC AGG ACA AGC CCT TCC TC	5'- GAG TGT GGG TGT GAA CTG CA	23	232
		5'- TTC TGC ACA GAG AGG GGA TCA	5'- GAG CCG ACA ACT CAT AGG GTA	24	165
13	**2DL5**	5'- GCG CTG TGG TGC CTC G	5'- GAC CAC TCA ATG GGG GAG C	25	214
		5'- TGC AGC TCC AGG AGC TCA	5'- GGG TCT GAC CAC TCA TAG GGT	26	191
14	**2DP1**	5'- GTC TGC CTG GCC CAG CT	5'- GTG TGA ACC CCG ACA TCT GTA C	27	205
		5'- CCA TCG GTC CCA TGA TGG	5'- CAC TGG GAG CTG ACA ACT GAT G	28	89
15	**2DS1**	5'- CAC TGG GAG CTG ACA ACT GAT G	5'- CTT CTC CAT CAG TCG CAT GAG	29	102
	control	5'- TGC CAA GTG GAG CAC CCA A	5'- GCA TCT TGC TCT GTG CAG AT	30	796

Figure 2-1: The list of KIR genotyping primers.

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 a 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.

For each sample, the master mix containing αQH2O 270ul/10X buffer 42.3ul/MgCl2 (50mM) 12.7ul dNTPs (25mM) 3.4ul/Taq Platinum 1.8ul was prepared. 150ng DNA was added into the master mix and 10ul of the mixture was added into the corresponding well in the plate where 2ul of each primer had been added. The amplification conditions are seen as below: The initial DNA denaturation was performed with one cycle of 94°C for 3 min. The PCR amplification included 5 cycles of 94°C for 15s, 65°C for 15s and 72°C for 30s, then 21 cycles of 94°C for 15s, 60°C for 15s and 72°C for 30s, followed by 5 cycles of 94°C for 15s, 55°C for 1min and 72°C for 2min, the whole amplification was finished by 1 cycle of 72°C for 7min. Here, the "stepdown" annealing temperature strategy is used to increase the specificity of the PCR. The schematic figure of KIR genotyping reading and scoring can be seen in **Figure 2-2** below.



Gene	Score1	Score2	Scoref	Lane	Size
2DL1				1	146
				2	330
2DL2				3	173
				4	151
2DL3				5	550
				6	800
2DL4				7	254
				8	288
2DS2				9	175
				10	240
2DS3				11	242
				12	190
2DS4				13	204
				14	197/219
2DS5				15	126
				16	178
3DL1				17	191
				18	186
3DL2				19	211
				20	130
3DS1				21	300
				22	180
3DL3				23	232
				24	165
2DL5				25	214
				26	191
2DP1				27	205
				28	89
2DS1				29	102

Figure 2-2: The schematic of KIR genotyping and scoring. The left figure indicates: for each KIR gene, there are two bands to show its presence or absence. In each lane there is also a control band to

show the PCR efficacy. The right figure is a scoring table to record the KIR genotyping results. Presence of each band in the correct size is recorded as 1, and the absence of the band is recorded as 0. For each sample, the gel picture is read by two persons respectively to reduce the reading error to the lowest level.

2. 4 Sequencing the locus of *KIR3DL1/S1*

See Chapter 4

2. 5 HLA typing

The genetic complexity of the HLA has required the use of various molecular typing methods to identify specific alleles. Sequence specific primers (SSP), and sequence-based typing (SBT) are the two methods that have been utilized over the last several years in the HLA community. The PCR-SSP method consists of one 96 well tray, with 95 primer mixes and one negative control, per sample designed to produce an intermediate/high resolution HLA typing. The SBT method is the most comprehensive method for a complete HLA typing which can provide the highest resolution possible. The purified amplicon from the genomic DNA encompassing exons1-5 for class I and exon 2 for class II is used as a template in forward and reverse sequencing reactions designed for either exons 2 and 3 of class I alleles or just exon 2 of class II alleles. After the sequencing reactions and subsequent purification, the products are loaded onto the sequencer to read and later on analysed by specific software.

2. 6 PCR and sequencing primer design

There are some principles for designing primers:

1. Primer length: the length should be between 18 and 30 nucleotides, with the optimal being 20-25 nucleotides. This length should be long enough for adequate specificity, and short enough for primers to bind easily to the template at the annealing temperature.

2. Annealing temperature or melting temperature(Tm): Tm denotes the temperature at which one half of the DNA duplex will dissociate to become single stranded. They should have a Tm between 60 and 70 degrees. The most widely used Wallace equation: Tm = 2(A+T) +

4(G+C), where A, C, G, and T are the numbers of each base in the primer. Sometimes the primer works poorly, especially for those primers shorter than 20 or longer than 25 nucleotides. A variety of online software can calculate Tm values more precisely.

Other guidelines for designing primers include: each primer should have a G/C content between 40 and 60 percent; repetitive G's or C's should especially be avoided; the 3'-end sequence of the primer should correspond to highly conserved regions of the target sequence; primers that can form 4 or more consecutive bonds with itself, or 8 or more bonds in total should be avoided. The primer sequence must not contain undesirable self-hybridization sequences.

The principles of designing sequencing primers are similar to these principles for PCR primers. One of the differences is that sequencing uses one primer, while PCR utilises two. And the sequencing primers should be purified before use. Additionally, good sequencing results require high quality primers, just as much as high quality templates.

Here in this study, we designed our sequencing primers with a special software CLC Main workbench 6. When we have no candidate primers that can meet all the criteria, we just relax the stringency of the selection requirements. On the other hand, when we have many options, we just design more primers aiming to obtain sufficient overlapping data to ensure a good sequence consensus.

What is noteworthy is that no computer programme or rule-of-thumb assessment can accurately predict either the success or failure of a primer. A primer that seems marginal may perform well, while another that appears to be perfect may not work at all. Overall, the test of a good primer is only in its use, and cannot be accurately predicted by these rules above.

2. 7 Quantification of CD4 counts and viral load

$CD3^+/CD4^+/CD8^+$ T lymphocyte percentages and true counts were determined by Flow Cytometry as following: 200ul of heparin-anticoagulated fresh whole blood was analysed using BD MultiTest IMK kit. Briefly, the sample was mixed with multitest antibodies and incubated for 15 minutes in the dark at room temperature before being transferred to BD True count tubes. 450ul of 1× BD Multitest lysing solution was added to the tube and incubated for another 15 minutes. Finally, $CD3^+$ /$CD4^+$ /$CD8^+$ T lymphocyte percentages and true counts were calculated using MultiSet^{TM} software.

HIV-1 plasma viral load was quantified by Nucleic Acid Sequence Based Amplification (NASBA) in Beijing You'an Hospital. An initial enzymatic amplification of the nucleic acid targets was followed by amplicon detection. The assay lower limit of detection was 50copies/ml. Samples kept in solution by any anticoagulant are acceptable for the NASBA assay.

2. 8 Statistical analyses

Allele and genotype frequencies were calculated by using SPSS 16 (SPSS, Inc. Chicago). Haplotype reconstruction, testing for Hardy-Weinberg equilibrium, and haplotype frequency estimations were performed using Arlequin version 3.11. A Chi-square test was used to examine differences in allele and genotype frequencies between different populations. All other statistical analyses were performed using GraphPad Prism 6.

Chapter 3 *KIR* genes distribution and KIR-HLA interactions, KIR potential footprints in the SM cohort.

3. 1 Introduction

Natural killer cells are crucial effector cells of the innate immune system. The activation of NK cells is determined by the balance between activating and inhibitory receptors through which the net signals are integrated to determine whether the function of NK cells is initiated or not (Parham 2005). Many of these receptors are relatively conserved and nonpolymorphic, for example, the NKG2D and NKG2A families (Lanier 1998; Moser, Byers et al. 2002); in contrast, as the main NK receptors, KIRs are quite diverse and polymorphic, with further complexity through their polymorphic HLA class I ligands. In this context, it was thought that the polymorphism of KIRs and KIR-HLA interactions are more likely to be responsible for the different reaction patterns in the immune response to specific pathogens, which in turn leads to different disease outcomes following infection (Jamil and Khakoo 2011).

Given the critical role of these receptors in the regulation of NK cell function, their associations with protection/susceptibility in human disease are not surprising. There has been accumulating evidence showing that KIRs and KIR-HLA interactions can play important roles in determining the outcome of HIV infection. Genetic and functional studies indicated that *KIR* genes, different haplotypes, allelic polymorphisms and different expression levels of KIRs, may all play a part in the association with HIV-1 infection outcomes, including susceptibility to HIV infection and the disease progression. Details of KIR's role in HIV-1 infection were reviewed in **Chapter 1** (1.5.6). Although there have been a few studies of the *KIR* gene frequencies in Chinese people, currently there are no reports showing the association between KIR and HIV in the Chinese population.

The SM cohort was established in 2004, when most of the HIV-infected former paid plasma donors had survived with HIV for more than 10 years in the absence of ART. These people joined a scheme for plasma donation between 1993 and 1995, within a relatively narrow time period. When large-scale screening for HIV infection was first instituted in 2004, they became aware of their HIV infection status; meanwhile, 149 premature deaths had been recorded in that village with symptoms compatible with HIV/AIDS before 2004. To some extent, having survived with HIV for more than ten years in the absence of ART, all of the individuals in the SM cohort could be thought of as "slow progressors" (Gillespie, Kaul et al. 2002; Zhang, Jiang et al. 2010; Kamya, Boulet et al. 2011). A previous study of the p17 region of *gag* and C2-V3 region of *env*, which included 89 individuals from a different group of paid plasma donors in Henan province (the same province where the SM village is located), suggested that the paid plasma donation associated HIV-1 subtype B′epidemic in China is monophyletic (Zhang, Chen et al. 2004). Based on the epidemiological history and further phylogenetic analysis of the HIV-1 *Gag, Pol*, and *Nef* proviral sequences, this outbreak of HIV infection in the paid plasma donors in the SM village was considered as a narrow-source infection (Dong, Zhang et al. 2011). Thus, the SM cohort provides us with a unique opportunity to study the host genetic factors that could affect the clinical outcome, because in this cohort the major factors such as viral strain, transmission route and timing of infection, which could affect the natural history of HIV-1, have been narrowly controlled. However, in this cohort, the "rapid progressors" are missing due to frailty bias, which is a common problem for such kinds of retrospective cohorts. In order to compensate for this loss, 252 healthy donors were recruited from a geographically adjacent village that were thought to have a similar genetic background to the HIV-1 infected individuals in the SM village. We know that it may not be a perfect situation to study HIV progression in this manner, but the

healthy controls can at least provide us with the genetic information as a background reference to see which *KIRs* have been lost or enriched in those surviving "slow progressors".

So our first hypothesis is that there are specific KIRs or KIR-HLA combinations which may be beneficial or protective against HIV-1 infection, thus when compared with the background level in the population, they may have become enriched in the "slow progressors"; on the other hand, if the effect is detrimental, a decreased frequency of specific KIRs or KIR-HLA combinations may be observed in the "slow progressors". The second hypothesis is that NK cells may exert immune pressure on the HIV-1 viral sequence through specific KIRs, such that we would be able to identify KIR associated "footprints" on HIV-1 evolution in this cohort.

There is strong evidence that CD8 T cells can exert considerable selection pressure on HIV sequences over the course of infection (Borrow, Lewicki et al. 1997; Cao, McNevin et al. 2003; Leslie, Pfafferott et al. 2004; Goonetilleke, Liu et al. 2009). Viral escape mutations arising within the epitopes can disrupt the binding of the viral peptide to HLA class I or impair recognition by the T cell receptor. Mutations in regions immediately flanking the epitope may affect antigen processing, facilitating escape from CD8 T cell response. About two-thirds of all non-envelope viral mutations detected in the chronic phases of HIV-1 infection are attributed to selection by CD8 T cell responses in HIV-1 infected patients (Allen, Altfeld et al. 2005); these kinds of "footprints" selected by CTL indicate the dominant influence of adaptive immunity on viral evolution. Can NK cells exert a similar kind of pressure and lead to "footprints" on HIV-1 evolution? If so, is this selection mediated through the KIRs?

Here, we take advantage of this unique cohort to answer these questions.

3. 2 Study subjects and methods

This study involved 261 patients from the SM cohort and 252 healthy controls from the adjacent village. DNA was extracted from each individual and KIR/HLA genotyping were performed as previously described (See Methods in **Chapter 2**). HIV-1 amino acid sequences (previously generated by Dr Yonghong Zhang) were used for KIR footprint analysis. The observed frequency for each KIR gene was determined by direct counting and verified using SPSS 16 (SPSS, Inc. Chicago). HLA class I allele and genotype frequencies were computed with the same statistical packages. The centromeric and telomeric motif frequencies and those of the HLA and KIR genes, as well as KIR–HLA compound genotypes were compared between groups using chi-squared or Fisher's exact tests as appropriate. P-values of <0.05 were considered significant.

3. 3 Results

3. 3. 1 *KIR* gene frequencies

All 15 *KIR* genes were present in the cohort. *KIR3DL3*, *KIR2DL4* and *KIR3DL2* are framework genes which were present in nearly all samples, except for two samples, SMP334 and SMP461, which consistently remained negative for *KIR2DL4* upon two repeats. A possible explanation is that the two samples may possess a novel *KIR2DL4* with a mismatch at the primer binding site, so they could not be amplified by the primers in the current protocol. The observed frequencies of *KIR2DL1*, *KIR2DL3*, *KIR2DS4*, *KIR2DP1* and *KIR3DL1* were greater than 90% in both the SM patient group and the healthy control group. In the SM patient group, their frequencies were 98.5%, 95.8%, 98.1%, 98.5% and 97.3%, respectively. The least frequent *KIR* gene was *KIR2DS3*, the frequency of which was 13.8% and 17.8% in the SM patient group and the healthy control group respectively. The frequencies of most activating genes were lower than 40%, except for *KIR2DS4*. Among the

inhibitory genes, the frequencies of the *KIR3DL* genes were generally higher than those of the *KIR2DL* genes. See **Table 3-1**.

Table 3-1: The comparison of *KIR* gene frequencies between the SM patient group and the healthy control group

***KIR* gene**	**SM patients %**	**Healthy control %**	**P**
	n=261	**n=252**	
KIR2DL1	98.5	100	0.124 F
KIR2DL2	22.6	21.8	0.915
KIR2DL3	95.8	99.2	**0.021 F**
KIR2DL4	99.2	100	0.499 F
KIR2DL5	39.5	42.9	0.435
KIR2DS1	34.9	37.3	0.566
KIR2DS2	21.5	20.6	0.82
KIR2DS3	13.8	17.9	0.207
KIR2DS4	98.1	97.2	0.571 F
KIR2DS5	28.7	29.4	0.875
KIR3DL1	97.3	94.1	0.068
KIR3DL2	100	100	-
KIR3DL3	100	100	-
KIR3DS1	31	38.1	0.096
KIR2DP1	98.5	100	0.124 F

Pearson Chi-Square was used to perform the comparison. F: Fisher's Exact Test. P<0.05 was significant.

The frequency of each *KIR* gene was similar between the two groups except for *KIR2DL3*, which had a significant lower frequency of 95.8% in the SM patient group than in the healthy control group, where it was 99.2%.

3. 3. 2 KIR haplotypes

An important component of KIR variation is that KIR haplotypes vary in gene content. Haplotype A comprises 9 *KIR* genes: *3DL3-2DL3-2DP1-2DL1-3DP1-2DL4-3DL1-2DS4-3DL2*, which is the shortest haplotype and contains only one activating gene, *KIR2DS4*. The remaining combinations of KIR genes can be assigned to Haplotype B. Haplotype A accounted for 50% of the SM patients, which was similar to that (51%) in the healthy controls. See **Figure 3-1**.

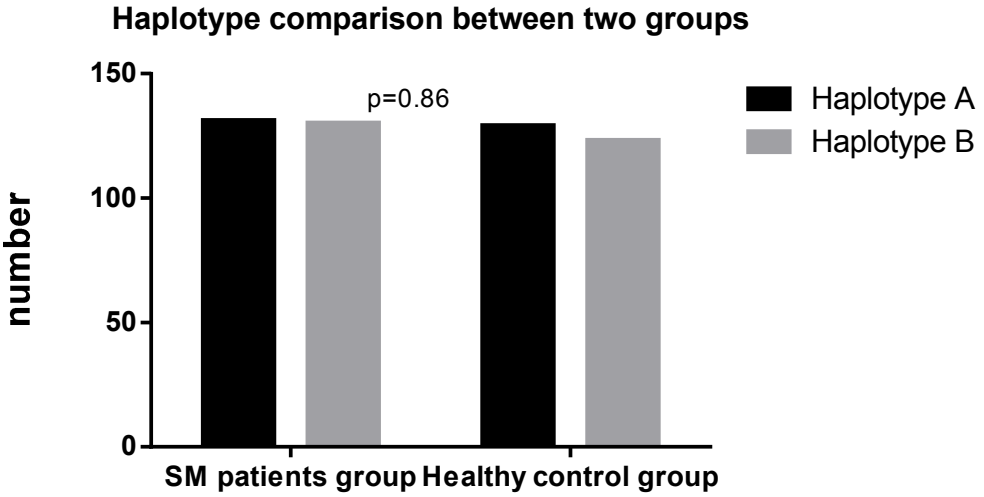


Figure 3-1: Haplotype A accounted for 50% of the SM patient group and 51% of the healthy control group respectively.

3. 3. 3 *KIR* gene frequencies compared to other population

Since the overall *KIR* gene frequencies were similar between the SM patient group and the healthy group, from the perspective of a demographic study, the two groups of individuals can be merged into one population and considered as Henan Chinese Han. Subsequently, the *KIR* gene frequencies from this merged population were compared to other populations. The *KIR* frequencies of the Henan Chinese Han population in this study were similar to those in other Chinese Han populations, meanwhile close to those of Japanese, but different from Caucasians. The frequency of *2DL3* in the Chinese Han populations was higher than that in Caucasians, while the frequencies of *2DL2*, *2DS2*, and *2DS3* genes were lower. See **Figure 3-2**.

observed frequency	3DL3	2DS2	2DL2	2DL3	2DP1	2DL1	2DL4	3DL1	3DS1	2DL5	2DS3	2DS5	2DS1	2DS4	3DL2
Henan Chinese Han(n=513)	1	0.21	0.22	0.97	0.99	0.99	1	0.96	0.35	0.41	0.16	0.29	0.36	0.98	1
a Zhejiang Chinese Han(n=104)	1	0.17	0.17	0.99	0.99	0.99	1	0.94	0.33	0.37	0.13	0.23	0.34	0.94	1
b Jiangshu Chinese Han(n=150)	1	0.25	0.21	1	1	1	1	0.93	0.4	0.45	0.21	0.31	0.47	0.93	1
c Japanese(n=239)	1	0.15	0.13	1	NT	0.99	1	0.95	0.46	0.49	0.17	0.32	0.46	0.87	0.99
d Caucasian(n=195)	1	0.5	0.49	0.88	0.98	0.97	1	0.95	0.4	0.53	0.28	0.36	0.37	0.95	1

Figure 3-2: The *KIR* gene frequencies in different populations. NT: not tested. a (Jiang, Zhu et al. 2005),b (Bao, Hou et al. 2010),c (Miyashita, Tsuchiya et al. 2006),d (Du, Gjertson et al. 2007).

3. 3. 4 *KIR* genotypes

In this study, 54 genotypes were identified, the most common genotype (observed 261 times) was *KIR3DL3-2DL3-2DP1-2DL1-2DL4-3DL1-2DS4-3DL2*, which belongs to Genotype ID1 group, http://www.allelefrequencies.net (Gonzalez-Galarza, Christmas et al. 2011), accounting for 51% of the observed population. See **Figure 3-3**.

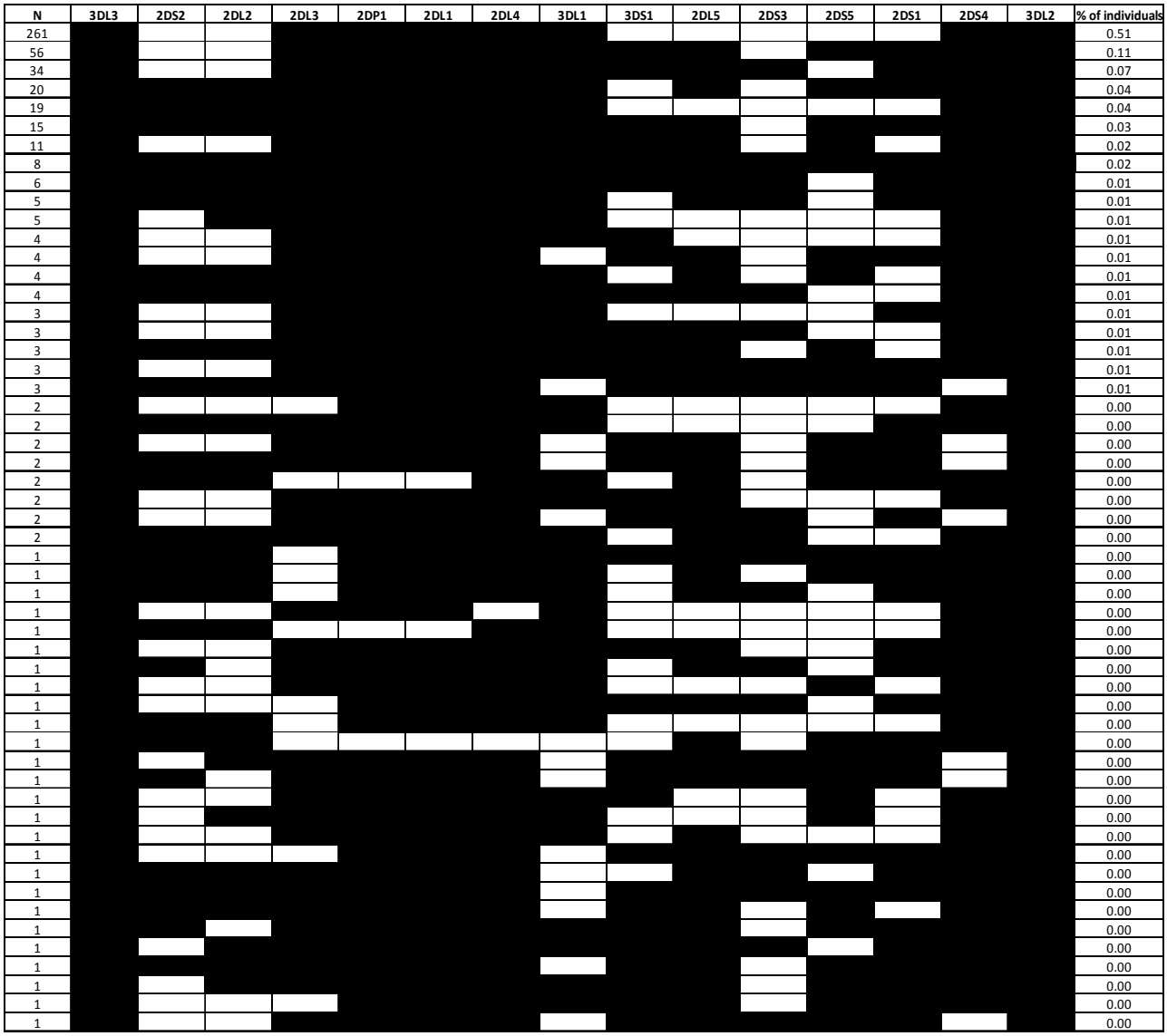


Figure 3-3: *KIR* genotypes observed in a population of Henan Chinese Han individuals (n=513). Filled boxes indicate the presence of a particular *KIR* gene; open boxes indicate the absence of this *KIR* gene. The number and percentage of individuals exhibiting each genotype are indicated.

3. 3. 5 HLA class I gene frequencies

We found that *HLA-A*02* was the predominant *HLA-A* allele in both groups, meanwhile *HLA-B*40* and *HLA-B*13* were very common *HLA-B* alleles in the studied population. Among *HLA-C* genes, *HLA-Cw*03* was the most frequent one (**Table 3-2**). Comparison of HLA class I frequencies showed only marginal differences: *HLA-A*26* was significantly

lower in the SM patient group than in the healthy controls (2.6% vs. 6.4%) and *HLA-Cw*02* was higher in the SM patient group (5.8%) compared to the control group (2.0%).

Table 3-2: HLA class I frequencies compared between the SM patients and the healthy controls

HLA class I	SM patients(n=233) %	Healthy controls(n=252) %	P
A*01	10.3	6.8	0.164
A*02	53.2	47.8	0.235
A*03	8.2	6.8	0.563
A*11	24.5	26.3	0.644
A*24	24.5	29.9	0.182
A*26	2.6	6.4	**0.045**
A*31	5.6	6.4	0.713
A*32	6.4	4.8	0.428
A*33	21	24.7	0.338
B*07	7.7	9.2	0.571
B*08	2.6	2	0.668
B*13	21	26.3	0.174
B*15	17.6	20.3	0.446
B*27	7.3	4	0.113
B*35	6.9	5.6	0.557
B*37	3.9	2.8	0.51
B*38	5.6	6.8	0.587
B*39	3.9	4	0.945
B*40	29.2	25.5	0.363
B*44	13.7	17.5	0.252
B*46	9.4	10	0.848
B*48	5.6	4.4	0.545
B*51	14.2	16.7	0.436
B*52	8.2	7.2	0.685
B*54	6.4	5.2	0.554
B*55	2.2	3.6	0.346
B*57	4.7	3.2	0.386
B*58	11.6	11.6	0.991
Cw*01	18.6	18.3	0.942
Cw*02	5.8	2	**0.032**
Cw*03	33.6	39	0.22
Cw*04	9.7	10.8	0.714
Cw*06	29.7	27.9	0.672
Cw*07	28.8	29.1	0.938
Cw*08	22.6	16.3	0.085
Cw*12	11.1	9.2	0.492
Cw*14	10.6	13.9	0.271
Cw*15	11.1	13.9	0.344

3. 3. 6 Serotypes and dimorphic groups

Serotypes and dimorphic groups of HLA class I were assigned to each sample: Bw4/Bw6 was characterised at http://www.dorak.info/hla/bw4bw6.html; C1/C2 was characterised at http://www.dorak.info/hla/c1c2.html. The frequencies of them were compared between the SM patient group and the healthy control group, but no differences were found between the two groups. See **Table 3-3**.

Table 3-3: Serotypes and dimorphic groups of HLA class I between the two groups

Serotypes	SM patients (%)	Healthy control (%)	p
Bw4	74.7	76.1	0.718
Bw6	74.7	76.1	0.718
Bw4/Bw4	22.8	23.9	0.764
Bw6/Bw6	22.8	23.9	0.764
Bw4/Bw6	51.9	52.2	0.954
C1	91.6	90.8	0.771
C2	51.3	51	0.942
C1/C1	48.7	49	0.942
C2/C2	8.4	9.2	0.771
C1/C2	42.9	41.8	0.811

3. 3. 7 Centromeric and telomeric motifs and genotypes

We adopted the recent techniques and terminologies used by Cooley et al (Cooley, Weisdorf et al. 2010) and Pyo et al (Pyo, Guethlein et al. 2010) to assign centromeric (cen) and telomeric (tel) motifs and genotypes to each of our samples. Comparisons of the group of genes present at the telomeric and centromeric parts of the KIR gene locus were performed between the two groups. Our data showed that the frequencies of most telomeric and centromeric motifs as well as genotypes were very similar between the SM patient group and healthy control group (**Table 3-4**). However, a significant difference was observed among

people carrying the telomeric genotype *B1/Bx* (other *Tel-B* genotypes except *Tel-B1*). It was significantly less frequent in the SM patient group (0.4%) compared to that in the healthy control group (2.8%).

Table 3-4: Centromeric and telomeric motifs and genotypes distribution between the SM patient group and the healthy control group

	SM patients (%)	Healthy controls (%)	P
Cen motifs			
cen-A	72.8	75.8	0.438
cen-B1	6.5	3.6	0.129
cen-B2	14.2	16.7	0.435
cen-B3	23	23.4	0.909
cen-Bx	6.1	4.4	0.371
Tel motifs			
tel-A	96.9	94.1	0.115
tel-B1	25.3	32.9	0.57
tel-Bx	16.1	12.7	0.275
Cen genotype			
c-A/A	52.5	52.4	0.98
c-A/B1	4.6	3.6	0.558
c-A/B2	13	16.7	0.246
c-A/Bx	2.7	3.2	0.741
c-B1/B1	0.4	0	1
c-B1/Bx	1.5	0	0.124
c-B2/B2	1.2	0	0.249
c-B3/B3	22.2	23	0.83
c-B3/Bx	0.8	0.4	1
c-Bx/Bx	1.2	0.8	1
Tel genotype			
t-A/A	59	57.1	0.67
t-A/B1	23	27.4	0.252
t-A/Bx	14.9	9.5	0.062
t-B1/B1	1.9	2.8	0.519
t-B1/Bx	0.4	2.8	**0.035**
t-Bx/Bx	0.8	0.4	1

Cen: centromeric; Tel: telomeric; *Cen-Bx*: the rest *Cen-B* genotypes except *Cen-B1*, *Cen-B2* and *Cen-B3*. *Tel-Bx*: the rest *Tel-B* genotypes except *Tel-B1*.

3. 3. 8 KIR-HLA interactions

In terms of the documented ligand-receptor relationships between KIR and HLA, we compared different combinations of KIR-HLA between the two groups. We found that the compound genotype *KIR3DS1 + HLA-Bw4* was relatively less frequent in the SM patient group (6.0%) than in the healthy control group (12%). See **Table 3-5**.

Table 3-5: KIR-HLA interactions between the SM patients group and the healthy control group

KIR-HLA compound	SM patients group (%)	Healthy control group (%)	P
3DS1+Bw4/x	25.3	29.9	0.263
3DL1+Bw4/x	73	71.3	0.687
3DL1S1+Bw4/x	74.3	75.7	0.713
3DS1+Bw4/Bw4	6	12	**0.023**
3DL1+Bw4/Bw4	22.3	22	0.831
3DL1S1+Bw4/Bw4	22.3	23.9	0.68
2DL2+C1/x	20.4	19.9	0.906
2DL3+C1/x	86.7	90	0.258
2DL2/3+C1/x	90.3	90	0.934
2DS2+C1/x	19.5	18.7	0.837
2DL2+C1/C1	10.2	10	0.937
2DL3+C1/C1	46.5	48.6	0.64
2DS2+C1/C1	9.3	9.6	0.92
2DL1+C2/x	50	51	0.828
2DS1+C2/x	22.6	19.1	0.355

Bw4/x : x represents *Bw4* or *Bw6*; *C1/x*: *x* represents *C1* or *C2*; *C2/x*: *x* represents *C1* or *C2*.

3. 3. 9 KIR footprints on HIV-1 evolution in the SM cohort

Amino acid sequences for *Gag, Nef* have been previously generated in this cohort (Dong, Zhang et al. 2011). To determine whether any individual *KIR* gene is capable of imposing an immune pressure on the HIV-1 sequence, we analysed viral amino acid sequences from those individuals with "KIR and Gag" (n=76) or "KIR and Nef" (n=80) respectively. We first identified the variation of residues at each amino acid position, and then we investigated

whether this change could be ascribed to the presence of a specific KIR gene or group of KIR genes. *KIR2DL4, KIR3DL2* and *KIR3DL3* were excluded because they are the framework genes. Due to very high frequencies (nearly 100%), *KIR2DL1* and *KIR2DS4* were also ruled out. *KIR3DL1* was ruled out because only one individual was negative for *KIR3DL1*. Eventually, *KIR2DL2*, *KIR2DL3*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5* and *KIR3DS1* were entered into the KIR footprint analyses (**Table 3-6**).

Table 3-6: KIR "footprints" on HIV-1 amino acid sequences in the SM cohort

VP	Res	KIR	Con	Variation	Fishers' P	Comments
Nef	68	*2DS5*	S	N	0.016	Presence of 2DS5
Nef	211	*2DS3*	R	H,L,P,S,V	0.03	Presence of 2DS3
Nef	202	*2DS3*	E	D,K	0.025	Presence of 2DS3
Nef	86	*2DS1*	E	D,G,K,N	0.028	Presence of 2DS1
Nef	220	*2DL2*	E	D,G,H,Q,R	0.015	Presence of 2DL2
Nef	135	*2DL2*	L	I,M	0.025	Presence of 2DL2
Nef	192	*2DL2*	S	N	0.025	Presence of 2DL2
Nef	193	*3DS1*	Q	L,R	0.03	Presence of 3DS1
Nef	181	*2DS5*	E	G,K	0.034	Lack of 2DS5
Nef	75	*2DL5*	D	A,E,N	0.008	Lack of 2DL5
Nef	75	*2DS1*	D	A,E,N	0.031	Lack of 2DS1
Nef	84	*2DL5*	E	D,K	0.042	Lack of 2DL5
Nef	223	*2DL5*	P	H,X	0.042	Lack of 2DL5
Gag	85	*3DS1*	I	L,M,V	0.022	Presence of 3DS1
Gag	268	*3DS1*	T	I,S,V	0.044	Presence of 3DS1
Gag	7	*2DS3*	K	N,R,S	0.023	Presence of 2DS3
Gag	114	*2DS2*	S	N,T	0.023	Presence of 2DS2
Gag	206	*2DS2*	V	A,I	0.03	Presence of 2DS2
Gag	99	*2DS1*	T	A,K,P,Q	0.019	Presence of 2DS1
Gag	206	*2DL2*	V	A,I	0.03	Presence of 2DL2
Gag	27	*2DL5*	V	I,L	0.04	Presence of 2DL5
Gag	108	*3DS1*	T	I,K,L,V,R,M,A	0.05	Lack of 3DS1
Gag	108	*2DS3*	T	I,K,L,V,R,M,A	0.039	Lack of 2DS3
Gag	115	*2DS2*	K	P,Q,R	0.027	Lack of 2DS2
Gag	211	*2DL5*	V	I	0.042	Lack of 2DL5
Gag	398	*2DL5*	R	K	0.04	Lack of 2DL5

VP: viral protein, Res: amino acid residue position in the SM cohort, Con: amino acid consensus at that position in the SM cohort, Variation: associated with presence or absence of specific *KIR* gene, the amino acid could change from the consensus one to the possible ones.

There are 12 Nef positions and 11 Gag positions shown to be associated with specific *KIRs*. A previous study in the same SM cohort has associated HLA class I with HIV sequence polymorphisms (Dong, Zhang et al. 2011). 140 single amino acid residues within Gag, RT, integrase, and Nef were shown to be associated with HLA class I molecules. We compared our KIR-associated amino acid positions to the previous HLA class I- associated positions, See **Table 3-7**.

Table 3-7: The comparison of KIR association positions and HLA class I-associated positions

VP	Res	KIR	HLA association	In the known CTL epitopes
Nef	68	*2DS5*		A2, B40
Nef	211	*2DS3*		
Nef	202	*2DS3*		
Nef	86	*2DS1*		
Nef	220	*2DL2*		
Nef	135	*2DL2*		Cw7
Nef	192	*2DL2*		
Nef	193	*3DS1*	*A1*	
Nef	181	*2DS5*		
Nef	75	*2DL5*		A2
Nef	75	*2DS1*		A2
Nef	84	*2DL5*		
Nef	223	*2DL5*		
Gag	85	*3DS1*		
Gag	268	*3DS1*		A2,A11,A24
Gag	7	*2DS3*	*A30,B15*	A30,A11,B27
Gag	114	*2DS2*		
Gag	206	*2DS2*		A2, B40
Gag	99	*2DS1*		
Gag	206	*2DL2*		A2,B40
Gag	27	*2DL5*	*B40,Cw3*	
Gag	108	*3DS1*	*A24,B15*	
Gag	108	*2DS3*	*A24,B15*	
Gag	115	*2DS2*		
Gag	211	*2DL5*	*A24*	
Gag	398	*2DL5*		

VP: viral protein, Res: amino acid residue, HLA association: the position is also associated with HLA class I molecules in the previous study. The information of known epitopes comes from the epitope maps in the Los Alamos database.

One Nef position has been associated with *HLA-A1* before, and 4 Gag positions (Gag7, 27, 108, 211) have been associated with different HLA class I molecules as above. For these overlapping viral protein positions, the immunological pressure from CTL could explain the HIV sequence polymorphisms, but for the rest of the associations, which were not ascribed previously to the pressure from HLA class I restricted CTL, the driving power for the amino acid sequence variations may be exerted by KIR-associated immune pressure.

Furthermore, we used the centromeric and telomeric data which contain groups of *KIR* genes to do the same analysis again, and this showed that the majority of the associations were with B motifs, which carry many of the activating KIRs. See **Table 3-8**.

Table 3-8: KIR motif associated mutations

	Mut	$P_{f\ [A/A\ vs.\ A/B\ vs.\ B/B]}$	$P_{a\ [A/x\ vs.\ B/B]}$	$P_{b\ [A/A\ vs.\ B/x]}$	Comment
Centromeric					
Nef	D75A	0.048	**0.027**	0.166	17% c-A/x vs 0% c-B/B (A motif associated mutation)
Nef	I196A	**0.007**	**0.003**	**0.03**	42% c-B/B vs 11% c-A/x (B motif associated mutation)
Nef	E84D	0.044	0.09	**0.013**	16% c-A/A vs. 0% c-B/x (A motif associated mutation)
Nef	P223X	**0.033**	0.093	**0.009**	23% c-A/A vs 2.7% c-B/x (A motif associated mutation)
Gag	V27I	0.079	0.153	**0.031**	20% c-A/A vs 2.8% c-B/x (A motif associated mutation)
Gag	V211I	0.057	**0.028**	0.07	24% c-A/x vs 3.8% c-B/B (A motif associated mutation)
Gag	R398K	0.064	0.089	**0.026**	15% c-A/A vs 0% c-B/x (A motif associated mutation)
Telomeric					
Nef	L108V	0.081	1	**0.041**	19% t-A/A vs 3% t-B/x (A motif associated mutation)
Nef	P223X	0.068	1	**0.022**	21% t-A/A vs 3% t-B/x (A motif associated mutation)
Gag	R57K	**0.031**	**0.027**	1	100% t-B/B vs 15% t-A/x (B motif associated mutation)
Gag	T268V	**0.005**	**0.013**	0.072	100% t-B/B vs 15% t-A/x (B motif associated mutation)

P_f: global p values comparing HIV-1 infected individuals carrying c-A/A, c-A/B, or c-B/B genotypes; P_a: p values comparing people with at least one A motif to those homozygotes for B motifs; P_b: p values comparing people with at least one B motif to those homozygotes for A motifs.

3. 4 Discussion

HIV infection is a complicated interaction between a polymorphic virus and a polymorphic host immune system. Sometimes it is difficult to discriminate between cause and effect of an observed immune response to the virus at a given time point. Compared to functional studies which are dynamic and interactional, the charm of immunogenetic epidemiological studies is that they can provide an *in vivo* population view of factors that affect the host response to the virus, leading to different outcomes in different populations, providing suggestions for subsequent *in vitro* functional studies.

Few data about the immunogenetic associations of HIV-1 infection in Chinese people were found in the literature. The unique advantages of the SM cohort involve several aspects: Firstly, almost all the individuals in the SM patient group were infected in a very narrow period, which was between 1993 and 1995 when the paid plasma donation scheme was prevalent in those rural area; Secondly, based on the analyses of the virus sequences (Dong, Zhang et al. 2011), we can confirm that the origin of the viral sequences was limited to a relatively narrow source, occurring only in individuals who had an evident plasma donation history, which strongly suggests that they were infected via the same blood contamination route. Hence, considering the usual dynamic interaction between virus and host, at least one side has been relatively "fixed", which offers us a unique advantage to evaluate how the other side, host factors, will shape the outcome of HIV infection. Different clinical phenotypes in the same infected population are the best outcome measures with which to study the underlying genetic determinants which are associated with the final outcome. Previous studies in the same cohort (Dong, Zhang et al. 2011) showed that CTL pressure has a major effect on inter-host HIV-1 viral diversity and is thought to represent a key element of viral control. However, the CTL pressure alone is not sufficient to explain the large heterogeneity

observed in the clinical manifestations of the HIV infection. Additional components of the immune system must be also involved in the control of HIV-1 disease. Moreover, HLA class I molecules, as the central part of the CTL immune response, are also the ligands for the KIRs, which highlights the potential role for KIRs in defence against HIV-1 infection.

The proportions of KIR gene frequencies have been compared between two groups trying to explore a possible causal relationship that may determine the HIV progression. In the ideal situation, it is the rapid progression group which should be compared with the slow progression group to see the differences. Unfortunately, this is a retrospective study; the rapid progressors had inevitably been lost from this cohort due to early death. In order to compensate for this loss, another healthy group in which the participants were from an adjacent village and considered to have a closely-related genetic background was recruited into this study. The rationale underlying this comparison is that the healthy group people could be considered as a surrogate of the previous total population in SM village: the similarities between the two populations could have been confirmed using SNP typing for other polymorphic genes in the Han Chinese. Following infection with HIV, some individuals died earlier, however many individuals progressed slowly until 10 years later when the first screening test was implemented in that village. To some extent, the slow progressor group could be considered as a subset of the previous total population, whilst the "missing" rapid progressor group would be considered to be another subset. The characteristics of the two groups may distribute at either side of a standard bell curve; at least the comparison between one subset and the overall population provided some insights to explore the KIR associations with outcome, even with reduced power.

The first investigation showed the profile of *KIR* gene distribution in the SM patient group and the healthy control group respectively. It was not surprising that almost all the *KIR* genes

had the similar frequency in both groups, except for *KIR2DL3*. After all, the population in both groups were from the same ethnic group, Chinese Han, and from a geographically adjacent area.

KIR2DL3 has been shown to play a role in enhancing the resolution of acute hepatitis C viral infection (Khakoo, Thio et al. 2004). The presumed mechanism underlying this observation is that the weaker inhibitory interaction of *KIR2DL3* with *C1*, but not the stronger inhibitory interaction of *KIR2DL2* and *KIR2DL1* with *C1* and *C2*, could allow penetrance of activating signals during viral infection. In our study, a significant difference of *KIR2DL3* frequency was also detected between the "slow progressors" group and the healthy control group. Since it is always necessary to incorporate the KIR ligand interactions into a biological model rather than just considering KIR alone, we further investigated *KIR2DL3*+ *C1C1* or *C1C2* between the two groups, but no further significant differences were found. So the initial difference has to be explained with caution. Moreover another concern is *KIR2DL3* itself. In the healthy controls group, the frequency of *KIR2DL3* was 99.2%, which was similar to that reported in other two Chinese Han population (Jiang, Zhu et al. 2005; Bao, Hou et al. 2010), 99% and 100%, respectively. However, in the SM patient group, the frequency of *KIR2DL3* (95.8%) was significantly lower. The *KIR* gene frequencies shown in this study were based on the typing method of SSP-PCR, which relies on sequence-specific primers to amplify the specific *KIR* genes. However these sequence-specific primers were designed based on the known alleles and they need to be updated periodically to ensure the ability to amplify all the possible alleles for each specific *KIR* gene in different populations. Even so, they may still miss amplifying some rare alleles. Under such circumstances, there might be some new *KIR2DL3* alleles in SM patients, which have variation in the binding positions of these "sequence-specific primers", in turn leading to a negative detection of this gene.

The power of this study can be defined as the probability of correctly concluding that the difference is genuine between two groups for specific KIR genes and gene combinations. Taking *KIR2DL3* as example, when we tried to show a true difference of gene frequencies between 95% and 99%, the sample size is required to be larger than 400 to maintain power higher than 80% (calculated by software PASS using Two Independent Proportions (Null Case) Power Analysis). However in the current study, in the context of a relatively small sample size, the power can reach only around 52%. Calculating another comparison of *KIR3DS1+Bw4/Bw4* (6% versus 12%， p=0.023)， we found that current sample size can provide a power of around 58%. Increasing sample size will decrease both type I error and type II error, consequently increasing power. According to the sample size power analysis, a future study requires the sample size at least greater than 400 cases in each group to ensure the analysis power is higher than 80%.

The main variation between KIR haplotypes is in the number of genes (Uhrberg, Valiante et al. 1997). The A haplotype can be identified by the presence of *KIR2DS4* as the only activating receptor gene, whereas B haplotypes have multiple activating receptors, but not *KIR2DS4* (Vilches, Pando et al. 2000). In general, the A haplotype is associated with an improved response to pathogens, whereas B haplotypes are associated with improved reproductive fitness (Lu, Zhang et al. 2008; Cheent and Khakoo 2009; Hiby, Apps et al. 2010). In our study, the A haplotype accounted for 50% of SM patients, which was a similar frequency to that (51%) in the healthy controls.

In our study population, the frequencies of homozygous *KIR3DL1*, heterozygous *KIR3DL1/KIR3DS1* and homozygous *KIR3DS1* are 65.1%, 30.6% and 3.9% respectively; $(65.1\%)^2+2*30.6\ \%+(3.9\%)^2=1$, so in this population, the frequencies of *KIR3DL1/3DS1* are in Hardy-Weinberg equilibrium.

The *KIR3DL1/S1* gene is distinct due to its diversity and varied expression patterns. It is the only KIR locus that encodes both inhibitory and activating allotypes. KIR3DL1 recognises *HLA-B* alleles with the Bw4 serological motif. Although KIR3DL1 and KIR3DS1 share a high degree of similarity and Bw4 was assumed to be the ligand for KIR3DS1, the direct binding of KIR3DS1 and Bw4 has not yet been demonstrated. However a number of epidemiological and functional studies support some sort of interaction between *KIR3DS1* and *HLA-B Bw4-80I* in the NK cell response to HIV infection. Maureen P. Martin et al. found that *KIR3DS1+HLA- B Bw4-80I* was protective against HIV progression (Martin, Gao et al. 2002); but other studies have failed to replicate this synergistic protective effect. Moreover, Gaudieri et al found that individuals with *KIR3DS1* and *Bw4-80I* actually exhibited an accelerated progression to AIDS (Gaudieri, DeSantis et al. 2005). O'Connell et al also found that in a small cohort of elite controllers, there was no correlation between *KIR/HLA* genotype and control of HIV replication by NK cells *in vitro* (O'Connell, Han et al. 2009). In our study, the frequency of *KIR3DS1+ Bw4* homozygotes was significantly lower in the SM "slow progressors" group (6%) compared to that (12%) in the healthy group. This observation suggested a loss of this compound in the "slow progressors", which implies that this combination has no protective effect against HIV progression. In our study, due to the mixture of 2-digit and 4-digit HLA typing results, we could not assign each *Bw4* motif to 80I or 80T subgroups, therefore, we were not able to investigate the effect of *KIR3DS1+Bw4-80I* in our study.

KIR3DL1 shows extensive polymorphism, and its variation has functional significance in terms of cell surface expression levels and inhibitory capacity, which has been shown to be relevant in a previous association study with HIV infection (Martin, Qi et al. 2007). But in our study, there was no correlation with *KIR3DL1/Bw4* genotype and HIV progression.

It is widely believed that CD8 T cell immune pressure is a driving force for HIV-1 evolution, and HLA class I molecules can impose a characteristic change on HIV-1 sequences. This immune pressure has been clearly shown at a population level over time (Moore, John et al. 2002). Given the interaction between HLA and KIR, it was assumed that KIR might be exerting a similar kind of immune pressure on HIV as do HLA class I molecules. A recent study (Alter, Heckerman et al. 2011) demonstrated that *KIR2DL2* positive NK cells can place immunological pressure on HIV-1, and the virus can evade such NK cell mediated immune pressure by selecting sequence polymorphisms. They used *KIR* genotypes and HIV-1 polymorphism to generate a group of footprints. Later, a selected polymorphism from an overlapping segment covering both *Vpu* and *Env* was constructed into a backbone virus, which was then used to infect cells that were co-cultured with autologous NK cells derived from *KIR2DL2* positive or negative individuals. The result showed that the wild-type virus was markedly inhibited by *KIR2DL2* positive NK cells; in contrast, the variant with the *KIR2DL2* selected mutation could not be inhibited.

In our study, we used the amino acid sequences of Gag, Nef and the *KIR* genotypes to identify potential KIR "footprints" on the HIV-1 evolution in our cohort. Based on the different amino acid sequences between single *KIR* gene positive and negative groups, the positions at which KIRs might have left footprints were determined. Some viral amino acid positions were described before in the same SM cohort from our group (Dong, Zhang et al. 2011), which have been associated with specific HLA class I molecules. To some extent, the variation of the virus could be ascribed to the immune pressure from HLA class I-restricted CTL, as it was reported that about two-thirds of all non-envelope viral mutations detected in the chronic phase are attributed to CD8 T cell responses in HIV-1-infected patients (Allen, Altfeld et al. 2005). Interestingly, for the remaining positions associated with KIRs, the

underlying driving pressure might come from *KIR*-associated pressure. Actually it is still difficult to explain these identified "footprints". First, some positions have been associated with two or more *KIRs*. For example, Nef75 was associated with *KIR2DL5* and *KIR2DS1*, and it was complicated to explain the relationship between an inhibitory *KIR* and an activating *KIR* gene, and viral variation. Second, some of the associated KIRs have no known ligands; in this context, it is difficult to speculate how they could exert selection pressure. Third, *KIR* genes are similar to each other, and linkage disequilibrium is quite common, so single gene association always need to be examined with caution. Lastly and most importantly, any genetic association finding needs to be confirmed by subsequent functional experiments.

We also collaborated with Mina John from Murdoch University and performed a similar statistical analysis looking for KIR footprints. Many positions were similar to those identified in our analyses. The results for individual *KIR* gene were less impressive as the Q and p values were only marginally significant. The Nef 75 was highlighted because it was the only association position (with *KIR2DL5*) which held up with a q-value below the cut off of 0.2.

Moreover, repeating the same analysis using the centromeric and telomeric data revealed that the presence of groups of *KIR* genes may be able to exert strong immune pressure on the virus forcing it to change key amino acids to survive. The majority of the associations were with B motifs which carry many activating *KIRs*, implying that *KIR* genes present on group B centromeric genotypes are likely to mount a greater immune pressure on HIV-1.

The confounders of this study might include limitations of the sample sizes used for the two populations. A relatively small sample size may be possible to generate some results of 'false positives', which is also known as type I error, could happen when we compared multiple *KIR* genes and different combinations of *HLA+KIR* between two groups. The probability of

observing a type I error is indicated by the P-value. Decreasing type I error will result in increasing type II error, and consequently decreasing power, but increasing sample size will decrease both type I error and type II error. In order to avoid 'false positives' as much as possible, future studies should include a larger sample size.

Although the absolute sample sizes were not as big as those reported in other studies, the "genetic association effect" could be regarded as magnified because other confounders such as ethnic group, founder virus, transmission route and infection duration have been filtered before we performed the statistical analysis.

Another confounding factor is HCV/HIV co-infection, which is likely to be high but this hasn't yet been fully investigated in our cohort. However, it is plausible that HCV/HIV co-infection will affect the present findings. It has been reported that *KIR3DS1* in conjunction with *HLA-Bw480I* is also protective in the setting of HCV (Lopez-Vazquez, Rodrigo et al. 2005). Future studies in this cohort will take this into consideration at the study design stage and aim to investigate the rate of HCV/HIV co-infection in the SM cohort.

Taken together, we have studied the polymorphism of KIRs at genotype, haplotype, ligand-receptor and centromeric/telomeric levels in this unique SM cohort. Through comparison with a healthy control group, some genetic associations were identified: the frequency of *KIR2DL3* was lower in the "slow progressors" group; the compound genotype of *KIR3DS1*+ *Bw4* homozygotes was significantly lower in the "slow progressors" group; additionally, group B centromeric genotypes (multiple activating genes) were shown to be likely to mount a greater immune pressure on HIV-1. In terms of KIR footprints, several amino acid positions were identified for which the substitution of an amino acid may be ascribed to the immune response from KIR specific NK cells rather than from HLA restricted CTL immune pressure.

It is becoming apparent that host genetic variation can exert significant selective pressure on the virus (Martin and Carrington 2013). The advantage of genetic epidemiological studies is that they can provide an *in vivo* population view of factors that affect the host response differentially across humans, giving meaning to subsequent functional studies to further investigate the molecular basis of their impact on AIDS pathogenesis and help clarify the relative contribution of the innate and adaptive immune response, which may lead in turn to the development of better therapies and vaccines.

Chapter 4 Sequencing the locus of *KIR3DL1/S1*

4. 1 Introduction

In the most recent Immuno Polymorphism Database (IPD)-KIR database (version 2.5.0) released in October 2013 (Robinson, Halliwell et al. 2013), *KIR3DL1* has been shown to be the most polymorphic gene apart from the framework gene *KIR3DL3*. It was assumed that *KIR3DS1* might originate from *KIR3DL1* through a recombination between a *3DL1* allele and an activating KIR gene, which replaced the inhibitory signalling domain of the 3DL1 allotype with the activating signalling domain of this activating KIR (Abi-Rached and Parham 2005). This seminal event is estimated to have occurred millions of years ago, before the separation of human and chimpanzee ancestors (Parham 2008). After the recombination, the ancestral *KIR3DS1* soon obtained several amino-acid substitutions in the extracellular domains that account for their different ligand-binding characteristics. Both of the two genes acquired additional polymorphisms later, but in very different ways (Norman, Abi-Rached et al. 2007).

The *KIR3DL1* and *KIR3DS1* genes share the same locus, and they share 97% similarities of nucleotides to one another. *KIR3DL1/S1* has 9 exons and 8 introns. The main difference between *KIR3DL1* and *KIR3DS1* is that there is a 2bp deletion in exon 8 of *KIR3DS1* that leads to a premature stop codon in exon 9, making exon 9 of *KIR3DS1* much shorter than that of *KIR3DL1* (**Figure 1-14**). This difference in the length of the region encoding the cytoplasmic tail in exon 9 leads to different lengths of the cytoplasmic tail (Dohring, Samaridis et al. 1996; Torkar, Norgate et al. 1998)(**Figure 4-1**), which can vary from 14 amino acid residues long (in some *KIR3DS1* alleles) to 108 amino acid residues long (in some KIR3DL1 proteins), therefore it in turn leads to different functions of these two genes. Generally, it is the different length of their cytoplasmic tails and their different linkages to

intracellular signalling pathways which determines the differences between *KIR3DL1* and *KIR3DS1*.

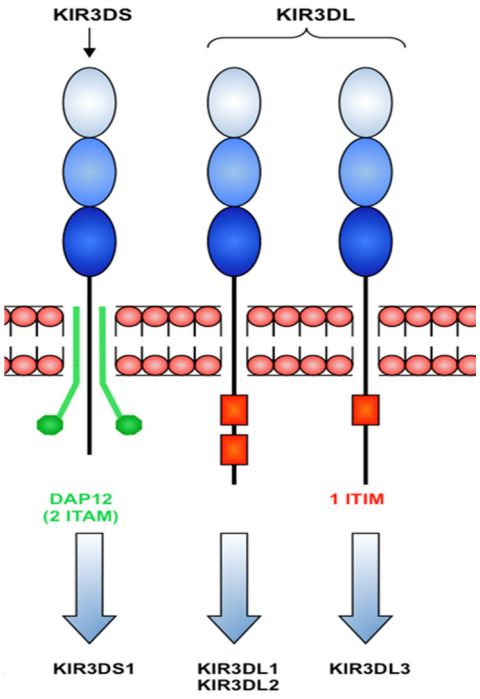


Figure 4-1: The different length of the cytoplasmic domain leads to different function between *KIR3DL1* and *KIR3DS1*. ITAM: immunoreceptor tyrosine-based activation motif. ITIM: immunoreceptor tyrosine-based inhibition motif. Adapted from http://www.ebi.ac.uk/ipd/kir/introduction.html

Among all the *KIR* genes, *KIR3DL1* and *KIR3DS1* play more prominent roles than other KIRs in HIV-1 infection, and this has been demonstrated in many studies (Martin, Gao et al. 2002; Qi, Martin et al. 2006; Alter, Martin et al. 2007; Martin, Qi et al. 2007; Boulet, Sharafi et al. 2008; Boulet, Song et al. 2010). KIR3DL1 uses Bw4 molecules as its ligands, although *KIR3DS1* shares nearly 97% similarity with *KIR3DL1*, the direct binding of KIR3DS1 with its putative Bw4 molecules has not yet been demonstrated. Even so, the compound genotype *KIR3DS1+ HLA-B Bw4 80I* has been shown to be protective against HIV-1 progression (Martin, Gao et al. 2002). It was interesting that without *KIR3DS1*, *HLA-B Bw4 80I* was not associated with any of the AIDS outcomes measured; meanwhile, *KIR3DS1* was significantly

associated with more rapid progression to AIDS in the absence of *HLA-B Bw4 80I* alleles, which suggested there might be an epistatic interaction between *KIR3DS1* and *Bw4 80I alleles* in HIV/AIDS progression. In terms of different expression levels, the KIR3DL1 allotypes can be divided further into two groups: KIR3DL1*l/x (lower expression and lower inhibitory activity) group and KIR3DL1*h/y (high expression and high inhibitory capacity) group, first assigned by Martin (Martin, Qi et al. 2007). In their studies, combined with the same ligands, the HLA-Bw4 80I allotypes, KIR3DL1*h/y allotypes have been shown to be protective against HIV, however no such kind of impact was observed with KIR3DL1*l/x allotypes, which aroused great interest to look into more details of this locus.

Currently in the newly released IPD-KIR database (Robinson, Halliwell et al. 2013), 92 known alleles of the *KIR3DL1* gene are described, which encode 62 different receptors. At least one of these alleles, *3DL1*004*, is poorly expressed at the cell surface because of a substitution that abrogates an important folding motif (Pando, Gardiner et al. 2003). Other *KIR3DL1* allelic products differ in their abundance at the cell surface. For example, *KIR3DL1*001*, **002*, **008, *015, *020* alleles are expressed at a high level, however, *KIR3DL1*005, *007, *009* are expressed at a low level (Gardiner, Guethlein et al. 2001; Yawata, Yawata et al. 2006). Such kind of allelic variation plays a role in determining the strength of the interaction, which in turn correlates with level of binding to HLA-B Bw4 ligands (Gardiner, Guethlein et al. 2001; Yawata, Yawata et al. 2006). Allelic variants can also differ in terms of the frequency of NK cells that express them. Disease association data are now accumulating and indicate that these differences are very significant in determining the outcome of infection (Parham 2005; Yawata, Yawata et al. 2006).

The name of each specific *KIR* gene is indicated before the separator *, and *KIR* alleles are named in a similar way to alleles of the HLA system (**Figure 4-2**). The first 3 digits after the

separator distinguish alleles differing in exon sequences which lead to non-synonymous changes. The next two digits represent alleles differing in exon sequences that lead to synonymous changes and the last two digits indicate alleles that only differ in an intron, promoter or other non-coding region.

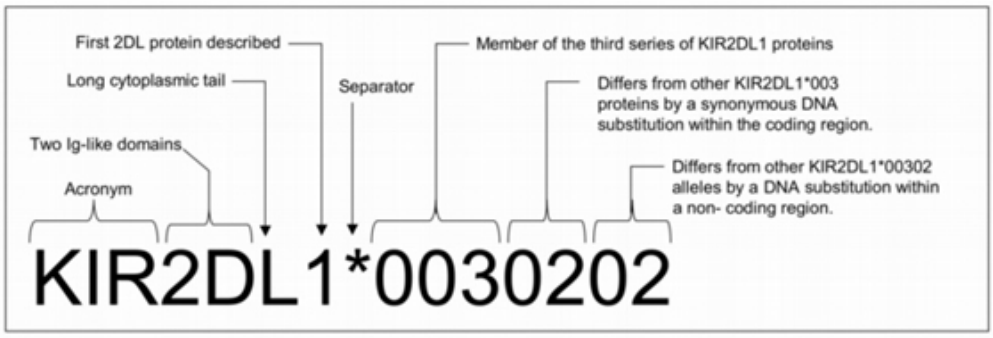


Figure 4-2: Nomenclature of *KIR* alleles. Adapted from http://www.ebi.ac.uk/ipd/kir/alleles.html

There are already some studies that have focussed on sequencing the *KIR3DL1/S1* locus, but most of them have concentrated on the coding DNA sequences (Halfpenny, Middleton et al. 2004; Belle, Hou et al. 2008). Recently some genome wide association studies have given us a hint that even SNP in the introns of a gene could have an impact on disease outcomes (Pereyra, Jia et al. 2010). These variants in noncoding regions, which may affect the level of transcription, translation and splicing, may also be important. In order to obtain complete information about this locus, it becomes more and more necessary to sequence the entire locus of *KIR3DL1/S1* which contains both the exons and introns.

The main barrier blocking the road to sequence the entire locus is the extremely high degree of similarity between *KIR3DL1* and *KIR3DS1*. Although using next generation sequencing techniques, we can easily generate large amounts of short DNA sequences from this locus,

the real difficulty is to know how to align them properly and analyse these sequences in a correct manner.

Here, the purpose of this study is to develop a protocol which is able to sequence the entire *KIR3DL1* and *KIR3DS1* locus from genomic DNA. The hypothesis underlying this development is that we would detect complete allelic diversity using this comprehensive sequencing strategy and thereby be able to evaluate the polymorphism of this locus at a six-digit level. For comparison and verification, we also used another exon-sequencing method to determine the allotypes of KIR3DL1 and KIR3DS1 for each sample.

4. 2 Methods

According to the *KIR* genotyping results in **Chapter 3**, the samples were divided into three groups: *KIR3DL1* homozygous group, *KIR3DL1/S1* dual positive group (here we called them heterozygous group), and *KIR3DS1* homozygous group, respectively. In terms of DNA quality, some samples were selected for the sequencing of the locus *KIR3DL1/S1*.

4. 2. 1 Long range *KIR3DL1/S1* amplification

A reaction volume of 50.2ul consisted of 150ng template DNA, 10ul 5× buffer (containing magnesium), 2.5ul each of 10μM forward and reverse primer, 2.5ul 10mM deoxyribonucleotide triphosphate (dNTP), 4ul dimethyl sulfoxide (DMSO), 0.7ul Expand Long range Enzyme mix (Roche Applied Sciences), and 28ul nuclease free water. Amplifications were carried out in a Q-cycler II Ultra gradient thermal cycler (Quanta biotech). The initial DNA denaturation was performed with one cycle of 92°C for 2 min. The PCR amplification included 5 cycles of 92°C for 10s, 52°C for 15s and 68°C for 14min, followed by 25 cycles of 92°C for 15s, 52°C for 15s and 68°C for 14min (+20s/cycle). 1% Agarose gel electrophoresis at 85V for 90min was used to confirm proper amplification of DNA. PCR reactions were then purified using ExoSAP-IT (Affymetrix) according to the

manufacturer's protocol. ExoSAP-IT utilizes two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase, to remove any unconsumed dNTPs and primers remaining in the PCR product mixture. Hence the treated PCR products were ready for use in DNA sequencing.

4. 2. 2 Sequencing

The BigDye Terminator v3.1 Cycle Sequencing Kit (life technologies) was used for sequencing. A reaction volume of 10ul consisted of 2ul template DNA, 5× buffer 2.075ul, BigDye terminator v3.1 0.25ul, nuclease free water 5.355ul, and 10μM sequencing primer 0.32ul. The PCR amplification included one cycle of 96°C for 1min, followed by 30 cycles of 96°C for 10s, 50°C for 5s and 60°C for 4min.

4. 2. 3 Post-sequencing purificaiton

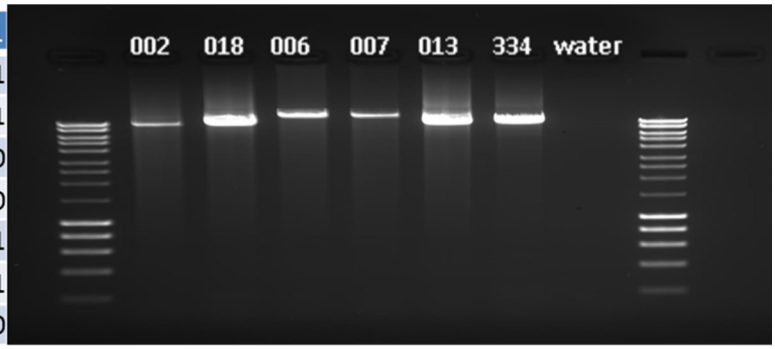
The post sequencing clean-up was done by using Sephadex filtration (Millipore, USA). The microtitre plate was prepared as following.

Add the G-50 powder (Sephadex) onto the black plate (MultiScreen Column Loader), then spray over to fill in the number of wells needed. The G-50 powder was then transferred into the corresponding wells of the MultiScreen plate (MAHVN4510). 300ul of water was added into each well containing the G-50 powder and the plate was allowed to sit at room temperature for at least 2 hours; then the G-50 plate was placed on a 96-well U or V bottom plate and centrifuged at 2300 rpm for 5min to remove water. 150ul of water was added twice and centrifuged in the same way to wash the column. After this, the G-50 plate was placed tightly onto a labelled sequencing plate; and the PCR product was added gently into the G-50 plate and centrifuged at 2300 rpm for 5 min. So the PCR product was filtered out into the corresponding well of the sequencing plate while excess BDT and primers were held back.

10ul of Hi-Di formamide was added into each well and the plate was sent for analysis on the ABI 3730XL sequencer.

In the beginning, two pairs of primers were designed and tested with the aim of amplifying the whole locus of *KIR3DL1/S1*. The first pair of primers: KIR3DL1S1_F1: 5' – AGTGAGAGCAATTTCCAGG-3' & KIR3DL1S1_R1: 5'- AACAGCATGAGGGAAGGT-3', has been shown to be able to amplify the *KIR3DL1/S1* locus no matter whether the samples contain only *KIR3DL1* and *KIR3DS1* or both, while another pair of primers failed to do so. The gel (**Figure 4-3**) below clearly showed the efficacy of the amplification by the first pair of primers.

SampleID	3DL1	3DS1
SMC002	0	1
SMC018	1	1
SMC006	1	0
SMC007	1	0
SMC013	1	1
SMP334	0	1
Water	0	0



1% Agarose gel; 60 minutes @110V

Figure 4-3: The loci of *KIR3DL1/S1* from six samples with different *KIR3DL1/KIR3DS1* genotyping results were successfully amplified by the pair of primers: KIR3DL1S1_F1+KIR3DL1S1_R1.

When the time of electrophoresis was elongated from 60 minutes to 105 minutes, in those samples possessing both *KIR3DL1* and *KIR3DS1* gene, the two bands representing the two genes could be clearly separated (**Figure 4-4**).

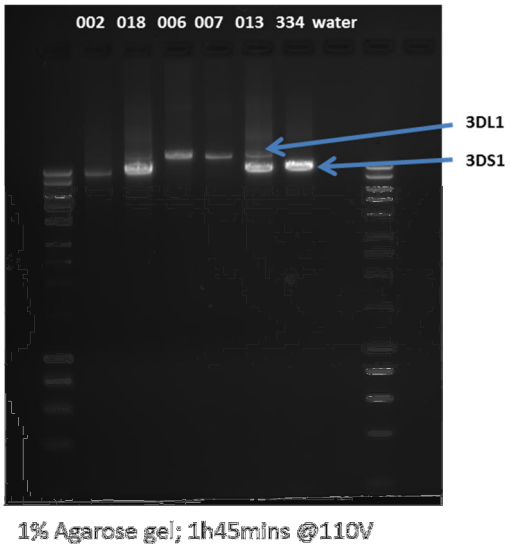


Figure 4-4: Longer time electrophoresis separated *KIR3DS1* from *KIR3DL1* in individuals possessing both of the two genes.

Although the locus had now been specifically amplified, the subsequent problem was that the amplification products from the heterozygous samples (possessing both *KIR3DL1* and *KIR3DS1*) could not be used directly as a template for the subsequent sequencing, because at many positions within the tracing sequence, it was difficult to differentiate *KIR3DL1* from *KIR3DS1*, and vice versa (**Figure 4-5**).

Figure 4-5: An example of tracing sequence obtained from a heterozygous sample. In the consensus bar at the bottom, it can be seen that at many positions, the reads were composed of a mixture of

sequences from both *KIR3DL1* and *KIR3DS1*. V represents A, C or G; R represents A or G. The exclusive sequences for *KIR3DL1* and *KIR3DS1* were aligned above to show how the mixture occurred.

Before the separation of *KIR3DL1* from *KIR3DS1* was finally achieved, we restricted the use of this pair of primers in only homozygous samples, and expected that the two alleles in these homozygous samples to be the same, otherwise we would still be confused by the coexistence of different reads. Meanwhile, other pairs of primers were tested, aiming at differentiating *KIR3DL1* from *KIR3DS1* in the heterozygous samples. The testing of different sets of *3DL1* and *3DS1* primers is shown below (**Figure 4-6**).

SampleID	3DL1	3DS1
SMP426	1	1
SMP437	1	1
SMP342	1	1
SM329	1	0
SM026	0	1

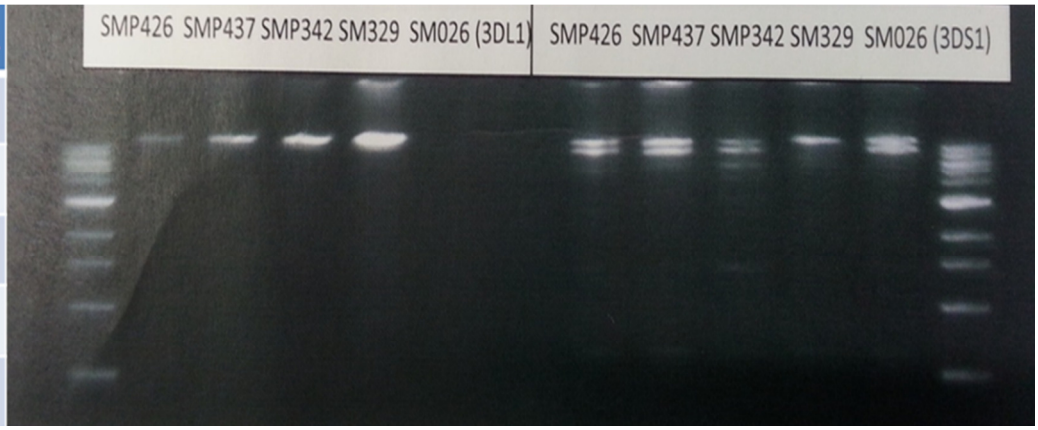
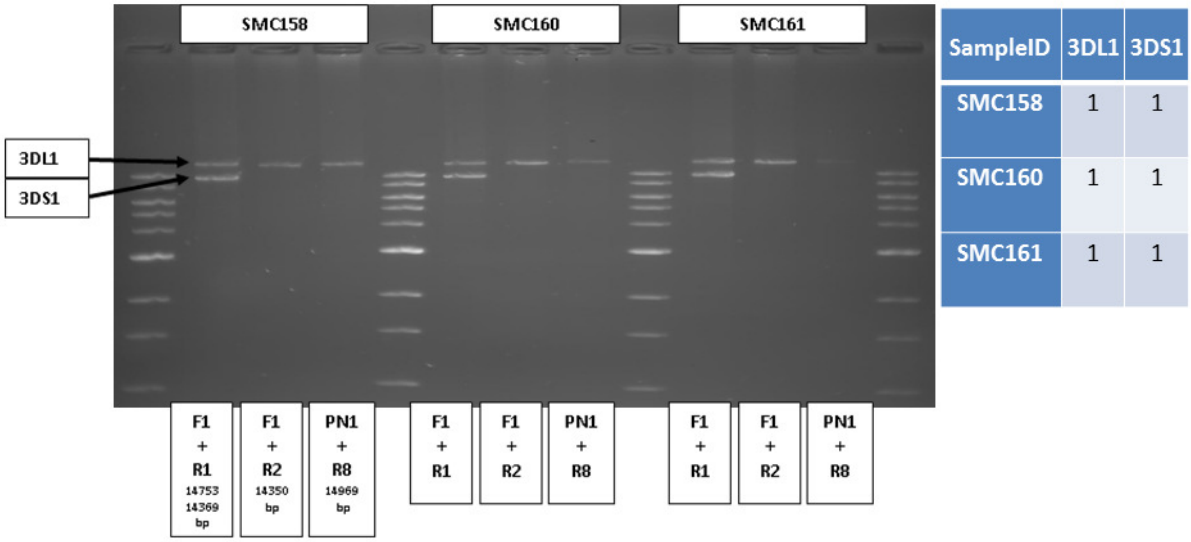


Figure 4-6: Comparison between two pairs of primers. The left (3DL1) half of the gel represents a pair of primers: *KIR3DL1S1*_F1: 5'- AGTGAGAGCAATTTCCAGG-3' + *KIR3DL1*_R2: 5'-GAACAGCACGTGGGTAAG-3' with an annealing temperature at 54.5°C which can pick up *KIR3DL1* exclusively from the heterozygous samples. In contrast, the right half (3DS1) represents a pair of primers: *KIR3DL1S1*_F1: 5'- AGTGAGAGCAATTTCCAGG-3' + *KIR3DS1*_R2: 5'-GTGGAACAGCACGTGTCTA-3' with an annealing temperature at 52°C which cannot separate the two genes in the same heterozygous samples.

After several times of trial and error, another pair of primers was finally shown to be effective to amplify *KIR3DS1* exclusively from the heterozygous samples (**Figure 4-7**).



SampleID	3DL1	3DS1
SMC158	1	1
SMC160	1	1
SMC161	1	1

Figure 4-7: Comparison of the 3 pairs of primers using the heterozygous samples. Here the pair of primers PN1: 5'- GAGTTTAAATCATTTGAACTGGTTCTG-3' + KIR3DS1_R8: 5'-GAACAGCACGTGTCTAAG-3' could amplify *KIR3DS1* exclusively from the heterozygous samples. F1+R1 is responsible for amplifying the locus but is not specific for either of the two genes; however F1+R2 is specific for *KIR3DL1*.

A panel of 31 sequencing primers was then formed into a recipe which could cover the full length of *KIR3DL1*. In this study, most of the amplifying and sequencing primers were designed by Dr Louis-Marie Yindom. The list of sequencing primers could be seen in **Table 4-1**. The binding position of each primer was shown in **Figure 4-8**.

Table 4-1: The list of primers for sequencing the full length genomic DNA of *KIR3DL1*

primer code	Primer sequences
KIR3DL1S1_F1	5'- AGT GAG AGC AAT TTC CAG G-3’
PN2	5'-ACT CCC TCC CTC GAT TCC C-3’
PN4	5'-CGA CAG GAC TTC CCT CCC A-3’
LYS15	5'- GAT CTC CAC TTC ATG CCC-3’
PN3	5'-GGC CTG GCT GCC AAG ACG-3’
PN5	5'-CTT CTG GGC ACT GGG AGT-3’
PN6	5'-ACA GTG AGA AGC CCA GAC R-3’
LYS2	5'- TCC CCA ATG ATG GCT ACA TTG-3’
LYS16	5'- TCC CAC TAA TCT CTT TCC TG-3’
PN7	5'-AGG AGA GAG ACA GAC ACG-3’
PN8	5'-TGT CCC AGT GAC AAT GAG AAC-3’
LYS3	5'- CAC CTA CAG ATG CCG TGT T-3’
PN9	5'-AAA GGT AGA AGG AGG AAA CAG AT-3’
PN10	5'-GGA AGC TCC TTA GCT AAG GAT T-3’
LYS17	5'- AGT GCT TCT CCT GCC TCA-3’
LYS4	5'- GGA GAT CAG AGG TTC CCT CAG-3’
LYS13	5'- AGT GAG CTG AGA TCA CAC-3’
LYS5	5'- AGA GTT TCC CTC CTT AG-3’
LYS14	5'- TCT CTG ATG ATG AGT GAT G-3’
LYS19	5'- GCT CTG TCA TGC AGG CT-3’
LYS7	5'- AGT GGA GGT TGC ATT GAG-3’
LYS8	5'- GAG AAC AGA GCT CAT GCA-3’
LYS9	5'- TCC AGG CTA SAG TGC AGT GTC-3’
LYS10	5'- CTG GTG AAA TGT GGT GCT GAT-3’
LYS11	5'- TTC TGT GAG CAT GAG ATC-3’
LYS12	5'- GAT AAG CAG CGA GTG ACA-3’
PN14	5'-CAC CTA CCT CGC TGT TGG-3’
PN11	5'-CTT GTC CGA AAG AGA TGC TGT AA-3’
PN12	5'-AAG CAA GAG AGA GGC ACC A-3’
PN13	5'-TGC AGG GAA CAG AAC AGC C-3’
LYS20	5'-CAT GTT TCA TAG GTT CA-3’

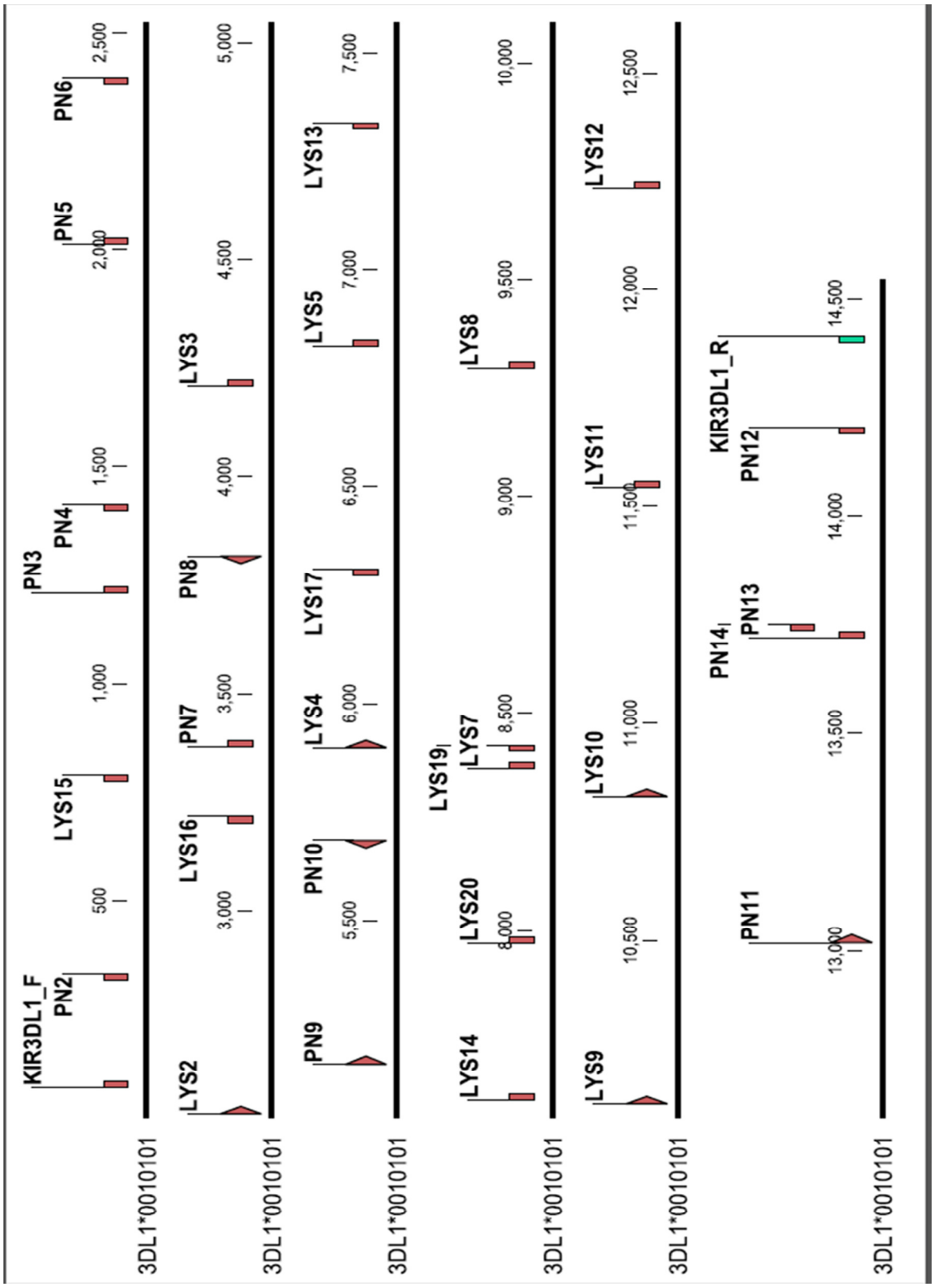


Figure 4-8: *KIR3DL1*0010101* is taken as the reference to show the binding positions of all the sequencing primers on the genomic DNA of *KIR3DL1*.

4. 2. 4 Another sequencing method (exons only)

The first step is to amplify all the exons, and then each of the exons was used as a template to perform the subsequent sequencing. Here, the PCR primers were also used as sequencing primers. PCR amplicons were purified by ExoSAP-IT (Affymetrix), directly sequenced in both directions using BigDye Terminator v3.1 then purified by Sephadex filtration, and analysed using an ABI 3730XL automated sequencer. The list of the primers is seen below (**Table 4-2**). Most of the primers were designed by Dr Paul J Norman.

Table 4-2: Primers list for amplification of the exons.

Primer code	target	Primer sequence	direction
PN1	exon 1	5'-GAGTTTAAATCATTTGAACTGGTTCTG-3'	F
PN2	exon 1	5'-ACTCCCTCCCTCGATTCCC-3'	R
PN3	exon 2	5'-GGCCTGGCTGCCAAGACG-3'	F
PN4	exon 2	5'-CGACAGGACTTCCCTCCCA-3'	R
PN5	exon 3	5'-CTTCTGGGCACTGGGAGT-3'	F
PN6	exon 3	5'-ACAGTGAGAAGCCCAGACR-3'	R
PN7	exon 4	5'-AGGAGAGAGACAGACACG-3'	F
PN8	exon 4	5'-TGTCCCAGTGACAATGAGAAC- 3'	R
PN9	exon 5	5'-AAAGGTAGAAGGAGGAAACAGAT-3'	F
PN10	exon 5	5'-GGAAGCTCCTTAGCTAAGGATT-3'	R
PN11	exon 7-9(L1)	5'-CTTGTCCGAAAGAGATGCTGTAA-3'	F
PN12	exon 7-9(L1)	5'-AAGCAAGAGAGAGGCACCA- 3'	R
PN13	exon 7-9(S1) shorter alternative	5'-TGCAGGGAACAGAACAGCC-3'	F
PN14	exon 7-9(S1) shorter alternative	5'-CACCTACCTCGCTGTTGG-3'	R
PN15	exon 7-9(S1)	5'- GCTTGTCCTAAAGAGACGC- 3'	F
PN16	exon 7-9(S1)	5'- GTAAGCAAGTGAGAGGCACA- 3'	R
LYS7	exon 6	5'- AGTGGAGGTTGCATTGAG - 3'	F

All PCR reactions were amplified with the S-96 Satellite Gradient Thermal Cycler from Quanta Biotech in a 20ul volume reaction mixture containing 2.6ul of 10X PCR buffer, 0.6ul of 50mM MgCl2, 0.2ul of 25mM dNTP, 0.4ul of 10uM forward and reverse primer respectively, 0.1ul of Platinum Taq polymerase, and 150ng template DNA. The PCR conditions were as follows: the initial DNA denaturation was performed with one cycle of 94°C for 2 min. The PCR amplification included 10 cycles of 94°C for 10s, 65°C for 1min and 72°C for 30s, followed by 20 cycles of 94°C for 10s, 61°C for 50s and 72°C for 30s.

4. 2. 5 Analyses

Chromatographs were aligned from each individual separately for manual inspection. All the sequencing tracing data were cleaned and verified, then assembled together. Locus-specific *KIR3DL1* and *KIR3DS1* libraries were created prior to analysis of the sequencing data. We went to the IPD-KIR database and the sequences of *KIR3DL1* and *KIR3DS1* in FASTA format were downloaded from FTP directory: ftp://ftp.ebi.ac.uk/pub/databases/ipd/kir/. Gen.fasta represents full-length sequences of the gene, and nuc.fasta represents coding sequences of the gene. Currently in the database there are 19 full-length sequences and 70 CDS sequences for *KIR3DL1*; and 5 full-length sequences and 16 CDS sequences for *KIR3DS1* (See **Appendix 1, 2, 3 and 4**). All of the downloaded sequences were imported into the software CLC Main workbench 6.7.1 and aligned to form the corresponding libraries. The sequences obtained from this study were then compared to the corresponding reference sequence to identify the subtypes and demonstrate the polymorphisms. Thus the sequencing results were interpreted and alleles were assigned. The software "CLC Main Workbench 6" (www.clcbio.com) was used to design the primers and manipulate all the trace sequences. Of course, the library should be updated with the latest versions of the IPD-KIR database as required.

4. 3 Results

4. 3. 1 The *KIR3DL1* alleles

4. 3. 1. 1 Exon sequencing results for *KIR3DL1*

Seventy-three samples from the SM patient cohort and the healthy controls were studied. All of them were Han Chinese. Previous PCR-SSP revealed that all of them were positive for *KIR3DL1* and 54 (74%) samples were positive for *KIR3DS1*. Ninety-two *KIR3DL1* alleles were identified and six types of *KIR3DL1* alleles were characterised in this Han Chinese population. The frequency of each allele was calculated and listed in **Table 4-3**. *KIR3DL1*01502* was the most common allele with a frequency of 71%, and *KIR3DL1*00501* was the second most common allele with a frequency of 12%.

Table 4-3: The frequency of *KIR3DL1* alleles in the selected samples

Allele	Number	Frequency (%)
3DL1*01502	65	71
3DL1*00501	11	12
3DL1*00701	10	11
3DL1*00101	3	3
3DL1*020	2	2
3DL1 New	1	1
total	92	100

12 genotypes of *KIR3DL1* have been identified. The frequencies of the genotypes are listed below in **Table 4-4**. *KIR3DL1*1502/----* is the most common genotype.

Table 4-4: The frequency of *KIR3DL1* genotypes in the selected samples

Allele 1	Allele 2	number	genotype frequency
KIR3DL1*01502	**----**	38	52.05%
KIR3DL1*01502	***KIR3DL1*01502***	11	15.07%
KIR3DL1*00701	**----**	7	9.59%
KIR3DL1*00501	**----**	5	6.85%
KIR3DL1*00501	***KIR3DL1*00501***	2	2.74%
KIR3DL1*00501	***KIR3DL1*01502***	2	2.74%
KIR3DL1*00701	***KIR3DL1*01502***	2	2.74%
KIR3DL1*00101	**----**	2	2.74%
KIR3DL1*00101	***KIR3DL1*00701***	1	1.37%
KIR3DL1*01502	***KIR3DL1*020***	1	1.37%
KIR3DL1*020	**----**	1	1.37%
KIR3DL1New	**----**	1	1.37%

---- indicates *KIR3DS1*.

4. 3. 1. 2 Full-length sequencing results of *KIR3DL1*

39 full genomic length sequences of *KIR3DL1* alleles were obtained from 35 samples. The sequence segments of each allele were assembled together and aligned to their most similar alleles in the IPD-KIR database (Robinson, Mistry et al. 2010) to show the polymorphisms. All of these full-length *KIR3DL1* alleles were shown to be novel, which means, compared to their most similar alleles in the database, each of them has at least one mutation. Most of the mutations are in the introns, and only one sample has mutations in exons.

The mutation patterns can be summarised into 14 types. The nucleotide sequence data of each new allele has been submitted to GenBank and the IPD-KIR database. Each of them has been

assigned an accession number and submission ID. Before submission to the database, each of the new alleles has been officially assigned a name by the World Health Organisation (WHO) Nomenclature Committee in September 2013.

There are 3 types of *3DL1*0050101*-like novel alleles, 2 types of *3DL1*0070101*-like novel alleles and 9 types of *3DL1*0150201*-like novel alleles identified in this study. There is also one new *KIR3DL1* gene isolated which has non-synonymous mutations within exons and was given a new name as *KIR3DL1*077*. The new names and the corresponding IWS submission IDs can be seen in **Table 4-5**.

Table 4-5: New names for these new *KIR3DL1* alleles and their IWS submission ID

New alleles	Names assigned by WHO	IWS submission ID
3DL1*00501N-01	*3DL1*0050103*	IWS40001826
3DL1*00501N-02	*3DL1*0050104*	IWS40001839
3DL1*00501N-03	*3DL1*0050105*	IWS40001841
3DL1*00701N-01	*3DL1*0070103*	IWS40001835
3DL1*00701N-02	*3DL1*0070104*	IWS40001837
3DL1*01502N-02	*3DL1*0150207*	IWS40001849
3DL1*01502N-03	*3DL1*0150204*	IWS40001843
3DL1*01502N-04	*3DL1*0150206*	IWS40001847
3DL1*01502N-05	*3DL1*0150205*	IWS40001849
3DL1*01502N-06	*3DL1*0150208*	IWS40001851
3DL1*01502N-07	*3DL1*0150209*	IWS40001853
3DL1*01502N-08	*3DL1*0150210*	IWS40001855
3DL1*New	*3DL1*077*	IWS40001861

Currently there are only two *3DL1*00501* alleles in the full length *KIR3DL1* database: *3DL1*0050101* and *3DL1*0050102*. So the new alleles were assigned names starting from *3DL1*0050103*.

3DL1*0050103 differs from *KIR3DL1*0050101* by a single point mutation from C to T at nucleotide position 6709 (C>T) in intron 5. ***3DL1*0050104*** differs from *KIR3DL1*0050101* with two changes at nucleotide positions 6709 (C>T) and 11365 (A>G) in introns 5 and 6, respectively. ***3DL1*0050105*** is similar to that of *KIR3DL1*0050101* except for two changes at position 6709 (C>T) and 13398 (G>A) of introns 5 and 7, respectively. See **Table 4-6**.

Table 4-6: The mutation positions of the three novel *KIR3DL1*0050101*-like alleles

Position	**6709**	**11365**	**13398**	**Number**
3DL1*0050101	**C**	**A**	**G**	
3DL1*00501N-01	T	-	-	2
3DL1*00501N-02	T	G	-	1
3DL1*00501N-03	T	-	A	1
Location	**Int 5**	**Int 6**	**Int 7**	

Number denotes the number of samples which contain the novel alleles. Int denotes Intron.

Currently in the full length *KIR3DL1* database there are only two *3DL1*00701* alleles: *3DL1*0070101* and *3DL1*0070102*. So the new alleles were assigned names starting from *3DL1*0070103*.

3DL1*0070103 differs from *KIR3DL1*0070101* by two point mutations: G to A at nucleotide position 11548 in intron 6 and T to G at position 13889 in the 3'UTR region. ***3DL1*0070104*** differs from *KIR3DL1*0070101* by a point mutation from T to G at position 13889 in the 3'UTR region of *KIR3DL1/S1* locus. See **Table 4-7**.

Table 4-7: The mutation positions of the two novel *KIR3DL1*0070101*-like alleles

Position	**11548**	**13889**	**Number**
3DL1*0070101	**G**	**T**	
3DL1*00701N-01	A	G	1
3DL1*00701N-02	-	G	2
Location	**Int 6**	**3' UTR**	

Number denotes the number of samples which contain the novel alleles. Int denotes Intron. UTR represents Untranslated Region.

Currently there have been reports of *3DL1*0150201*, *3DL1*0150202*, and *3DL1*0150203* in the full-length KIR3DL1 database. So the new alleles were assigned names starting from *3DL1*0150204*.

3DL1*0150204 has 4 point mutations: 3037 (G>A), 4115 (A>G), 6053 (G>C) and 8034 (A>G) compared to *KIR3DL1*0150201*. ***3DL1*0150205*** differs from *KIR3DL1*0150201* at five nucleotide positions: 1796 (G>A) in intron 2, 3037 (G>A) in intron 3, 4115 (A>G) in intron 4, 6053 (G>C) in intron 5, and 8034 (A>G) in intron 5. ***3DL1*0150206*** has 6 mutations: 3037 (G>A), 4115 (A>G), 6053 (G>C), 6751 (G>A), 8034 (A>G), 13489 (T>G) compared to *KIR3DL1*0150201*. ***3DL1*0150207*** differs from *KIR3DL1*0150201* at five nucleotide positions: 1796 (G>A) in intron 2, 3037 (G>A) in intron 3, 4115 (A>G) in intron 4, 6053 (G>C) in intron 5, and 8034 (A>G) in intron 5. ***3DL1*0150208*** possesses 5 SNPs at nucleotide positions 3037 (G>A), 4115 (A>G), 6053 (G>C), 8034 (A>G), and 9446 (T>C). ***3DL1*0150209*** is characterised with 7 SNPs at -163 (G>C), 3037 (G>A), 4115 (A>G), 6053 (G>C), 8034 (A>G), 10723 (C>A), and 10747 (C>G) compared to *KIR3DL1*0150201*. ***3DL1*0150210*** has 7 point mutations at 3037 (G>A), 4115 (A>G), 6053 (G>C), 8034 (A>G), 9446 (T>C), 10723 (C>A), 10747 (C>G).

The new *KIR3DL1* gene has been assigned a new name, *3DL1*077*, which has 2 non-synonymous mutations in exon 5 and six intronic changes. See **Table 4-8**.

Table 4-8: The mutation positions of the 7 novel *KIR3DL1*0150201*-like alleles and the new KIR gene: *KIR3DL1*077*

Position	-163	1289	1796	2910	3037	3356	4115	5110	5116	5144	6053	6751	N
3DL1*0150201	G	T	G	C	G	C	A	A	A	C	G	G	
3DL1*01502N-02	-	-	A	-	A	-	G	-	-	-	C	-	2
3DL1*01502N-03	-	-	-	-	A	-	G	-	-	-	C	-	20
3DL1*01502N-04	-	-	-	-	A	-	G	-	-	-	C	A	1
3DL1*01502N-05	-	-	-	-	A	-	G	-	-	-	C	-	1
3DL1*01502N-06	-	-	-	-	A	-	G	-	-	-	C	-	5
3DL1*01502N-07	C	-	-	-	A	-	G	-	-	-	C	-	1
3DL1*01502N-08	-	-	-	-	A	-	G	-	-	-	C	-	1
3DL1*077	-	-	-	-	A	-	G	C	T	-	C	-	1
Location	5' UTR	Int 2	Int 2	Int 3	Int 3	Ex 4	Int 4	Ex 5	Ex 5	Ex 5	Int 5	Int 5	32

To be continued with the table below

Position	7806	8034	8190	9446	9536	10723	10747	12624	12733	13489	N
3DL1*0150201	A	A	A	T	C	C	C	T	G	T	
3DL1*01502N-02	-	G	-	-	-	-	-	-	-	-	2
3DL1*01502N-03	-	G	-	-	-	-	-	-	-	-	20
3DL1*01502N-04	-	G	-	-	-	-	-	-	-	G	1
3DL1*01502N-05	-	G	C	-	-	-	-	-	-	-	1
3DL1*01502N-06	-	G	-	C	-	-	-	-	-	-	5
3DL1*01502N-07	-	G	-	-	-	A	G	-	-	-	1
3DL1*01502N-08	-	G	-	C	-	A	G	-	-	-	1
3DL1*077	-	G	-	-	-	A	G	-	-	-	1
Location	Int 5	Int 5	Int 5	Int 6	Int 6	Int 6	Int 6	Int 6	Int 6	Int 8	32

N denotes the number of samples which contain the novel alleles. Int represents Intron. Ex represents Exon. UTR represents Untranslated Region.

4. 3. 2 The *KIR3DS1* alleles

4. 3. 2. 1 Exon sequencing results for *KIR3DS1*

51 samples from the SM patient cohort and the healthy controls were used for the study. All of them were Han Chinese. Previous PCR-SSP revealed that all of them were positive for *KIR3DS1* and 40 (78%) samples were also positive for *KIR3DL1*. Nearly all of the *KIR3DS1*

alleles were *KIR3DS1*01301* (**Figure 4-9**), there was only one *KIR3DS1* allele which had a mutation in exon 5 and was different from *KIR3DS1*01301* (**Figure 4-10**).

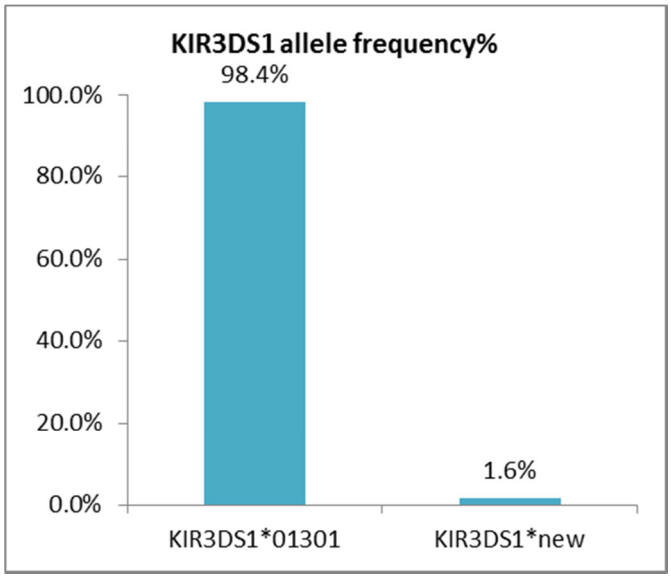


Figure 4-9: *KIR3DS1* allele frequencies in the tested samples.

KIR3DS1 new allele differs from *KIR3DS1*01301* by a point mutation from G to C at position 775 (CDS sequence) which leads to an amino acid change from Glycine to Arginine at position 259.

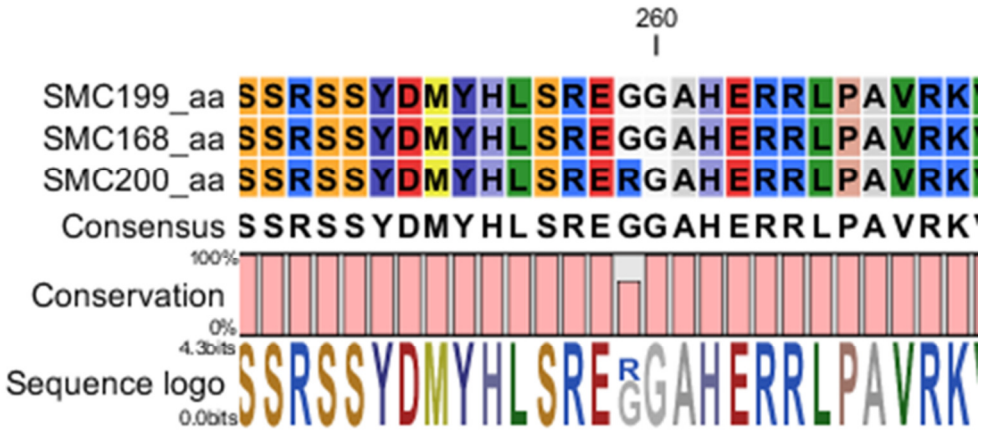


Figure 4-10: The amino acid sequence of the new *KIR3DS1* allele (SMC200), named *KIR3DS1*059* was aligned to *KIR3DS1*01301*. It was shown that at position 259, Glycine was substituted by Arginine.

4. 3. 2. 2 Full-length sequencing results of *KIR3DS1*

21 full genomic length sequences of *KIR3DS1* alleles were obtained from 35 samples. Eleven types of *KIR3DS1* alleles were identified. *KIR3DS1*0130101* was the most common allele (6/21), which accounted for 28% of all the alleles. *KIR3DS1*0130103* was the second most common allele (4/21), accounting for 19% of all the alleles. Similar to the analyses of *KIR3DL1* full-length sequences, there were also new *KIR3DS1* alleles identified. Each of the new alleles has been officially assigned a name by the WHO Nomenclature Committee in September 2013. Details can be seen in **Table 4-9**.

Table 4-9: New names for these new *KIR3DS1* alleles and their IWS submission ID

New alleles	Names assigned by WHO	IWS number
3DS1*01301N-01	*3DS1*0130104*	IWS40001828
3DS1*01301N-02	*3DS1*0130109*	IWS40001838
3DS1*01301N-03	*3DS1*0130105*	IWS40001830
3DS1*01301N-04	*3DS1*0130106*	IWS40001832
3DS1*01301N-05	*3DS1*0130110*	IWS40001840
3DS1*01301N-06	*3DS1*0130107*	IWS40001834
3DS1*01301N-07	*3DS1*0130111*	IWS40001842
3DS1*01301N-08	*3DS1*0130108*	IWS40001836
3DS1*new	*3DS1*059*	IWS40001844

All the new alleles are most similar to *KIR3DS1*0130101*; since there have been three K*IR3DS1*01301* alleles in the IPD-KIR database, the new names have to start from *KIR3DS1*0130104*.

3DS1*0130104 is identical to *KIR3DS1*0130101* except for changes at positions 1768 (C>T) and 12617 (C>A); ***3DS1*0130105*** differs from *KIR3DS1*0130101* with a single point mutation at position 6739 (G>T) of intron 5; ***3DS1*0130106*** is similar to *KIR3DS1*0130101* except for a SNP at 7211(G>T) in intron 5. ***3DS1*0130107*** differs from *KIR3DS1*0130101* at nucleotide position 8922 (A>G) in intron 5. ***3DS1*0130108*** has 3 changes at 12548 (C>G) of intron 6, 13804 (C>G) of intron 6, and 13819 (G>A) of intron 7. ***3DS1*0130109*** differs

from *KIR3DS1*0130101* at nucleotide position 5238 (A>G) of intron 4. ***3DS1*0130110*** is characterised with two nucleotide changes at 7322(G>T) in intron 5 and 12617 (C>A) in intron 6. ***3DS1*059*** is identical to *KIR3DS1*0130101* except for a non-synonymous mutation in exon 5 at position 5586 (G>C) and another change at the 3'UTR region 14255 (G>A). See **Table 4-10**.

Table 4-10: The mutation positions of these new *KIR3DS1* alleles. The sequence of *KIR3DS1*0130101* is demonstrated as the reference sequence.

Chromosomal positions	**1768**	**5238**	**5586**	**6739**	**7211**	**7322**	**8922**
3DS1*0130101	**C**	**A**	**G**	**G**	**A**	**G**	**A**
3DS1*01301N-01	T	-	-	-	-	-	-
3DS1*01301N-02	-	G	-	-	-	-	-
3DS1*01301N-03	-	-	-	T	-	-	-
3DS1*01301N-04	-	-	-	-	G	-	-
3DS1*01301N-05	-	-	-	-	-	T	-
3DS1*01301N-06	-	-	-	-	-	-	G
3DS1*01301N-07	-	-	-	-	-	-	-
3DS1*01301N-08	-	-	-	-	-	-	-
3DS1*new	-	-	C	-	-	-	-
Location	**Int 2**	**Int 4**	**Ex 5**	**Int 5**	**Int 5**	**Int 5**	**Int 5**

To be continued with the table below

Chromosomal positions	**10837**	**12548**	**12617**	**13804**	**13819**	**14066**	**14255**	**N**
3DS1*0130101	**C**	**C**	**C**	**C**	**G**	**C**	**G**	
3DS1*01301N-01	-	-	A	-	-	-	-	1
3DS1*01301N-02	-	-	-	-	-	-	-	1
3DS1*01301N-03	-	-	-	-	-	-	-	1
3DS1*01301N-04	-	-	-	-	-	-	-	1
3DS1*01301N-05	-	-	A	-	-	-	-	1
3DS1*01301N-06	-	-	-	-	-	-	-	2
3DS1*01301N-07	T	-	-	-	-	G	-	1
3DS1*01301N-08	-	G	-	G	A	-	-	2
3DS1*new	-	-	-	-	-	-	A	1
Location	**Int 6**	**Int 6**	**Int 6**	**Int 7**	**Int 7**	**3'UTR**	**3'UTR**	**11**

Below are some examples of the new alleles from the tested samples (**Figure 4-11/4-12/4-13/4-14**).

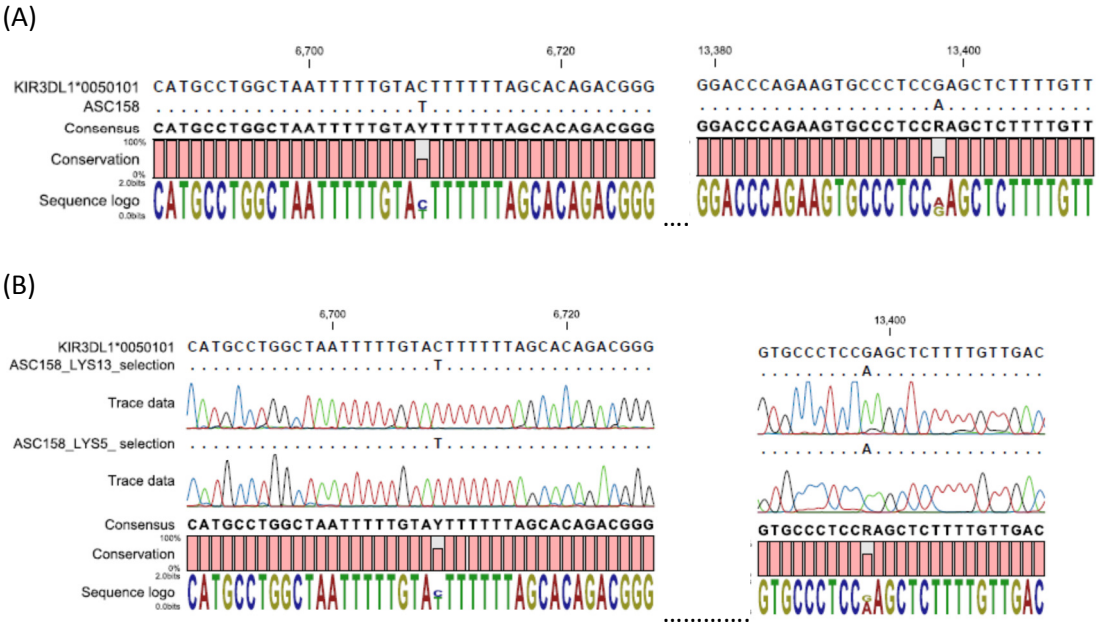
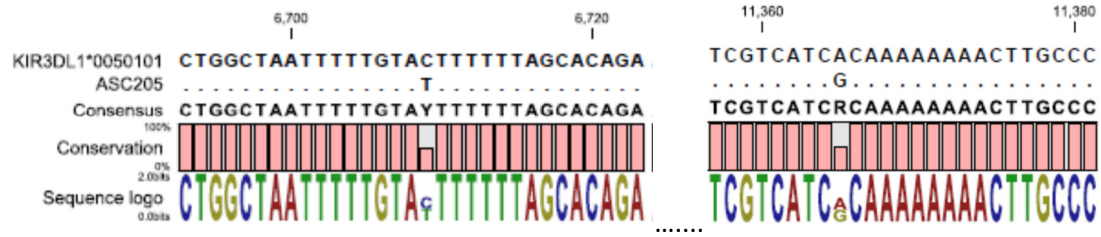


Figure 4-11: Partial alignment of *3DL1*0050105* allele showing the regions around the mutations. (A) Nucleotide alignment with conservation bars and consensus sequence; (B) Electrophorogram alignment showing the mutations at position 6709 from C to T and at position 13398 from G to A respectively.

(A)



(B)

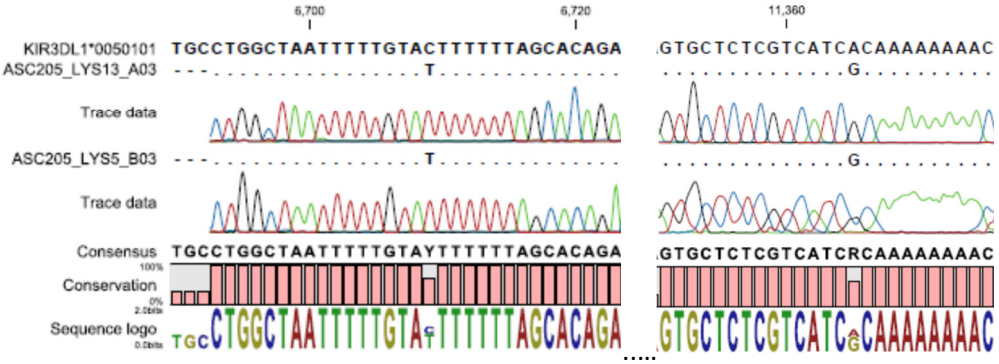
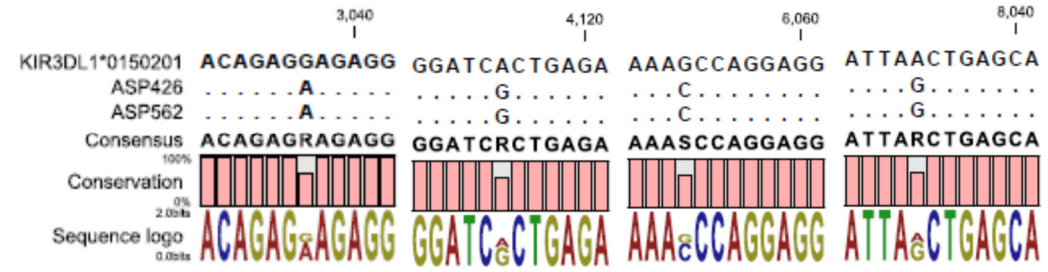


Figure 4-12: Partial alignment of *3DL1*0050104* allele showing the regions around the mutations. (A) Nucleotide alignment with conservation bars and consensus sequence; (B) Electrophorogram alignment showing the mutations at position 6709 from C to T and at position 11365 from A to G respectively.

(A)



(B)

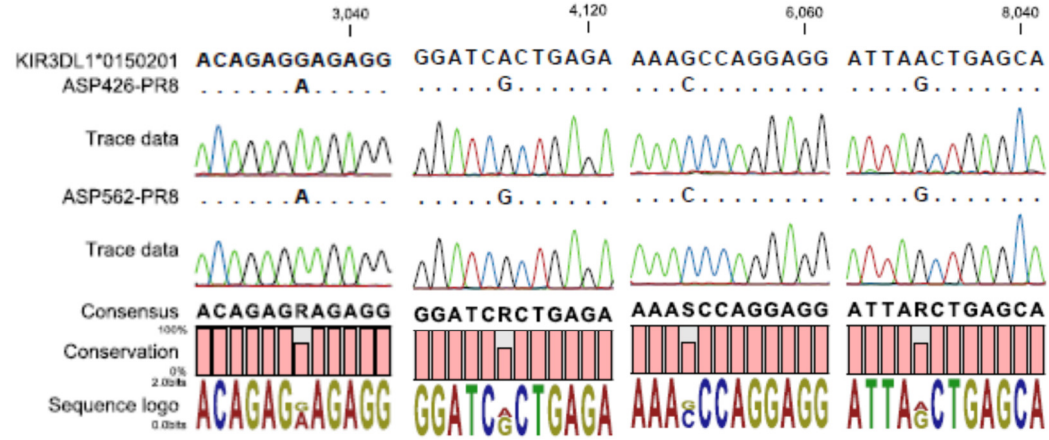
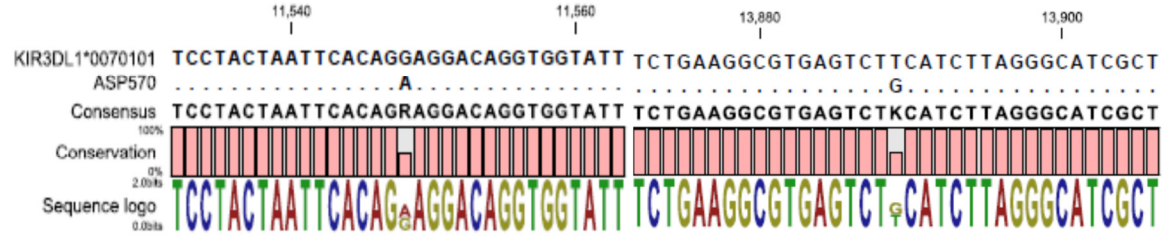


Figure 4-13: Partial alignment of *3DL1*0150204* allele showing the regions around the mutations. (A) Nucleotide alignment with conservation bars and consensus sequence; (B) Electrophorogram alignment showing the mutations at position 3037 from G to A; position 4115 from A to G; position 6053 from G to C and at position 8034 from A to G respectively.

(A)



(B)

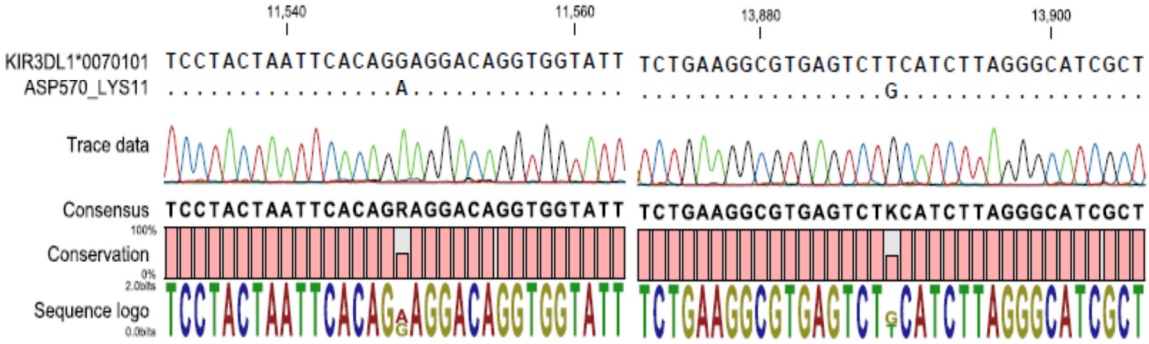


Figure 4-14: Partial alignment of *3DL1*0070103* allele showing the regions around the mutations. (A) Nucleotide alignment with conservation bars and consensus sequence; (B) Electrophorogram alignment showing the mutations at position 11548 from G to A and at position 13889 from T to G respectively.

4. 4 Discussion

Here, we reported a novel method to sequence the entire locus of *KIR3DL1/S1*. Two specific pairs of primers (F1+R2 and PN1+R8 in **Figure 4-7**) have been successfully designed and tested to be able to amplify *KIR3DL1* and *KIR3DS1* exclusively, not only from the homozygous samples but also from the heterozygous samples. Using this novel sequencing method, we have been able to show the polymorphism of this locus at a six-digit level. Overall, 12 new *KIR3DL1* alleles, 1 new *KIR3DL1* gene, 8 new *KIR3DS1* alleles and 1 new *KIR3DS1* gene have been identified in this study.

The first pair of primers (F1+R1) targeted the consensus area of both *KIR3DL1* and *KIR3DS1*, so they were able to amplify the locus no matter whether each of these two genes was present or absent. As can be seen from the gel picture (**Figure 4-4**), when we increased the time of electrophoresis, the two genes could be separated from each other. Of course, we could cut the gel and elute the band of DNA we needed, but the amount of work and exposure time under UV would have increased. Therefore, the one-step exclusive amplification of each KIR3D gene at the same *KIR3DL1/S1* locus via using specific primers becomes the perfect solution. This was the first challenge in this study and it was achieved successfully. The second challenge was to design a panel of sequencing primers, which could cover the full-length of *KIR3DL1* and *KIR3DS1*, especially for the long intron areas. Some of the PCR primers can also be used as sequencing primers, but some of them cannot. Because PCR is intrinsically an exponential process, and it is usually carried out well beyond completion, even rather poor primers will produce amplification in a PCR reaction. Sequencing, however, is strictly linear, and is much more unforgiving of poor primers. After several rounds of trial and error, finally a panel of reliable sequencing primers has been established for each KIR3D gene. Since this is a new method to sequence the entire locus of *KIR3DL1/S1*, it is not

surprising to find that most of the alleles we identified are regarded as new alleles because in the full-length genomic DAN database for *KIR3DL1* and *KIR3DS1*, the number of alleles is limited. Since this method was proven to be effective and highly-efficient, along with our on-going sequencing work, we believe that more and more new alleles will be identified and the database of the full-length alleles of both *KIR3DL1* and *KIR3DS1* will be greatly enlarged.

An investigation involving 28 populations (Norman, Abi-Rached et al. 2007) demonstrated that *KIR3DL1* is highly variable and has split into two distinctive lineages, identified by *KIR3DL1*005* and *KIR3DL1*015*, which later diversified into many variants. The most common allotype, KIR3DL1*015, accounts for 34% of all the observed 3DL1 allotypes. Compared to our observation, we find that in Henan Han population (both the SM patients and the healthy controls), *KIR3DL1*01502* is the predominant *KIR3DL1* subtype, which accounts for 71% of all the isolated alleles. *KIR3DL1*00501* is the second most common allele, which accounts for 12% of all the isolated alleles.

In the same study (Norman, Abi-Rached et al. 2007), *KIR3DS1*013* is demonstrated as dominant, which accounts for 97% of the *KIR3DS1* alleles isolated from all the populations; other *KIR3DS1* alleles are rare and restricted to a single population. The 6 additional KIR3DS1 allotypes differ from *KIR3DS1*013* by only single substitutions. In this sense, *KIR3DS1* is homogeneous. The same distribution of *KIR3DS1* alleles was observed in our study: *KIR3DS1*013* is dominant, accounting for 98% of all the *KIR3DS1* alleles we isolated. There is only one different *KIR3DS1* allele which has a mutation in exon 5 leading to a non-synonymous mutation at amino acid level, causing Glycine to be substituted by Arginine. This novel *KIR3DS1* was later assigned a name as *KIR3DS1*059*.

The profile of *KIR3DL1* and *KIR3DS1* have been investigated in a variety of populations, however the data on allelic diversity for the locus of *KIR3DL1/S1* are limited. The allele

frequencies of *KIR3DL1* in this study are similar to the findings in a recent report about Chinese Han population (Tao, He et al. 2013), in which *3DL1*01502* is the most common allele with a frequency of 63.67% and *3DL1*00501* also occupies the second common place with a frequency of 16.8%. In contrast, the frequencies of *3DL1*01502* are 29.50% and 45.70% in the Taiwan Han population and Japanese population, respectively (Yawata, Yawata et al. 2006; Wu, Zhao et al. 2009). The distribution of *KIR3DL1* in our study is also different from that of Caucasian and African-American populations (Trundley, Frebel et al. 2007; Jiang, Hou et al. 2010).

Putting these data together, we can see that *KIR3DL1/S1* consists of 3 lineages of alleles: *3DL1*005*-like, *3DL1*015*-like and *3DS1*, which are present in all human populations. Among them, *KIR3DL1* is highly variable and has greatly diversified during human evolution; however, *KIR3DS1* is practically invariant. Since it has been estimated that the three lineages of alleles became separated millions of years ago, they have been maintained by balancing selection; in this sense, *KIR3DL1*005, KIR3DL1*015* and *KIR3DS1*013* must have been beneficial for the survival and propagation of the human species.

So what are the differences among the three lineages? Obviously, the main difference is that *KIR3DS1*013* is an activating receptor. Then what is the difference between the two inhibitory allele groups, *KIR3DL1*015* and *KIR3DL1*005*? Firstly, *KIR3DL1*015* is expressed at a higher level on the cell surface than *KIR3DL1*005* (Gardiner, Guethlein et al. 2001; Yawata, Yawata et al. 2006); Secondly, they show differential reactivity with Bw4 allotypes and different associations with disease. For example, KIR3DL1*015-like allotypes combine more effectively with HLA-B*57 than with HLA-B*27 in determining disease progression in HIV, in contrast, KIR3DL1*005-like allotypes work better with HLA-B*27 than HLA-B*57 (Martin, Qi et al. 2007).

The new *KIR3DL1* gene has been assigned a new name, *3DL1*077*, which has 2 non-synonymous mutations in exon 5 and differs from *KIR3DL1*0150201* by one point mutation from A to C at position 5110 (CDS sequence) and another from A to T at position 5116, leading to an amino acid change from lysine to threonine at position 233, and another amino acid change from glutamine to leucine at position 235. Exon 5 encodes D2 domain for KIR3D molecules.

The first crystal structure of KIR3DL1, was determined in complex with HLA-B*5701 which provides a template to examine the impact of sequence and correlate this to peptide-HLA specificity. It was suggested that a 'hotspot' existed within the D1–D2 domains, consisting of loops 165–167, 199–201 and 278–282, all of which converged to form an comprehensive network that centred on Glu 282. Mutation within the three loops may alter the conformation of neighbouring residues within this hotspot region, consequently affecting receptor specificity (Vivian, Duncan et al. 2011). The two D2 domain variation within KIR3DL1*077 were at positions 233 and 235, which don't fall into this 'hotspot'. Whether these mutations affect the affinity of the interface between the D2 domain of KIR3DL1 and its ligand HLA-Bw4 molecules needs further investigation. Meanwhile it is noteworthy that the D2 domain and associated HLA molecules seem to have co-evolved to form a highly complementary binding interface.

Four positions that differ between KIR3DL1 and KIR3DS1 map to the KIR3DL1*001–pHLA-B*5701 interface, consequently affecting the interaction (Sharma, Bastard et al. 2009; O'Connor, Yamada et al. 2011). Whether the mutation from Glycine to Arginine existing in Domain 2 for KIR3DS1*059 alters the binding ability with Peptide-HLA Bw4 complex also needs further study.

The main purpose for this study was to develop a new method for sequencing the entire locus of *KIR3DL1/S1*, so in the beginning, we just selected samples randomly based on the *KIR3DL1* and *KIR3DS1* genotyping results and the DNA quality. This is why we analysed the data in terms of Henan Han population instead of comparing the frequencies of *KIR3DL1* subtypes between the SM patient cohort and the healthy controls. Comparison of *KIR3DS1* seems impossible because nearly all of the tested samples are *KIR3DS1*013*. The rationale for comparison of *KIR3DL1* is that different *KIR3DL1* alleles are expressed at different levels which could in turn determine different functions of KIR3DL1^{+} NK cells. If *KIR3DL1* alone, or together with its ligands, does play a role in determining the outcome of HIV-1 infection, those beneficial *KIR3DL1* alleles may be enriched in the "slow progressors" group compared with the healthy controls group. In the latter group, the frequencies of corresponding *KIR3DL1* alleles could be considered as a baseline level. Another issue is that the clinical relevance of *KIR3DL1/S1* 6-digit polymorphism has yet to be defined. If such kind of sequence based subtyping for this locus is shown to be important in clinical settings, then improved typing methods and analysis may warrant further investigation.

Taken together, the work we have done in this study has offered us the opportunity to identify the extent of polymorphism for this *KIR3DL1/S1* locus at both 4-digit and 6-digit levels. Especially the latter one, which represents a novel method to sequence the locus, will enable us to develop profound insights into these two similar genes.

Chapter 5 The role of KIRs in acute HIV-1 infection

5. 1 Introduction

Early events during acute HIV-1 infection are crucial in determining the subsequent disease progression (McMichael, Borrow et al. 2010). Mounting evidence demonstrates that antiviral immune responses induced soon after HIV-1 infection, prior to the induction of adaptive immune responses, are crucial to the early control of viral infection (Alter, Teigen et al. 2007; Fellay, Shianna et al. 2007).

Among the early effector cells, NK cells represent a unique set of cells that do not express specific antigen receptors but are regulated by the cluster of signals induced from their inhibitory and activating receptors (Fauci, Mavilio et al. 2005; Alter and Altfeld 2009). It has been shown that NK cell numbers are elevated during acute HIV-1 infection, with an expansion of $CD56^{dim}CD16^{pos}$ NK cells and an early depletion of $CD56^{bright}CD16^{neg}$ NK cells (Alter, Teigen et al. 2005). $CD56^{dim}$ NK cells produce low levels of cytokines (Cooper, Fehniger et al. 2001) but are potent mediators of ADCC, LAK activity and natural cytotoxicity. The $CD56^{dim}$ NK-cell subset has high-level expression of KIRs whereas $CD56^{bright}$ NK cells have high expression of CD94/NKG2A (Voss, Daley et al. 1998; André, Spertini et al. 2000). It was also shown that during acute infection, a NK cell population expressing *KIR3DL1* and/or *KIR3DS1* expanded in individuals simultaneously expressing *HLA-Bw4 80IIe* (Alter, Rihn et al. 2009). Several studies have indicated the involvement of NK cells in the control of HIV-1 (Martin, Gao et al. 2002; Qi, Martin et al. 2006; Martin, Qi et al. 2007). However, the timing of the antiviral effects of NK cells remains uncertain.

From previous GWAS in HIV-1 infected subjects (Fellay, Shianna et al. 2007; Pereyra, Jia et al. 2010) , we know that one SNP: -35C upstream of the *HLA-C* gene, was associated with protection and was also associated with increased *HLA-C* expression on the surface of T cells

(Thomas, Apps et al. 2009). Intriguingly, the protective effect of this SNP could not be assigned to a specific *HLA-C* allele or phylogenetically related subgroup (Thomas, Apps et al. 2009), implicating a potential NK cell rather than CD8-dependent mechanism. Since *HLA-C* alleles serve as ligands for KIR2D receptors, there is a possibility that this protective effect is NK-cell-dependent through the interaction of HLA-C with its KIR ligands.

Here, taking advantage of a valuable acute HIV-1 infection cohort which was established prospectively in 2006 in a Chinese MSM (men who have sex with men) population, we combined the KIR genotyping results and clinical data to show the role of KIRs in the outcome of acute HIV-1 infection.

5. 2 Methods

5. 2. 1 Study population

This acute HIV-1 infection cohort was an open, ongoing, longitudinal cohort recruited from HIV-1 seronegative MSM, who were provided with risk-education counselling before being tested. The recruitment started from October 2006 at You'an hospital in Beijing. After recruitment, these HIV-negative men were monitored every 2 months for HIV-RNA and plasma HIV antibodies, as well as any suspicious symptoms of acute HIV-1 infection. It is important to realise that an agreed definition of acute HIV infection has not been universally accepted. The most common operational way to diagnose acute HIV-1 infection is based on the detection of HIV-1 replication in the absence of HIV antibodies (Keele, Giorgi et al. 2008). So in this cohort acute HIV-1 infection was defined as: HIV-RNA positive, meanwhile, HIV antibodies negative or indeterminate. After seroconversion, clinical and laboratory measurements (CD4 counts, viral load, blood routine tests etc.) were taken at week1, 2, 4, 8 and 12, and then every 3 months. None of the subjects were treated with antiretroviral therapy during acute HIV-1 infection. The study was approved by Beijing

You'an Hospital Research Ethics Committee, and written informed consent was obtained from each individual.

The roadmap for establishing the acute HIV-1 infection cohort can be seen below in **Figure 5-1**.

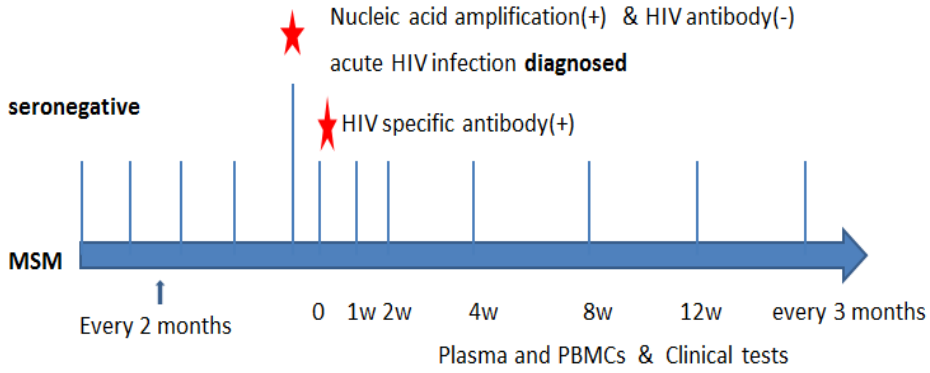
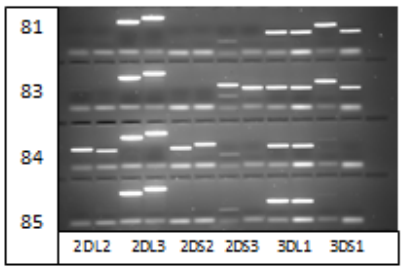


Figure 5-1: The roadmap for establishing the acute HIV-1 infection cohort.

Three pairs of KIR genes (*KIR2DL2/2DS2, KIR2DL3/2DS3*, and *KIR3DL1/3DS1*) were genotyped. We selected these genes based on their promising results in **Chapter 3**. In this cohort, we obtained 245 DNA samples, but some of them had poor DNA quality. We succeeded in genotyping 216 samples. An example of a schematic gel picture and result interpretation is shown in **Figure 5-2**.



5-2 a

sample	2DL2	2DL3	2DS2	2DS3	3DL1	3DS1
81	0	1	0	0	1	1
83	0	1	0	1	1	1
84	1	1	1	0	1	0
85	0	1	0	0	1	0

5-2 b

Figure 5-2: The schematic figure for 6 *KIR* gene typing. 5-2 a: each row represents one sample. Each *KIR* gene is amplified by two pairs of sequence-specific primers; presence of specific bands indicates that the sample is positive for the gene under investigation. If there are discrepancies between the two lanes for one *KIR* gene, this sample will be repeated. 5-2 b: Example of the result interpretation sheet.

5. 2. 2 Laboratory reagents

Standard HIV ELISA (Abbott recombinant HIV-1/2 third generation, Vironostika HIV Uni-Form II plus O; Abbott, Chicago, IL) and Western blot analysis (Genelabs, Redwood City, CA; HIV Blot 2.2, AE2029) were used for antibody tests. Plasma HIV-1 RNA copies were measured by a quantitative reverse transcriptase–polymerase chain reaction HIV-1 RNA test (Roche Cobas Amplicor HIV-1 monitor test). CD4 and CD8 T cell counts were done in a routine clinical way.

5. 3 Results

5. 3. 1 KIR genotyping results.

Table 5-1: The KIR typing results (n=216) in acute HIV-1 infection cohort

ID	2DL2	2DL3	2DS2	2DS3	3DL1	3DS1	ID	2DL2	2DL3	2DS2	2DS3	3DL1	3DS1	ID	2DL2	2DL3	2DS2	2DS3	3DL1	3DS1
YA-1	0	1	0	0	1	0	YA-185	1	1	1	1	1	1	YA-339	0	1	0	0	1	0
YA-4	0	1	0	0	1	0	YA-187	1	1	1	0	1	1	YA-340	0	1	0	0	1	0
YA-10	0	1	0	1	1	1	YA-191	0	1	0	0	0	1	YA-341	0	1	0	1	1	1
YA-11	0	1	0	0	1	0	YA-192	0	1	0	0	1	0	YA-342	1	1	1	0	1	0
YA-40	0	1	0	0	1	0	YA-193	0	1	0	0	1	0	YA-345	0	1	0	0	1	0
YA-42	0	1	0	1	1	1	YA-198	1	0	1	0	0	1	YA-347	1	1	1	1	1	1
YA-44	0	1	0	0	1	0	YA-200	1	1	1	0	1	1	YA-348	0	1	0	0	1	0
YA-45	1	1	1	0	1	1	YA-203	0	1	0	0	1	0	YA-349	0	1	0	0	1	0
YA-46	0	1	0	0	1	0	YA-207	0	1	0	1	1	1	YA-350	0	1	0	0	1	0
YA-48	0	1	0	1	1	1	YA-208	0	1	0	0	1	1	YA-352	1	1	1	0	1	0
YA-50	0	1	0	0	1	0	YA-210	0	1	0	0	1	0	YA-353	0	1	0	1	1	1
YA-51	0	1	0	0	1	0	YA-214	0	1	0	0	1	0	YA-355	0	1	0	0	1	0
YA-54	1	1	1	0	1	0	YA-216	0	1	0	0	1	0	YA-357	1	1	1	0	1	0
YA-55	0	1	0	0	1	1	YA-220	0	1	0	0	1	0	YA-358	1	1	1	0	1	1
YA-58	0	1	0	0	1	0	YA-221	0	1	0	0	1	0	YA-360	0	1	0	0	1	1
YA-60	1	0	1	0	1	0	YA-222	0	1	0	0	1	0	YA-361	0	1	0	0	1	1
YA-61	0	1	0	0	1	1	YA-223	0	1	0	0	1	0	YA-363	0	1	0	1	1	1
YA-63	0	1	0	0	1	0	YA-224	0	1	0	0	1	0	YA-365	1	1	1	0	1	0
YA-65	0	1	0	0	1	0	YA-228	0	1	0	1	1	1	YA-368	1	1	1	0	1	0
YA-67	0	1	0	1	1	1	YA-229	0	1	0	0	1	1	YA-369	1	1	1	1	1	1
YA-68	0	1	0	0	1	0	YA-233	0	1	0	0	1	1	YA-374	0	1	0	0	1	0
YA-70	1	1	1	0	1	0	YA-234	0	1	0	0	1	1	YA-377	0	1	0	1	1	1
YA-75	0	1	0	0	1	0	YA-237	1	1	1	0	0	1	YA-378	0	1	0	0	1	1
YA-78	0	1	0	0	1	1	YA-238	0	1	0	0	1	0	YA-379	1	1	1	0	1	0
YA-81	0	1	0	0	1	1	YA-241	0	1	0	0	1	0	YA-381	0	1	0	0	1	0
YA-82	0	1	0	0	1	0	YA-242	0	1	0	0	1	0	YA-382	0	1	0	0	1	0
YA-83	0	1	0	1	1	1	YA-247	0	1	0	0	0	1	YA-384	0	1	0	0	1	0
YA-84	1	1	1	0	1	0	YA-248	0	1	0	0	1	0	YA-386	0	1	0	1	1	1
YA-85	0	1	0	0	1	0	YA-249	0	1	0	0	1	0	YA-387	0	1	0	0	1	0
YA-86	0	1	0	1	1	1	YA-250	1	1	1	1	1	0	YA-388	0	1	0	0	1	1
YA-87	0	1	0	0	1	0	YA-251	0	1	0	0	1	1	YA-390	0	1	0	1	1	1
YA-88	1	1	1	0	1	1	YA-253	0	1	0	0	1	0	YA-391	1	1	1	0	1	0
YA-89	0	1	0	0	1	0	YA-254	0	1	0	0	1	1	YA-392	0	1	0	0	1	0
YA-90	0	1	0	0	1	0	YA-257	0	1	0	0	1	0	YA-393	0	1	0	0	1	0
YA-91	0	1	0	0	1	1	YA-258	0	1	0	0	1	0	YA-394	0	1	0	0	1	0
YA-92	0	1	0	0	1	0	YA-260	0	1	0	0	1	0	YA-395	1	1	1	0	1	1
YA-93	0	1	0	0	1	1	YA-262	0	1	0	0	1	0	YA-396	0	1	0	0	1	0
YA-94	0	1	0	0	1	0	YA-266	0	1	0	0	1	0	YA-398	0	1	0	0	1	0
YA-96	1	1	1	1	0	1	YA-268	0	1	0	0	1	0	YA-399	0	1	0	0	1	1
YA-97	0	1	0	0	1	0	YA-269	0	1	0	0	1	1	YA-400	0	1	0	0	1	0
YA-98	1	1	1	0	1	1	YA-271	0	1	0	0	1	0	YA-401	0	1	0	0	1	1
YA-99	0	1	0	0	1	0	YA-275	0	1	0	0	1	0	YA-403	1	1	1	1	1	0
YA-100	0	1	0	0	1	1	YA-278	0	1	0	0	1	0	YA-405	0	1	0	0	1	0
YA-105	0	1	0	0	1	0	YA-279	0	1	0	0	1	1	YA-406	0	1	0	0	1	0
YA-107	0	1	0	0	1	0	YA-280	0	1	0	0	1	0	YA-407	0	1	0	0	1	0
YA-112	0	1	0	0	1	1	YA-281	1	1	1	1	1	1	YA-408	0	1	0	0	1	1
YA-117	0	1	0	0	1	1	YA-282	0	1	0	1	1	1	YA-409	0	1	0	0	1	0
YA-118	0	1	0	0	1	1	YA-284	1	1	1	1	1	0	YA-410	0	1	0	0	1	1
YA-119	0	1	0	0	1	1	YA-285	0	1	0	0	1	0	YA-411	0	1	0	0	1	1
YA-126	0	1	0	0	1	0	YA-289	0	1	0	0	1	1	YA-412	0	1	0	0	1	1
YA-127	0	1	0	0	1	0	YA-291	0	1	0	0	1	0	YA-414	0	1	0	0	1	0
YA-129	0	1	0	0	1	1	YA-292	0	1	0	0	1	0	YA-415	0	1	0	0	1	0
YA-139	0	1	0	0	1	0	YA-294	0	1	0	1	1	1	YA-417	0	1	0	0	1	0
YA-141	0	1	0	0	1	1	YA-295	0	1	0	0	1	0	YA-418	0	1	0	0	1	1
YA-142	0	1	0	0	0	1	YA-296	0	1	0	0	1	1	YA-420	0	1	0	0	1	0
YA-147	1	1	1	0	1	1	YA-299	0	1	0	0	1	1	YA-421	0	1	0	0	1	0
YA-149	0	1	0	0	1	1	YA-302	0	1	0	0	1	1	YA-422	0	1	0	1	1	1
YA-155	0	1	0	0	1	0	YA-303	1	1	1	1	1	1	YA-423	0	1	0	0	1	0
YA-158	0	1	0	0	1	0	YA-304	0	1	0	0	1	0	YA-424	0	1	0	0	1	1
YA-159	0	1	0	0	1	0	YA-306	1	1	1	1	0	1	YA-425	0	1	0	0	1	1
YA-161	0	1	0	0	1	0	YA-308	0	1	0	0	1	0	YA-426	1	1	1	0	1	0
YA-162	0	1	0	0	1	0	YA-309	0	1	0	0	1	0	YA-427	0	1	0	0	1	0
YA-163	0	1	0	0	1	0	YA-312	0	1	0	0	1	0	YA-428	0	1	0	0	1	1
YA-165	0	1	0	0	1	0	YA-317	0	1	0	1	1	1	YA-430	0	1	0	0	1	0
YA-166	0	1	0	0	1	1	YA-319	1	0	1	1	1	0	YA-431	0	1	0	0	0	1
YA-169	0	1	0	0	1	0	YA-321	0	1	0	0	1	0	YA-432	0	1	0	0	1	0
YA-172	1	1	1	0	1	0	YA-324	0	1	0	0	1	0	YA-433	0	1	0	0	1	1
YA-174	0	1	0	0	1	0	YA-325	0	1	0	0	1	1	YA-434	0	1	0	0	1	0
YA-176	0	1	0	0	1	0	YA-327	0	1	0	0	1	0	YA-435	1	1	1	1	1	1
YA-179	0	1	0	0	1	0	YA-328	0	1	0	0	1	1	YA-439	1	1	1	1	1	1
YA-183	0	1	0	0	1	1	YA-332	0	1	0	0	1	0	YA-442	0	1	0	0	1	1
YA-184	1	1	1	0	1	0	YA-336	0	1	0	0	1	0	YA-444	0	1	0	0	1	1

1 indicates presence; 0 indicates absence.

5. 3. 2 General information for this study population

5. 3. 2. 1 All 216 patients were men. The median age is 30 (youngest 18, oldest 66), 95% of the individuals were from the Chinese Han population. The characteristics of the study population were shown in **Table 5-2**.

Table 5-2: Characteristics of the study population

	Number of subjects	Percentage (%)	Median age (IQR)
Gender			
Male	216	100%	
Age	203		30(25-38)
Ethnic group	199		
Chinese Han	188	94.50%	
Manchu	6	3.00%	
Mongolian	1	0.50%	
Korean	1	0.50%	
Zhuang	1	0.50%	
Hui	1	0.50%	
Miao	1	0.50%	

IQR: interquartile range.

5. 3. 2. 2 Only 81 patients in this study population had the results of HIV-1 subtypes.

Table 5-3: Subtypes of HIV-1 in acute HIV-1 infection cohort

Subtypes	Number of individuals	Percentage (%)
CRF01_AE	41	50.60%
B	27	33.30%
B/C	9	11.10%
C	2	2.50%
CRF01_AE/B	1	1.20%
D/C	1	1.20%
Total	81	100%

5. 3. 3 The comparison of KIR gene frequencies within the 3 groups

Among the 216 patients, the frequencies of the 6 KIR genes were as following*:* *KIR2DL2* 17.1%, *KIR2DS*2 17.1%; KIR2DL3 98.6%, *KIR2DS3* 14.4%; *KIR3DL1* 96.3%, *KIR3DS1* 40.7%. They were compared with the corresponding *KIR* gene frequencies in the SM cohort (**Table 5-4**).

Table 5-4: KIR gene frequencies comparison in different study population

KIR gene frequencies (%)	**Healthy**	**HIV infected**		
	Control (n=252)	**Acute (n=216)**	**Slow progressors (n=261)**	P
KIR2DL2	21.8	17.1	22.6	0.294
KIR2DS2	20.6	17.1	21.5	0.467
KIR2DL3	99.2	98.6	95.8	0.019
KIR2DS3	17.9	14.4	13.8	0.393
KIR3DL1	94.1	96.3	97.3	0.165
KIR3DS1	38.1	40.7	31	0.070

Control: the healthy control individuals; Acute: individuals in the acute HIV-1 infection cohort; Slow progressors: the HIV-1 infected individuals in the SM cohort. Chi-Square tests were used and P<0.05 was considered significant.

The frequency of *KIR2DL3* was significantly different among the 3 groups (P=0.019), and the P value for *KIR3DS1* was close to 0.05; In order to further explore the differences, pair-wise comparisons were done for 2 of the *KIR* genes, and the results were represented graphically in **Figure 5-3**.

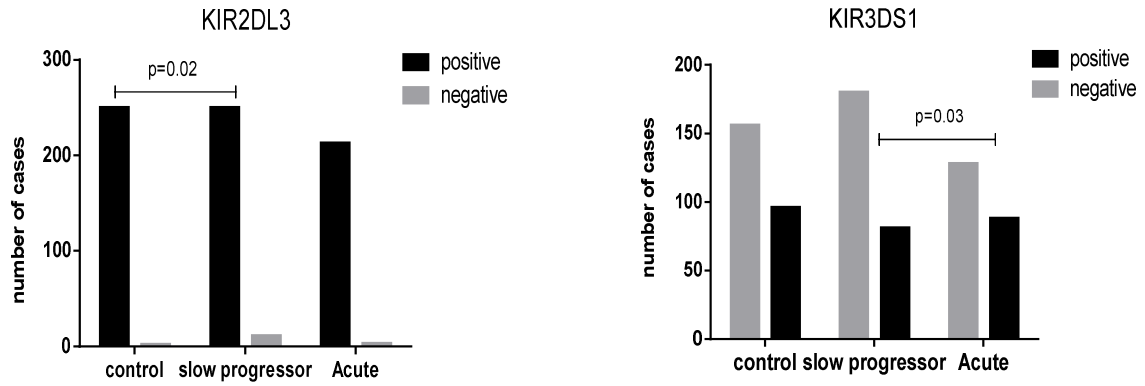


Figure 5-3: Pair-wise comparison for *KIR2DL3* and *KIR3DS1* among the 3 study groups.

From the pair-wise comparison above, we found that for *KIR2DL3* the significant difference was actually seen between the healthy controls and the slow progressors rather than among the 3 groups. As for *KIR3DS1*, interestingly we found that the frequency in slow progressors group (31%) was significantly lower than that in the acute infection group (40.7%) (P=0.03).

4. To some extent, the acute cohort could be considered as a combination of the future "rapid progressors" and "slow progressors". In order to show the potential role of *KIR* genes on HIV disease progression, we compared the 6 gene frequencies between acute group and slow progressors group: only *KIR3DS1* was shown significantly different between the two groups (**Table 5-5**).

Table 5-5: *KIR* gene comparison between acute HIV-1 infected patients and slow progressors in the SM cohort

Gene frequency	Acute (n=216)	Slow progressors(n=261)	P
KIR2DL2	17.1	22.6	0.17
KIR2DS2	17.1	21.5	0.25
KIR2DL3	98.6	95.8	0.10
KIR2DS3	14.4	13.8	0.89
KIR3DL1	96.3	97.3	0.60
KIR3DS1	40.7	31	0.03

All individuals in the SM cohort were Han Chinese, which 95% of patients in the acute HIV-1 cohort were Han Chinese, and 5% (11/216) were from other ethnic groups. The difference observed with *KIR3DS1* remained significant even when the 11 individuals from minority ethnic groups were excluded: acute cohort (n=205) 40.5% versus slow progressors (n=261) 31%, P=0.03.

5. 3. 4 The correlation of KIR with the clinical data including CD4 counts and viral load

We further explored the role of the *KIR* genes using acute CD4 loss and viral set point level to evaluate their potential impacts on disease progression and inhibition of viral replication.

For each patient, there were a series of longitudinal measurements of CD4 counts and HIV-RNA levels. We firstly cleaned up the clinical data in chronological order and tried to demonstrate the HIV-RNA curves to see if there was a relatively consistent pattern, which may help us define the set point. However the curves were quite variable among different patients. Some examples are shown below in **Figure 5-4**.

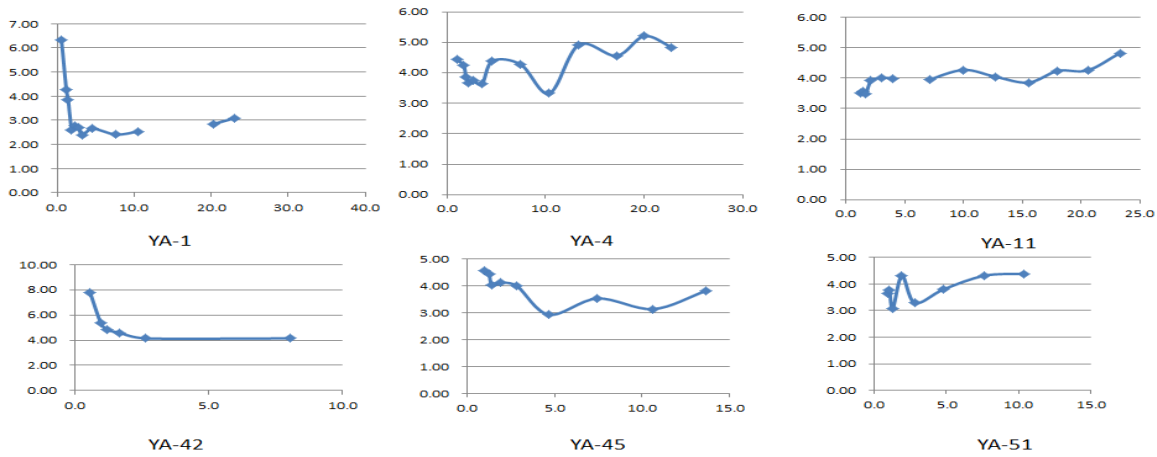


Figure 5-4: HIV-RNA curves in acute HIV-1 infection. The horizontal axis represents the weeks from the onset of HIV-1 infection; the vertical axis represents the log of the HIV-RNA level.

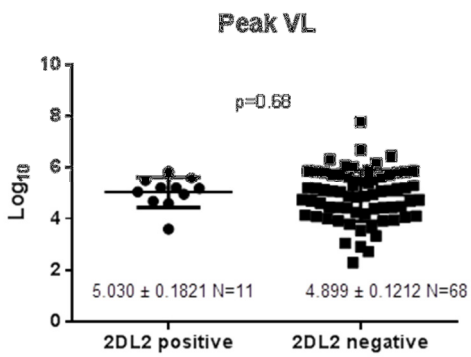
We can see that there were no consistent HIV-RNA change patterns to help us define an accurate setpoint for each patient. So in this study, based on the definitions used in the literatures (Mellors, Kingsley et al. 1995; Mellors, Rinaldo et al. 1996), we chose the HIV-RNA measurement value at 3 months and 6 months recorded as SP3 (set point at 3 months) and SP6(set point at 6 months) respectively to perform the analysis.

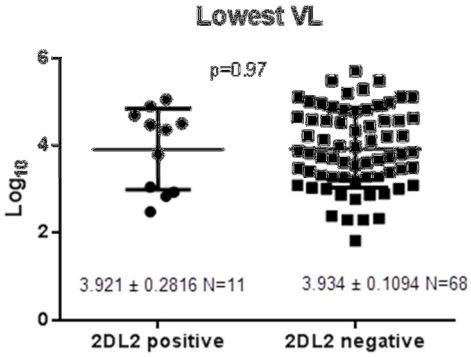
As for CD4 counts, we calculated the average CD4 counts in the first 3 months , in the second 3 months and in the first 6 months respectively to do the analysis.

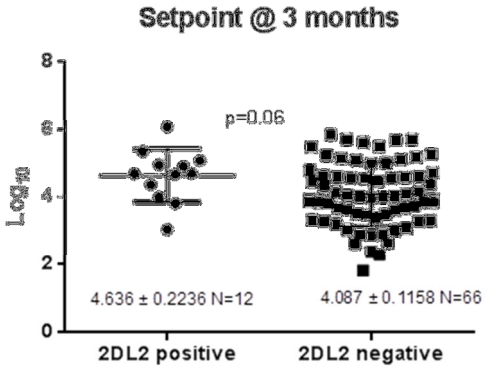
Patients were divided into different groups in terms of specific *KIR* gene positive or negative, thus the average CD4 counts and HIV-RNA levels at different time points were compared between the two groups to show the associations of *KIR* with different clinical outcomes following acute HIV-1 infection.

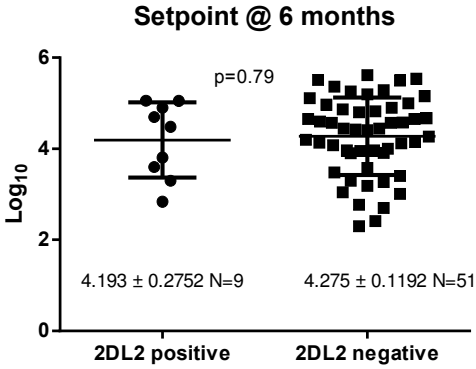
The multiple comparisons for each *KIR* gene were demonstrated below:

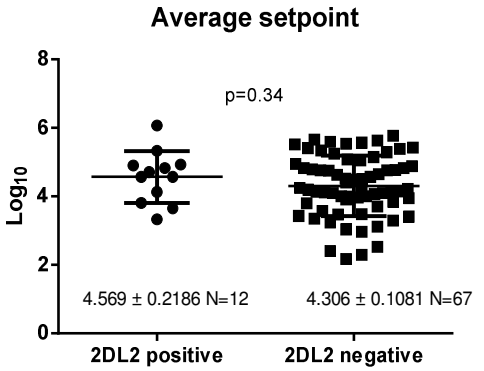
KIR2DL2

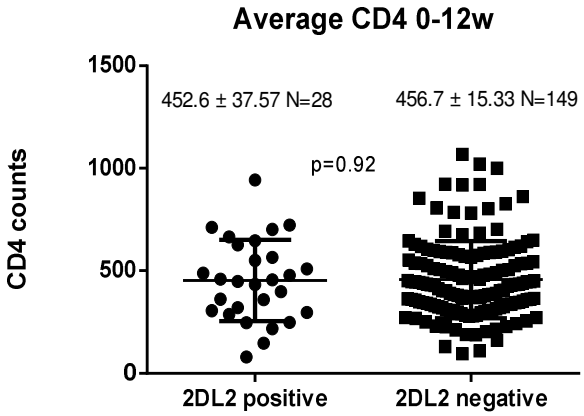

Peak VL
p=0.68
Log10
10
8
6
4
2
0
5.030 ± 0.1821 N=11
4.899 ± 0.1212 N=68
2DL2 positive
2DL2 negative

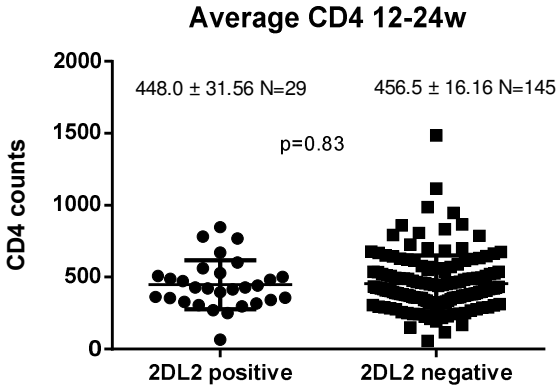

Lowest VL
p=0.97
Log10
6
4
2
0
3.921 ± 0.2816 N=11
3.934 ± 0.1094 N=68
2DL2 positive
2DL2 negative

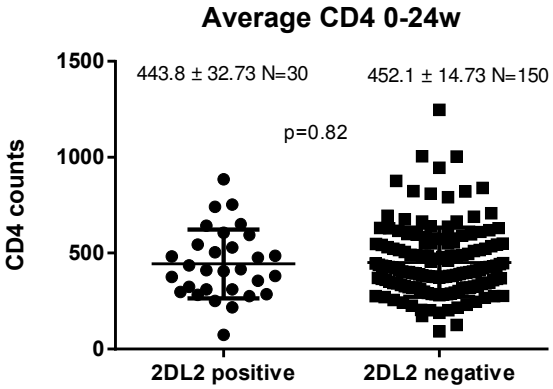

Setpoint @ 3 months
p=0.06
Log10
8
6
4
2
0
4.636 ± 0.2236 N=12
4.087 ± 0.1158 N=66
2DL2 positive
2DL2 negative


Setpoint @ 6 months
p=0.79
Log10
6
4
2
0
4.193 ± 0.2752 N=9
4.275 ± 0.1192 N=51
2DL2 positive
2DL2 negative


Average setpoint
p=0.34
Log10
8
6
4
2
0
4.569 ± 0.2186 N=12
4.306 ± 0.1081 N=67
2DL2 positive
2DL2 negative


Average CD4 0-12w
CD4 counts
1500
1000
500
0
452.6 ± 37.57 N=28
456.7 ± 15.33 N=149
p=0.92
2DL2 positive
2DL2 negative


Average CD4 12-24w
CD4 counts
2000
1500
1000
500
0
448.0 ± 31.56 N=29
456.5 ± 16.16 N=145
p=0.83
2DL2 positive
2DL2 negative


Average CD4 0-24w
CD4 counts
1500
1000
500
0
443.8 ± 32.73 N=30
452.1 ± 14.73 N=150
p=0.82
2DL2 positive
2DL2 negative

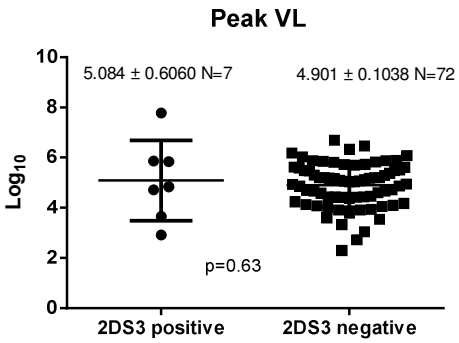
KIR2DL3

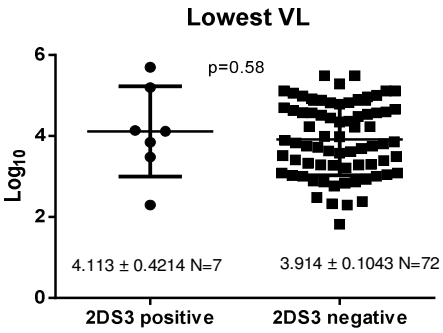
It was impossible to make any comparison because there were only 2 negative *KIR2DL3* individuals in the study population.

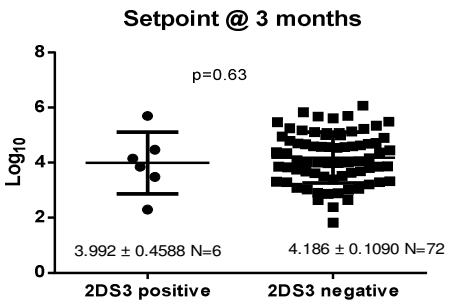
KIR2DS2

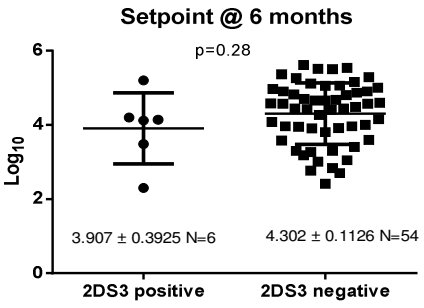
KIR2DS2 is in strong linkage disequilibrium with KIR2DL2 (Wn=0.976, p<0.001); in this cohort, all the *KIR2DS2* positive individuals were also *KIR2DL2* positive. Therefore the comparisons were exactly the same as those for *KIR2DL2*.

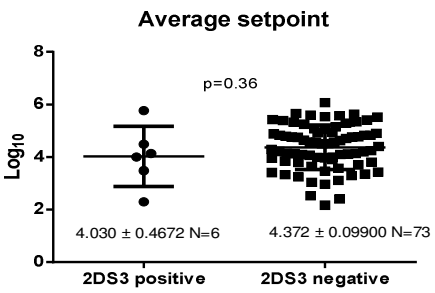
KIR2DS3

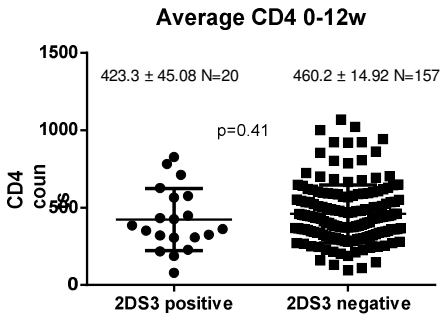


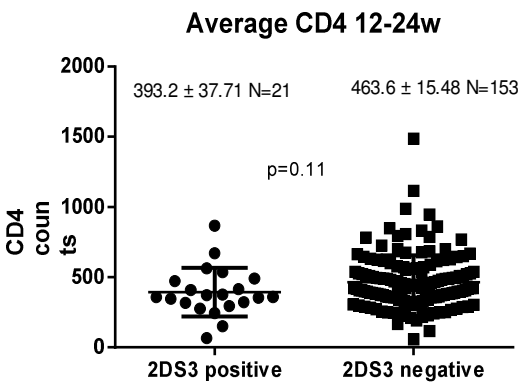


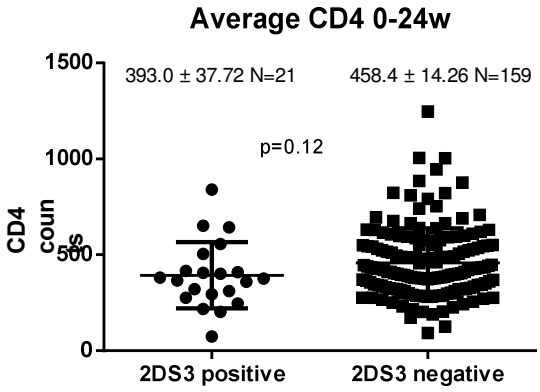






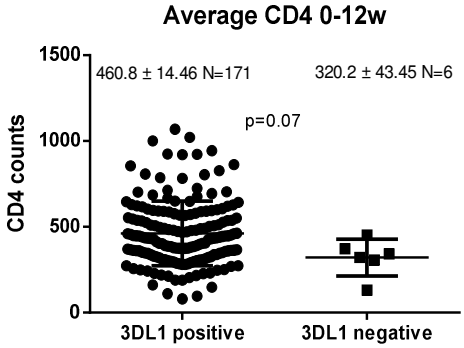


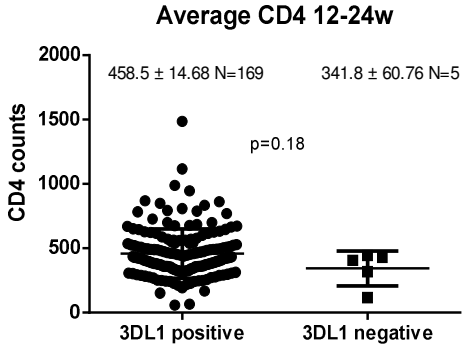


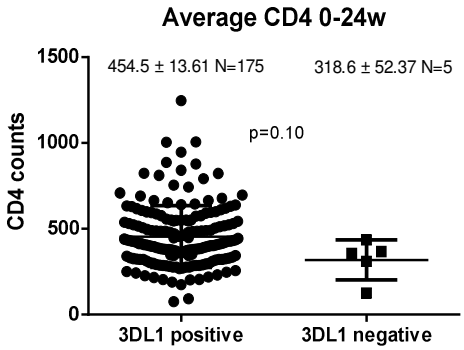


KIR3DL1

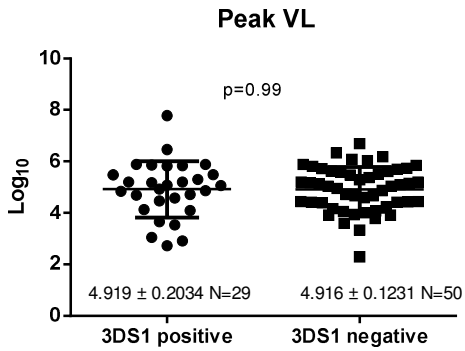
There were only 7 *KIR3DL1* negative individuals in the study population, and only 2 of them had HIV-RNA data. Therefore here only CD4 counts were compared.

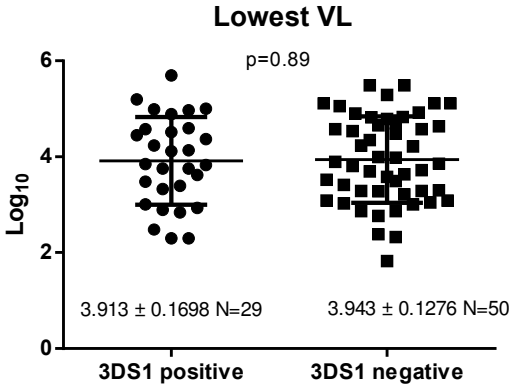


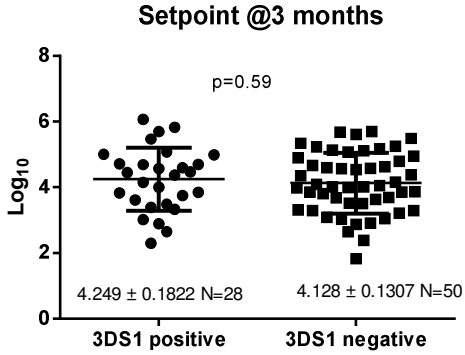


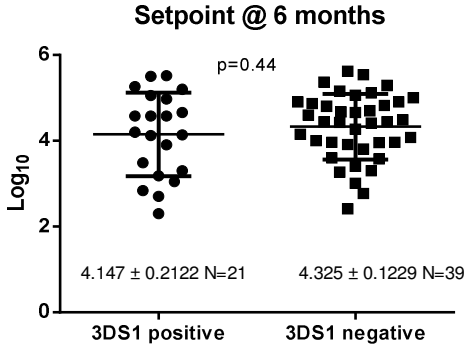


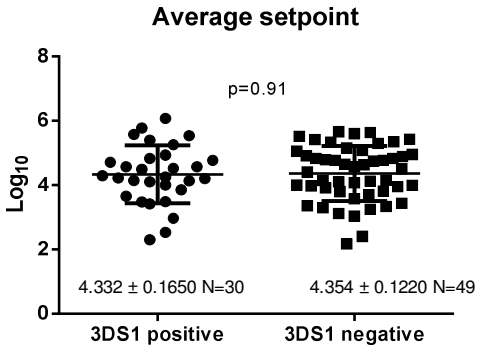
KIR3DS1

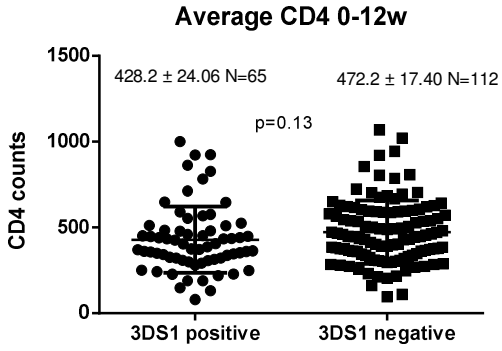

Peak VL
p=0.99
Log10
4.919 ± 0.2034 N=29
4.916 ± 0.1231 N=50
3DS1 positive
3DS1 negative

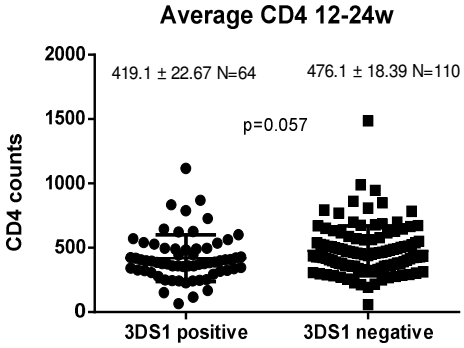

Lowest VL
p=0.89
Log10
3.913 ± 0.1698 N=29
3.943 ± 0.1276 N=50
3DS1 positive
3DS1 negative

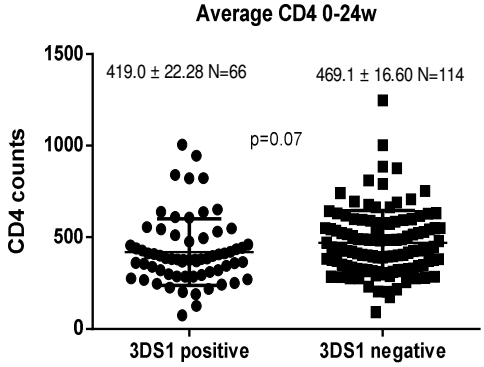

Setpoint @3 months
p=0.59
Log10
4.249 ± 0.1822 N=28
4.128 ± 0.1307 N=50
3DS1 positive
3DS1 negative


Setpoint @ 6 months
p=0.44
Log10
4.147 ± 0.2122 N=21
4.325 ± 0.1229 N=39
3DS1 positive
3DS1 negative


Average setpoint
p=0.91
Log10
4.332 ± 0.1650 N=30
4.354 ± 0.1220 N=49
3DS1 positive
3DS1 negative


Average CD4 0-12w
428.2 ± 24.06 N=65
472.2 ± 17.40 N=112
p=0.13
CD4 counts
3DS1 positive
3DS1 negative


Average CD4 12-24w
419.1 ± 22.67 N=64
476.1 ± 18.39 N=110
p=0.057
CD4 counts
3DS1 positive
3DS1 negative


Average CD4 0-24w
419.0 ± 22.28 N=66
469.1 ± 16.60 N=114
p=0.07
CD4 counts
3DS1 positive
3DS1 negative

All these comparison results can be summarised in the table below:

Table 5-6: The associations of each KIR with different clinical outcomes following acute HIV-1 infection

P value	Peak VL	Lowest VL	Set point(3 months)	Set point(6 months)	Average set point	Average CD4 counts(0-12w)	Average CD4 counts(12-24w)	Average CD4 counts(0-24w)
2DL2$^+$/2DL2$^-$	0.68	0.97	0.06	0.79	0.34	0.92	0.83	0.82
2DL3$^+$/2DL3$^-$	Only 2 *KIR2DL3* negative individuals							
2DS2$^+$/2DS2$^-$	0.68	0.97	0.06	0.79	0.34	0.92	0.83	0.82
2DS3$^+$/2DS3$^-$	0.63	0.58	0.63	0.28	0.36	0.41	0.11	0.12
3DL1$^+$/3DL1$^-$	Only 7 *KIR3DL1* negative individuals							
3DS1$^+$/3DS1$^-$	0.99	0.89	0.59	0.44	0.91	0.13	0.06	0.07

Peak VL: the highest viral load among all the results; Lowest VL: the lowest Viral load in all the results; The viraemia RNA level usually settles to a stable level approximately 3-6 months from the onset of the infection, here set point (3 months) is the HIV-RNA level at month 3, set point (6 months) is HIV-RNA level at month 6, the average set point is the mean value of the two. Average CD4 counts (0-12w) is the arithmetic mean of all CD4 counts within the first 3 months; 12-24w denotes the second 3 months, and the average CD4 count (0-24w) is the arithmetic mean in the 6 months. *KIR2DL2* is in strong linkage disequilibrium with *KIR2DS2* (Wn=0.976, P<0.001); in this cohort, all the individuals whose *KIR2DL2* are positive are also positive for *KIR2DS2*. Those P values close to 0.05 are denoted in orange.

5. 4 Discussion

Epidemiological studies demonstrate some HLA class I alleles are protective against HIV-1 disease progression (Carrington and O'Brien 2003), suggesting that CD8 T cells play a crucial role in the control of viral replication; meanwhile it has also been shown that the protective effect could be enhanced by the co-expression of specific *KIR* alleles. For example, *KIR3DS1, KIR3DL1*004* and KIR3DL1hi allotypes have all been demonstrated to be associated with protective effects in combination with *HLA-Bw4* alleles (Martin, Gao et al. 2002; Martin, Qi et al. 2007). These associations strongly implicate that part of the protective effect of *HLA* class I alleles may be modulated by NK cells through particular KIR, potentially at the early stage of infection before the induction of adaptive immune responses, rather than through CD8+ T cells alone.

Luckily we could get access to a valuable acute HIV-1 infection cohort to study potential associations with specific KIRs and clinical outcome. This cohort was established prospectively in a high-risk MSM population and the longitudinal CD4 counts and HIV-RNA levels for most patients from the onset of HIV-1 infection were well recorded, which provided us with reliable clinical information to evaluate the associations of specific *KIRs* with HIV-1 progression and viral control. We focused our analyses on 6 *KIR* genes which were selected on the basis that these 6 genes have been the most widely reported to be associated with the outcome of HIV-1 and other viral infections. This allowed us to reduce the number of multiple comparisons for our statistical analysis as well as tailor the *KIR* genotyping to the small amounts of DNA that were available from the acute cohort.

We first compared the 6 *KIR* gene frequencies in this acute HIV-1 cohort with those in our two previous groups (healthy control group and slow progressors group) that we identified in the SM cohort: *KIR2DL3* again showed significant differences among the 3 groups, but

when we further did the pair-wise comparison, the acute HIV-1 infection cohort was not involved in the significant differences.

It was interesting to find that the frequency of *KIR3DS1* was significantly lower in the slow progressors group (31%) than in the acute group (40.7%). Generally speaking, the acute HIV-1 infection cohort could be considered as a combination of all the future "rapid progressors" and "slow progressors". Therefore it was easy to understand that at the early stages of HIV-1 infection, the *KIR3DS1* frequency (40.7%) was close to that in the healthy controls (38.1%). We propose that in the SM cohort, rapid progressors with *KIR3DS1* died before the cohort was recruited; in those remaining slow progressors, the *KIR3DS1* frequency was shown to be significantly lower (31%), which implies that *KIR3DS1* might be detrimental to survival with HIV infection. This finding is consistent with an earlier observation (Martin, Gao et al. 2002) in which *KIR3DS1* was associated with delayed progression only when found in combination with *HLA-B Bw4-80I*; in contrast, *KIR3DS1* by itself was associated with rapid progression to AIDS (RH=1.23, P=0.03). Most individuals (95%) in the acute HIV cohort were Chinese Han, which was comparable to those in SM cohort (100% Chinese Han). Even when we excluded the 11 cases that were from minority ethnic groups, the difference was still significant. Although all the study subjects in the acute HIV-1 cohort were men, based on previous data, there should be no difference for *KIR* gene frequency according to gender. Therefore the significant difference in *KIR3DS1* frequency between the acute patients and slow progressors strongly implies that *KIR3DS1* plays a role in HIV-1 disease progression.

Using the longitudinal measurements of CD4 counts and HIV-RNA levels, we further evaluated the effects of the 6 *KIR* genes on disease progression and viral suppression. The "set point" indicates the viral load when plasma viraemia has settled from its peak to a stable level (Daar, Moudgil et al. 1991; Schacker, Collier et al. 1996), which in the absence of ART

is maintained by the balance between virus turnover and the immune responses. This steady state has been considered as an important indicator of disease progression (Mellors, Kingsley et al. 1995; Mellors, Rinaldo et al. 1996). Time to the establishment of set point is variable among patients: in one study (Huang, Chen et al. 2012), the cumulative frequency of patients whose viral load measurements reached the steady state at days 30, 60, 90, and 120 was 7.9%, 57.9%, 76.3%, and 100%, respectively. The widely-accepted duration for the set point is 3-6 months from the onset of the infection. In our study, set points values after 3 months and 6 months together with the highest and lowest viral load levels were taken into account respectively to evaluate the role of *KIRs* on viral control, but no significant differences were found between specific *KIR* positive and negative groups. Neither was any significant difference found when we utilised CD4 counts for evaluation. Only one trend (P=0.06) was observed, that *KIR2DL2* and/or *KIR2DS2* (they are in strong linkage disequilibrium with each other) were associated with viral load at set point (3 months). In addition, another trend was that *KIR3DS1* might have an association with disease progression (P=0.057): compared to the negative patients, *KIR3DS1* positive patients seemed to have lower CD4 counts. Therefore, taken together with the significantly different *KIR3DS1* gene frequency between acute patients and the slow progressors, our data strongly suggests that *KIR3DS1* has an involvement in key processes that determine the outcome of the HIV infection.

When we evaluate the outcome of HIV infection, inevitably there is a range of miscellaneous factors that could exert impacts on viral replication and subsequent disease progression. Apart from the variable human immunogenetic factors, the most important determinant is the nature of the infecting virus. There were different HIV-1 subtypes in this acute cohort: about 50.6% individuals were infected with CRF01_AE and about 33.3% individuals had subtype B. CRF01_AE represents a putative subtype of an A/E recombinant that originated in Central

Africa but has spread epidemically in Asia. CRF01_AE has spread rapidly in China since the early 2000s. A recent molecular epidemiology survey in Beijing identified CRF01_AE (40.4%) as the most dominant strain. In the MSM population, CRF01_AE accounted for 56.8% of all infections (unpublished data from Beijing CDC).

The properties of the virus, such as co-receptor use and replication capacity, may influence progression rates. Growing evidences have been accumulating to suggest that viral subtype is an important contributing factor (Kanki, Hamel et al. 1999; Kaleebu, French et al. 2002; Baeten, Chohan et al. 2007). Individuals infected with subtypes C, D are 8 times more likely to develop AIDS than individuals infected with subtype A (Kanki, Hamel et al. 1999) . In Uganda, where subtypes A and D are most prevalent, subtype D is associated with faster disease progression compared with subtype A (Kiwanuka, Laeyendecker et al. 2008).

The finding that subtype A progresses less rapidly than subtype D raises the question of whether other HIV-1 subtypes also are associated with different rates of disease progression. In terms of local HIV epidemiology, it would be interesting to compare progression rates in Brazil, where subtype B and subtype F co-circulate along with B-F recombinants, and in Thailand, where B and CRF01_AE predominate. In China, where subtype B and subtype C co-circulate, comparison of progression rates would be of particular interest because subtype C accounts for the largest number of infections worldwide.

In 2013, an analysis of 3364 seroconverters with known HIV-1 subtypes from the CASCADE collaboration showed that: compared with subtype B, the CD4 count at seroconversion was significantly higher for subtype CRF01 and lower for subtype C (Touloumi, Pantazis et al. 2013). Subsequent CD4 decline was significantly slower for subtypes A and CRF02 and marginally slower for subtype C compared with B. In an adjusted analysis, the median viral load set point and time to clinical AIDS/death did not differ significantly by subtype,

although all subtypes, except C, tended to have lower levels compared with B. Since in this acute HIV infection cohort, the majority of patients were infected with CRF01_AE, there may be differences from the SM cohort which was infected with subtype B'.

The evidence as to whether the route of infection affects disease progression is conflicting. Before HAART era, one large meta-analysis covering 13,000 HIV-1 infected individuals failed to find a survival difference according to route of exposure (2000). However, another meta-analysis showed a higher mortality in injecting drug users than in people infected by other routes (Porter, Babiker et al. 2003). But we must be careful to evaluate the issue of disease progression in injecting drug users, due to the high rate of death from other causes.

Obviously there are some limitations for this study. Firstly, we have not studied the HLA-KIR interactions in the acute cohort due to lack of available HLA data currently, but it is definitely worth doing this in the future to investigate the possible synergetic effects between the two loci. Secondly, although the acute HIV-1 infection cohort is a valuable resource, the population size is not large enough to allow more stratified analyses. Moreover, the profile of *KIR* genes and their associated molecules is quite complicated: for an individual, possessing one specific *KIR* gene doesn't necessarily mean that the gene is definitely expressed on the surface of circulating NK cells. So it is noteworthy that a genetic study only shows the direction of an association, subsequent functional experiments are always needed to confirm the finding and try to explore the underlying mechanisms.

Overall our findings provide new insights into the impact of *KIRs* in acute HIV-1 infection. *KIR3DS1* appears more likely to be involved into HIV-1 disease progression rather than viral control, and *KIR3DS1* on its own seems to be detrimental to the clinical outcome of HIV

infection. More studies are needed to explore the role of this mysterious and elusive "activating" *KIR* gene in HIV infection.

Chapter 6 General discussion and future plans

NK cells are important components of the innate immune system, representing the first line of defence against viral infection (Lanier 1998). Among the human NK receptor families, only the *KIR* genes are highly polymorphic and consequently could explain the differential responses to viral infections between individuals. The *KIRs* are a multigene family and the diversity within KIR system is present at the level of the locus, of the allele, of the haplotype, of the HLA ligand, and within KIR expression patterns. The expression of KIRs on NK cells is stochastic and variegated (Shilling, Guethlein et al. 2002; Yawata, Yawata et al. 2008). It is not yet clear which of these variable factors are most important to account for the influence of KIR polymorphisms on the host immune responses to infection.

Previous genetic and functional studies have indicated that different *KIR* genes, different haplotypes, allelic polymorphisms and different expression levels of KIRs, may all play a part in the association with HIV-1 infection outcomes, including susceptibility to HIV infection and subsequent disease progression, as can be seen in the table below:

Table 6-1: The genetic and functional association studies of KIRs in HIV infection.

Genetic studies

KIR and HLA	Effect	References
KIR3DS1+HLA-Bw4 80I	Slower progression to AIDS	(Martin, Gao et al. 2002) (Gaudieri, DeSantis et al. 2005)
KIR3DL1+HLA-Bw4	Slower progression to AIDS	(Martin, Qi et al. 2007)
Co-expression of *3DL1*h/*y* and *B*57*	Reduced risk of progressing to AIDS	(Boulet, Kleyman et al. 2008)

Homozygosity for *KIR3DS1*	Relative resistance to HIV infection	(Boulet, Sharafi et al. 2008)
KIR3DL1*h/*y* with *HLA-B*57	Slow time to AIDS and low viral load	(Kamya, Boulet et al. 2011)
Increase in *KIR3DS1* count	Lower viral set point	(Pelak, Need et al. 2011)
KIR2DS2	Associated with faster progression to AIDS	(Gaudieri, DeSantis et al. 2005)
Haplotype B KIRs	Associated with lower CD4 T cell counts	(Jennes, Verheyden et al. 2011)

Functional studies

Specific phenotype	Effect	References
KIR3DS1(+) /KIR3DL1(+) with HLA-B Bw4 80I	KIR3DS1(+) /KIR3DL1(+) NK cells expand in acute HIV-1 infection in the presence of HLA-B Bw4 80I	(Alter, Rihn et al. 2009)
KIR3DS1$^+$ NK cells in an HLA-B Bw4 80I dependent manner	Inhibit HIV-1 replication *in vitro* more potently	(Alter, Martin et al. 2007)
Changes in the HLA class I presented peptide	Amino acid changes in HIV-1 encoded peptides can significantly modulate the binding of these inhibitory KIRs and result in the activation of primary KIR$^+$ NK cells	(Hansasuta, Dong et al. 2004) (Thananchai, Gillespie et al. 2007) (Thananchai, Makadzange et al. 2009) (Alter, Heckerman et al. 2011)

In order to explain the protective effect of an inhibitory KIR, a model based on NK cell development was proposed. During NK cell development, this model suggests that NK cells need to obtain an inhibitory signal through an inhibitory KIR to become functionally competent (Kim, Poursine-Laurent et al. 2005; Kim, Sunwoo et al. 2008; Elliott, Wahle et al.

2010; Elliott and Yokoyama 2011). Thus different inhibitory KIRs with different functional avidities may stimulate particularly potent NK cell responses. This model helps to explain the protective effect of *KIR3DL1* alleles associated with higher KIR3DL1 expression in the context of HLA-Bw4 and of the SNP rs9264942 associated with higher expression of HLA-C molecules. The SNP rs9264942 showed the most significant association with viral load control in a GWAS in European Americans (Pereyra, Jia et al. 2010) and was also associated with the level of HLA-C expression (Thomas, Apps et al. 2009). Increasing HLA-C cell surface expression was further shown to be associated with protection against multiple outcomes in both African and European Americans (Apps, Qi et al. 2013). Corrah *et al* re-evaluated the relationship between this SNP and HLA-C expression level and proposed that it was the particularly low expression of the 35T-associated *HLA-Cw*07* allele that largely accounts for the low HLA-C expression associated with the -35 T allele, and possibly to the relatively high risk of disease progression (Corrah, Goonetilleke et al. 2011). On the other hand, Blais et al (Blais, Zhang et al. 2012) showed that the C allele was also associated with delayed disease progression in the SM cohort and was linked with greater selective pressure through HLA-C molecules on HIV sequence variation, which could not be accounted for by associations with any particular HLA-C allele.

An alternative explanation for the protective effect of inhibitory KIRs might be that the inhibitory KIRs could enhance the protective HLA-B-restricted responses to HIV-1. If so, the mechanism is not understood, but could derive from KIR expression on HLA-B-restricted T-cells that in some way enhances their function.

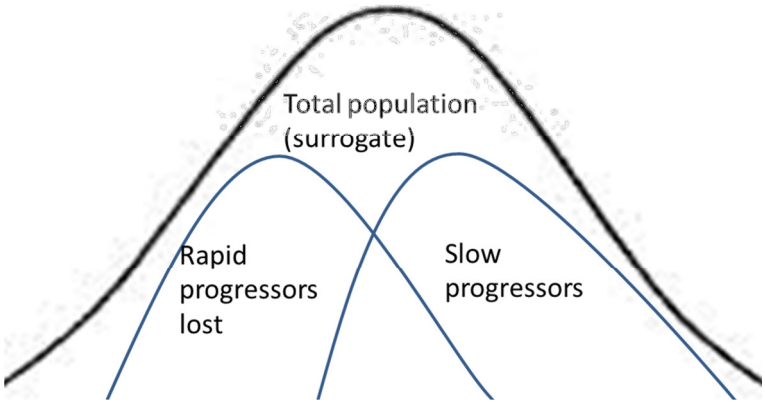
HIV infection is a highly complex and dynamic interaction between a polymorphic virus and a polymorphic host immune system. Multiple factors, including viral subtype, HIV-1 RNA level, degree of immune activation, age, co-infections, socioeconomic status, and host

genetics, all make a contribution to determining progression rate. The SM cohort provides us with a unique opportunity to study the host genetic factors because some major factors such as viral strain, transmission route and timing of infection have been narrowly controlled in this cohort, as a consequence of the manner of infection in these former plasma donors.

In this project, we started to look at the polymorphism of KIRs in this chronic HIV-1 infected cohort, evaluating the impact of KIR and KIR-HLA interactions in terms of HIV-1 infection progression (**Chapter 3**).

The *KIR* gene frequencies comparison was made between the "slow progressor" group and a healthy control group (from an adjacent village) instead of the ideal group of "rapid progressor" group. Because this is a retrospective study, the rapid progressors have inevitably been lost from this cohort over the first 10 years of infection before samples were collected due to premature death. The similarities between the two Han Chinese populations have been confirmed for other polymorphic genes such as HLA class I (Rai, Zhang et al. 2013). The rationale underlying this comparison is that the healthy group people could be considered as a surrogate of the previous total population of the SM village: to some extent, the "slow progressor" group could be considered as a subset of the previous total population. So the comparison between one subset and the overall population provides some insights to explore the KIR associations with outcome, even though the power of the study is reduced. A schematic of this rationale is presented below.

Figure 6-1: The relationship between the compared populations



It was not surprising to find that the *KIR* gene frequencies in the SM patient cohort were similar to those in the healthy controls, except for *KIR2DL3*. *KIR2DL3* and *KIR2DL2* segregate as alleles of a single genetic locus, which is often referred to as *KIR2DL2/L3* (Uhrberg, Parham et al. 2002; Robinson, Mistry et al. 2010). The weaker affinity between *KIR2DL3* and *HLA-C1* compared to *KIR2DL2* and *HLA-C1* is thought to underlie the mechanism of the observation (Khakoo, Thio et al. 2004) that increased resolution of acute hepatitis C viral infection was associated with homozygous *KIR2DL3* and *HLA-C1*, which was the first major demonstration of the role of *KIR2DL3* in a human viral infection.

HLA class I provides ligands for the KIR receptors. KIR molecules regulate the activity of NK and some T cells through interaction with specific HLA class I molecules on target cells. Because HLA class I alleles are under continuous selection pressure from infectious disease morbidity and mortality, the KIR locus must also evolve to maintain and enhance beneficial interaction with HLA class I (Khakoo, Rajalingam et al. 2000). In order for a functional interaction, both the ligands and receptors gene must coexist in the same individual, even though they are encoded on different chromosomes. However, further analysis did not demonstrate any differences in *KIR2DL3+HLA-C1/C1* or *KIR2DL3+HLA-C1/x* between the SM patient group and the healthy control group.

Activating KIRs have been associated with several diseases (Kulkarni, Martin et al. 2008) and the *KIR3DS1* gene has drawn particular attention in HIV disease pathogenesis (Martin, Gao et al. 2002; Gaudieri, DeSantis et al. 2005; Qi, Martin et al. 2006; Alter, Martin et al. 2007; Barbour, Sriram et al. 2007; Long, Ndhlovu et al. 2008). Although most genetic association studies agree that *KIR3DS1* is probably protective against HIV, our finding is not consistent with this because the combination of *KIR3DS1+Bw4/Bw4* was not shown to be enriched in the protected "slow progressor" group, instead, it was lower than that of the background level. The reason for the discrepancy between our findings and previous studies is not clear but may relate to other factors in this cohort, such as the particular infecting clade or co-infections with other blood-borne viruses. It is also possible that there are different alleles of KIR3DS1 in the Chinese population, leading to our studies to define KIR3DS1 in our population by sequence analysis (**Chapter 5**).

The study in **Chapter 3** focused on chronic HIV-1 infection to see whether there would be any *KIR* gene or *KIR/HLA* gene combination enriched in the "slow progressor" group. Actually, as the crucial component of innate immune response, the KIR-modulated NK cells should exert their "protective" role in the acute phase of HIV infection through a rapid and efficient innate immune response. On the basis of this presumption, another possible explanation for any protective or detrimental effect of KIRs in HIV disease progression might be due to the impact of specific KIR-mediated NK cells or even KIR-expressing T cells in controlling multiple acute opportunistic infections, consequently determining the final outcome of HIV disease.

In this context, we further studied the role of KIRs in another Chinese population, in a recently-established acute HIV-1 infection cohort (**Chapter 5**), to see the impact of KIRs in terms of HIV replication control and disease progression (characterised by CD4 loss). This

cohort was a prospective on-going cohort, which provided detailed clinical data for each acute HIV-1 infected patient, enabling us to use these clinical parameters such as series of CD4 counts and HIV-1 viral load results for our analysis. A few trends were identified in this study, which was limited in both participant numbers and the extent of the KIR typing we could perform: it is also important to note that these patients differed from the SM cohort in route of transmission (largely through homosexual sex) and infecting clade. One finding was that *KIR2DL2* (*KIR2DS2* cannot be ruled out due to the high linkage disequilibrium between them) may have a detrimental impact on the control of HIV-1 replication (the set point at 3 months was higher in the *KIR2DL2* positive patients compared with *KIR2DL2* negative patients, P=0.06). A previous genetic association study (Gaudieri, DeSantis et al. 2005) has revealed a detrimental effect of *KIR2DL2* and *KIR2DS2* on CD4 T cell decline and progression to AIDS. However, a recent functional study (Alter, Heckerman et al. 2011) demonstrated that *KIR2DL2* positive NK cells can place immunological pressure on HIV-1, and the virus can evade such NK cell mediated immune pressure by selecting sequence polymorphisms, although the authors could not rule out involvement of *KIR2DS2*.

Another trend was that *KIR3DS1* might have a detrimental impact on HIV-1 disease progression in the acute phase (the average CD4 counts in the first 24 weeks in the *KIR3DS1* positive patients was lower than that in the *KIR3DS1* negative patients, P=0.07). Combining the finding that *KIR3DS1* was significantly lower in the "slow progressor" group (31%) in the SM cohort than in this acute group (40.7%), we concluded that *KIR3DS1* might be detrimental to survival with HIV infection. This finding is consistent with an earlier observation (Martin, Gao et al. 2002) in which *KIR3DS1* by itself was associated with rapid progression to AIDS (RH=1.23, P=0.03).

However the role of *KIR3DS1* in the outcome of HIV infection is still controversial. A study of 191 individuals (Gaudieri, DeSantis et al. 2005) showed that *KIR3DS1* itself and the compound genotype of *KIR3DS1+Bw4-80I* was associated with rapid progression to AIDS. Another study (Barbour, Sriram et al. 2007) involving 255 individuals indicated that *KIR3DS1* independently associated with higher CD4 T cell counts, but had no effect on viral load levels. The concept of the synergistic effect on HIV-1 disease between *KIR3DS1* and *HLA-Bw4 80I* (Martin, Gao et al. 2002) was also questioned by a study (Barbour, Sriram et al. 2007), which indicated that *KIR3DS1* and *HLA-Bw4 80I* might independently exert a protective effect on the course of HIV-1 infection.

In our study (**Chapter 5**), *KIR3DS1* appears more likely to be involved into HIV-1 disease progression in the Han Chinese rather than viral control, and *KIR3DS1* on its own seems to be detrimental to the clinical outcome of HIV infection. These putative findings in our genetic association study need to be confirmed or rejected in larger studies and explored by downstream functional studies. Several factors may complicate the interpretation of genetic association studies with functional studies. First, a direct interaction between KIR3DS1 and HLA-Bw4 molecules has not been demonstrated to date, and many studies have failed to show any binding of KIR3DS1 to HLA class I molecules (Gardiner, Guethlein et al. 2001; Carr, Rosen et al. 2007; O'Connor, Yamada et al. 2011; Vivian, Duncan et al. 2011). Second, for those individuals expressing both KIR3DS1 and KIR3DL1 receptors, a relatively common situation, it is hard to evaluate their independent influences on NK cell function. For example, KIR3DS1 might also be involved in the licensing of NK cells, and in contrast to KIR3DL1, KIR3DS1 might lead to decreased responsiveness of NK cells, so the combined effect in heterozygotes may be hard to measure. Third, among individuals, there are also *KIR3DL1/3DS1* copy number variations which may have a functional impact (Pelak, Need et

al. 2011). Fourth, linkage disequilibrium always needs to be taken into consideration since KIR genes are located very close to each other. Finally, amino acid changes in HIV-1-encoded peptides can significantly modulate the binding of inhibitory KIRs to peptide-HLA complex, consequently affecting the function of KIR^+ NK cells, so the infecting virus needs to be taken into consideration.

As mentioned above, the direct interaction between KIR3DS1 and HLA-Bw4 80I has yet to be shown. The affinity of activating KIRs is lower than that of their inhibitory counterparts and might be modified by a stress peptide generated during infection or a viral peptide presented by HLA class I. One murine model has shown that Ly49p-mediated protection in MCMV infection needs a third, as yet undefined, protein to mediate the interaction between Ly49p NK cell receptor and its putative ligand H2Dk (Lee, Girard et al. 2001). Meanwhile, *KIR3DS1* in conjunction with *HLA-Bw4 80I* is also protective in the setting of HCV (Lopez-Vazquez, Rodrigo et al. 2005). Overall, these findings suggest that a third molecule induced during viral infection rather than the viral peptide itself might affect the affinity of KIR3DS1 for its putative ligand HLA-Bw4 80I, but the search for this third molecule is still underway.

When we discuss our putative conclusions here, we must explain the results with caution. With regard to statistical power, the sample size of the SM cohort is not sufficient to show significant differences in gene frequencies. Taking *KIR2DL3* as an example, when we tried to show a true difference of gene frequencies between 95% and 99%, the required sample size should be larger than 400:400 to maintain power higher than 80% (calculated by software PASS using Two Independent Proportions (Null Case) Power Analysis). In order to avoid "false positives" as much as possible, future studies should enrol a larger sample size of participants. According to our sample size power calculation, a future study requires a sample

size of at least greater than 400 cases in each group to ensure the analysis power is higher than 80%.

Another potential confounding factor in our studies is the impact of co-infection acquired through blood-borne virus transmission, particularly HCV. In the US and Western Europe, among HIV-infected persons, HCV prevalence is 72% to 95% among injection drug users (IDU), 1% to 12% in men who have sex with men (MSM), and 9% to 27% in heterosexuals (Alter 2006). In the SM cohort, HCV/HIV co-infection is likely to be high (assumed to be 100%) but this hasn't yet been fully investigated in the SM cohort. It has been well established that HIV has a negative impact on the natural history of HCV, including a higher rate of viral persistence, increased viral load, and more rapid progression to fibrosis, end-stage liver disease, and death (Bica, McGovern et al. 2001; Weber, Sabin et al. 2006). However the influence of HCV on HIV disease progression is still a matter of debate and remains an unresolved issue (Bica, McGovern et al. 2001). HCV does not seem to have a major impact on the natural history of HIV infection. In studies before the HAART era, HCV co-infection had no effect on HIV progression (Dorrucci, Pezzotti et al. 1995). The reason might be that the high mortality from AIDS-related causes predominated and masked any other impacts on mortality. In the HAART era, Greub et al. (Greub, Ledergerber et al. 2000) reported that HCV positivity was associated with a higher risk of death or developing an AIDS-related illness in a cohort of 3,111 Swiss HIV^+ patients starting HAART. On the other hand, two large American series consisting of more than 12,000 patients (Sulkowski, Moore et al. 2002; Sullivan, Hanson et al. 2006) failed to confirm such an effect. How HCV may affect the course of HIV infection is also unclear. One proposed mechanism suggests that the HCV effect may be mediated by increased immune activation and $CD4^+$ T cell apoptosis in

untreated subjects (Kovacs, Al-Harthi et al. 2008; Gonzalez, Falconer et al. 2009; Korner, Kramer et al. 2009; Kovacs, Karim et al. 2010).

It is plausible that HCV/HIV co-infection in the SM cohort could affect the present findings, since the impact of KIR on HCV disease may be distinct from that affecting HIV progression. It has been reported that *KIR3DS1* in conjunction with *HLA-Bw4 80I* is also protective in the setting of HCV (Lopez-Vazquez, Rodrigo et al. 2005).We recommend that future studies in our cohort take this into consideration at the study design stage with the plan to investigate the rate of HCV/HIV co-infection in the SM cohort.

In the cohort population study (**Chapter 3**), the frequencies of activating *KIRs* were generally lower than the inhibitory *KIRs* except for *KIR2DS4*, which is the only activating *KIR* gene in the A haplotype. The inhibitory KIRs are relatively well studied. Ligands have been defined for many of inhibitory KIRs and biological specificity has been demonstrated. In contrast, the functional relevance of activating KIRs is less obvious: most of them have no identified ligands and those that could bind HLA seem to bind with a lower affinity than their inhibitory KIR counterparts. From an evolutionary viewpoint, it is assumed that activating KIRs may arise from their inhibitory counterparts and then further diversify by recombination and duplication events (Abi-Rached and Parham 2005). This was initially regarded as a circular process, in that the biological pressure has driven the formation of activating KIRs but once established and subjected to changing environmental conditions, detrimental effects become apparent, and these activating KIRs are then selected again.

Another important component of KIR variation is that *KIR* haplotypes vary in gene content (Uhrberg, Valiante et al. 1997; Wilson, Torkar et al. 2000). Generally, *KIR* haplotypes are determined by segregation analysis in family studies. But because this was not feasible in our cohort, several assumptions were also made in determining the haplotypes based on

published gene frequencies and patterns of linkage disequilibrium between pairs of *KIR* genes: 1) *3DL3, 3DP1, 2DL4* and *3DL2* are present on all haplotypes; 2) if *2DL1* is present, *2DP1* is always present; 3)*3DS1* segregates as an allele of *3DL1*; 4)*2DL2* and *2DL3* segregate as alleles of a single locus. In **Chapter 3**, the A haplotype accounted for 51% of all the observed population, further motif analyses showed that one combination motif *Tel-B1/Bx* was significantly lower in the SM patient group.

CD8 T cells are known to be able to exert extreme selection pressure on HIV sequences over the course of infection (Borrow, Lewicki et al. 1997; Price, Goulder et al. 1997; Kelleher, Long et al. 2001; Cao, McNevin et al. 2003; Brumme, Brumme et al. 2008). However HIV-1 virus can escape such kind of immunological pressure through mutations arising within the HLA class I-restricted CD8 T cell epitopes, which can disrupt binding of a viral peptide to HLA class I, or impair recognition by the T cell receptor. The mutation can also lie in regions immediately flanking the epitope, which may affect antigen processing. About two-thirds of all non-envelope viral mutations detected in the chronic phase are attributed to CD8 T cell responses in HIV-1-infected patients (Allen, Altfeld et al. 2005).

The sequence changes in the peptides presented by HLA class I molecules have an important influence on the affinity of the binding between KIRs and their respective HLA class I ligands, and potentially on NK cell recognition of HIV-1-infected cells. If the HLA class I – presented peptide impacts the subsequent NK cell responses, the virus might evolve to either escape NK cell recognition by activating KIRs and/or repress NK cell function by increasing binding of inhibitory KIRs. In other words, peptides that bind to HLA class I molecules but not KIRs can disrupt KIR-mediated inhibition of NK cell activation. Peptides conferring weak recognition by KIRs can antagonize the inhibitory effect of other peptides inducing

strong KIR binding to the HLA class I ligand, and trigger NK cell degranulation (Fadda, Borhis et al. 2010).

In our study in **Chapter 3**, several viral amino acid sequences were associated with specific KIRs. Some positions have been previously associated with HLA class I molecules, where the variation could be ascribed to the immune pressure from the HLA class I restricted CTL. However, the remainder of these positions seem to associate only with KIRs and the underlying driving force could originate from KIR-associated immune pressure, which we have described as "KIR footprints". The amino acid position of Nef75 was the strongest candidate position because it was the only association (with *2DL5*) holding up with a q value below 0.2 (P=0.008), which showed: at position Nef75, all the 33 *KIR2DL5* positive individuals have the consensus amino acid: aspartic acid. In contrast, for 43 *KIR2DL5* negative individuals, 31 of them demonstrated the aspartic acid, whereas the virus in 12 of them showed a change to alanine, glutamic acid or asparagine. From the perspective of a functional study, it is really difficult to explore this kind of genetic association, not least because the ligand for *KIR2DL5* is still unknown. More complicated is that Nef75 has also been shown to be associated with *KIR2DS1*. Is there any interaction between the two *KIRs*? Or are both of them just markers due to linkage disequilibrium with other determinants? These questions definitely need future studies to resolve the issues.

The locus of *KIR3DL1/S1* occupies the focus of the study of KIRs due to its extensive associations with different disease outcomes. It was thought that *KIR3DS1* might originate from *KIR3DL1* (Abi-Rached and Parham 2005); they share a high degree of similarity with each other, and only a few amino-acid substitutions in the extracellular domains lead to the different ligand-binding characteristics (Norman, Abi-Rached et al. 2007). In **Chapter 4**, a novel method of sequencing the locus for *KIR3DL1/S1* was established, which enabled us to

identify new alleles of *KIR3DL1* and *KIR3DS1* in the Chinese Han population. Although most of the mutations are in the noncoding regions, current reports have shown that even these variants in noncoding regions may affect the level of transcription, translation and splicing, which in turn may have further biological effects.

There are some limitations to our studies that we should acknowledge. First, although the SM patient cohort is a unique one with many confounders filtered before analyses, the cohort size is still small, especially for further stratified analyses. Our results need to be substantiated in other larger Han Chinese cohorts. Second, the clinical data could not be utilised in the study of the chronic cohort because later on some of the infected individuals started antiretroviral therapy without clear clinical indications, making clinical data difficult to interpret. Third, due to the limitation of DNA amounts, we could not combine HLA data for our analyses in the acute cohort. Since the acute HIV-1 cohort is an on-going cohort, the HLA-KIR interaction study would be a very important component of future analyses. Finally, phenotypic and functional studies are required in tandem to validate the associations found using genetic studies, but due to the lack of commercially available reagents to specifically assess the expression of most activating KIRs and some inhibitory KIRs, the role played by these receptors in the NK cell response to HIV-1 infection has largely been limited to the genetic association level. The development of better research tools to characterise KIR-HLA interactions at a functional level is urgently needed to deepen our insights into this field.

In conclusion, in this study the role of KIRs and KIR/HLA interactions were evaluated in acute and chronic HIV-1 infection in two distinct Han Chinese cohorts. It is recognised that the area of KIR/HLA associations is very complex, and reported studies in different populations have generated conflicting results. The study of the two most polymorphic gene systems in the human genome is really complicated and on the basis of our current

understanding, the results cannot be explained in a straightforward manner. The associations could be interactive, dynamic, and on most occasions, they are susceptible to confounding from linkage disequilibrium interference, together with the different levels of polymorphism. It is obvious that a single model is not sufficient to explain the elusive KIR-HLA interaction and its impact on determining HIV-1 replication and disease progression. Some mechanisms could only be explained under specific circumstances. Future studies require larger cohorts so that the subsequent stratified analyses could still have sufficient statistical power. Prospective cohorts from well-defined populations, studied with elaborative design and detailed follow-up will be important to answer these complicated questions. A better understanding of the interaction between KIR-modulated-NK cell function and HIV-1 infection will allow identifying novel approaches to restore and/or enhance NK cell function in HIV-1 infected patients, and potentially harness antiviral immune responses against HIV-1.

Future plans

1. To obtain the HLA data from the acute HIV-1 infection cohort (**Chapter 5**) and show the possible association of HLA-KIR interaction with HIV-1 replication or disease progression.

2. To apply the novel method of sequencing the locus *KIR3DL1/S1* in other populations to further test the efficacy, meanwhile to identify more *KIR3DL1* and *KIR3DS1* alleles.

3. Based on the KIR footprints identified, subsequent functional studies could be designed to test the possible mechanisms of these associations.

Appendix

Appendix 1 The current full-length alleles of *KIR3DL1* in IPD-KIR database.

Allele	Length(bp)
KIR3DL1*0010101	14546
KIR3DL1*0010102	14546
KIR3DL1*002	14549
KIR3DL1*00401	14566
KIR3DL1*0050101	14547
KIR3DL1*0050102	14547
KIR3DL1*0070101	14550
KIR3DL1*0070102	14550
KIR3DL1*008	14549
KIR3DL1*0150201	14549
KIR3DL1*0150202	14550
KIR3DL1*0150203	14549
KIR3DL1*01701	14549
KIR3DL1*025	14549
KIR3DL1*0290101	14549
KIR3DL1*0290102	14549
KIR3DL1*059	16902
KIR3DL1*062	14549
KIR3DL1*063	14564
Total number	**19**

Appendix 2 The current alleles of *KIR3DL1* containing only sequences of exons in IPD-KIR database.

Allele	length(bp)
KIR3DL1*0010101	1335
KIR3DL1*0010102	1158
KIR3DL1*00102	1254
KIR3DL1*002	1335
KIR3DL1*00401	1335
KIR3DL1*00402	1335
KIR3DL1*00403	1302
KIR3DL1*0050101	1335
KIR3DL1*0050102	1335
KIR3DL1*00502	1335
KIR3DL1*006	1335
KIR3DL1*0070101	1335
KIR3DL1*0070102	1335
KIR3DL1*008	1335
KIR3DL1*009	1335
KIR3DL1*01501	1335
KIR3DL1*0150201	1335
KIR3DL1*0150202	1335
KIR3DL1*0150203	1335
KIR3DL1*01503	1335
KIR3DL1*016	1335
KIR3DL1*01701	1335
KIR3DL1*01702	1265
KIR3DL1*018	1260
KIR3DL1*019	1335
KIR3DL1*020	1335
KIR3DL1*021	1335
KIR3DL1*022	1335
KIR3DL1*023	1335
KIR3DL1*024N	1334
KIR3DL1*025	1335
KIR3DL1*026	1335
KIR3DL1*027	1335
KIR3DL1*028	1335
KIR3DL1*0290101	1335
KIR3DL1*0290102	1335
KIR3DL1*030	1335
KIR3DL1*03101	1335

KIR3DL1*03102	1335
KIR3DL1*032	1335
KIR3DL1*033	1335
KIR3DL1*034	1335
KIR3DL1*035	1335
KIR3DL1*036	1335
KIR3DL1*037	1335
KIR3DL1*038	1335
KIR3DL1*039	1335
KIR3DL1*040	1335
KIR3DL1*041	1335
KIR3DL1*042	1335
KIR3DL1*043	1335
KIR3DL1*044	1335
KIR3DL1*051	1335
KIR3DL1*052	1335
KIR3DL1*053	1335
KIR3DL1*054	1335
KIR3DL1*056	1335
KIR3DL1*057	1000
KIR3DL1*059	1335
KIR3DL1*060	1335
KIR3DL1*061	1335
KIR3DL1*062	1335
KIR3DL1*063	1335
KIR3DL1*064	1302
KIR3DL1*065	1335
KIR3DL1*066	1335
KIR3DL1*067	1335
KIR3DL1*068	1335
KIR3DL1*072	1335
KIR3DL1*073	1302
Total number	**70**

Appendix 3 The current full-length alleles of *KIR3DS1* in IPD-KIR database.

Allele	Length(bp)
KIR3DS1*0130101	14932
KIR3DS1*0130102	14933
KIR3DS1*0130103	14932
KIR3DS1*014	5245
KIR3DS1*055	14932
Total number	**5**

Appendix 4 The current alleles of *KIR3DS1* containing only sequences of exons in IPD-KIR database.

Allele	Length(bp)
KIR3DS1*010	1164
KIR3DS1*011	1149
KIR3DS1*012	1149
KIR3DS1*0130101	1165
KIR3DS1*0130102	1165
KIR3DS1*0130103	1165
KIR3DS1*01302	1165
KIR3DS1*014	1165
KIR3DS1*045	1165
KIR3DS1*046	1165
KIR3DS1*047	1165
KIR3DS1*048	1165
KIR3DS1*049N	1164
KIR3DS1*050	1149
KIR3DS1*055	1165
KIR3DS1*058	1165
Total number	**16**

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