Innate Immune Mechanisms in Limiting HIV-1 Pathogenesis among South African adults and mother-infant pairs

by

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DECLARATION

This study presents original work by the author and this dissertation has never been submitted to this or any other university. As the candidate's supervisor, I agree to the submission of this dissertation.

The research described in this dissertation was done independently at HIV Pathogenesis Programme Laboratory and Hasso Plattner Research Laboratory, KwaZulu Natal, South Africa under the supervision of Dr. William Henry Carr. Another part of this work was done at the National Cancer Institute, National Institute of Health, Frederick MD, USA under the supervision of Dr. Arman Bashirova, Dr. Pat Bartman and Dr. Mary Carrington.

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BONGIWE NDLOVU DATE

9 February 2012

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DR. WILLIAM CARR DATE

DEDICATION

I dedicate this dissertation to my late mother who encouraged me in my educational and writing pursuits. She had been a pillar of strength and without her love and support, all would have been in vain.

To my family and friends: Thank you!!!

PUBLICATIONS/ PRESENTATIONS

1. **Ndlovu BG**, Danaviah S, Moodley E, Viljoen J, Newell M-L, Ndung'u T, Gao X, Carrington M and Carr WH (2010). The use of Dried Blood Spots for the Determination of Killer Immunoglobulin-Like Receptor (KIR) and HLA Gene Repertoires among South Africans. *HIV Pathogenesis Scientific Retreat, 21 October 2010*

2. **Ndlovu BG**, Danaviah S, Moodley E, Viljoen J, Newell M-L, Ndung'u T, Gao X, Carrington M and Carr WH (2009). The use of Dried Blood Spots for the Determination of Killer Immunoglobulin-Like Receptor (KIR) and HLA Gene Repertoires among South Africans.*South African Immunologists Society meeting, 09 December 2009*

3. **Ndlovu BG**, Danaviah S, Moodley E, Ghebremichael M, Bland R, Newell ML, Ndung'u T and Carr WH (2011). Use of dried blood spots for the determination of genetic variation of interleukin-10, killer immunoglobulin-like receptor and HLA class-I genes. *Journal of Tissue Antigens;* 1-9

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral
AW1	Wash buffer 1
AW2	Wash buffer 2
AE	Elution buffer
CCR5	Chemokine (CC motif) receptor 5
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
CXCR4	Chemokine (CXC motif) receptor 4
DAP 12	Adaptor protein 12
DNA	Deoxy-ribose nucleic acid
DBS	Dried blood spot
dNTP	Deoxynucleotide Triphosphate
dATP	Deoxyadenosine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dCTP	Deoxycytidine triphosphate

DMSO	Dimethyl sufoxide
EDTA	Ethylenediamine-tetraacetic acid
gDNA	Genomic deoxyribose nucleic acid
GSSP	Group sequencing-specific primers
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus subtype 1
HIV-MTCT	Human Immunodeficiency virus- mother to child transmission
HLA	Human leukocyte antigen
HLA-DRB1	Human leukocyte antigen-DRB1
HLA-A	Human leukocyte antigen-A locus
HLA-B	Human leukocyte antigen-B locus
HLA-C	Human leukocyte antigen-C locus
HLA-C1	Human leukocyte antigen-C group 1 allotype
HLA-C2	Human leukocyte antigen-C group 2 allotype
HLA-C1/C2	Human leukocyte antigen-C group 1/2 allotype
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12

IFN-γ	Interferon-gamma
ITAMs	Immunoreceptor tyrosine-based activating motifs
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
KIR	Killer cell immunoglobulin-like receptor
KIR2DL1	Two Immunoglobulin-like long cytoplasmic domains
KIR2DS1	Two Immunoglobulin-like short cytoplasmic domains
KIR3DL1	Three Immunoglobulin-like long cytoplasmic domains
KIR3DS1	Three Immunoglobulin-like short cytoplasmic domains
MDA	Multiple displacement amplification
MHC	Major histocompatibility complex
MIP 1-α	Macrophage inflammatory protein 1-alpha
MIP-1β	Macrophage inflammatory protein 1-βeta
NK	Natural killer cell
PBS	Phosphate Buffered saline
PCR	Polymerase chain reaction
PMTCT	Prevention of HIV-1 mother to child transmission
RANTES	Regulated upon Activation, Normal T-cell expressed and Secreted
RNA	Ribose nucleic acid

RT-PCR	Real time- Polymerase chain reaction
SBT-PCR	Sequence-based Typing-Polymerase chain Reaction
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
SSP-PCR	Sequence specific primer-polymerase chain reaction
TE	Tris-ethylenediamine-tetraacetic acid buffer
TNF-α	Tumour necrotic factor alpha
UV	Ultraviolet
WGA	Whole genome amplification
wgaDNA	Whole genome amplified DNA
WHO	World Health Organisation

ETHICAL APPROVAL

This study is a substudy of the HIV-1 vertical transmission study cohort. Ethical Approval was obtained under research protocol T050/01 approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal, Durban, South Africa, and the Internal Review Board of Massachusetts General Hospital.

ABSTRACT

This study was conducted to investigate the role of natural killer cell surface receptors, KIRs and their cognate HLA ligands in preventing HIV-1 acquisition and disease progression in HIV-1 exposed infants. Using DBS stored for 8 years from 21 pregnant South African women we evaluated 3 methods of gDNA extraction with and without whole genome amplification (WGA) to characterize immune-related genes: *IL-10*, *KIR* and *HLA* class I. However, IL-10 SNP typing was only for testing the quality of gDNA. QIAamp DNA mini kit yielded the highest gDNA quality ($p<0.05$; Wilcoxon Signed Rank Test) with sufficient yield for subsequent analyses. In contrast, WGA was not reliable for SSP-PCR analysis of *KIR2DL1*, *KIR2DS1*, *KIR2DL5*, and *KIR2DL3* or high resolution HLA genotyping using a sequence-based approach. A cohort of 370 infants; 124 HIV-1 perinatally infected, 120 exposed uninfected and 126 unexposed healthy infants was used for KIR and HLA genotyping. After adjustment for viral load and multiple comparisons, the frequency of *HLA-Cw*04:01* allele was likely to be associated with susceptibility to mother-to-child acquisition of HIV-1 in exposed infected (EI) infants ($p=0.05$; Logistic Regression analysis). *HLA-A*23:01* was likely to be associated with decreased CD4 T lymphocyte count in HIV-1 infected infants ($p=0.01$; ANOVA), whereas *HLA-B*81* tended to be associated with higher CD4 T lymphocyte count ($p=0.04$, ANOVA). We speculate that *HLA-Cw*04:01* interacts with *KIR2DL1* and inhibit NK cell responses which predispose the infants to HIV-1 infection. *KIR2DS1* and *KIR2DL5* were both associated with faster HIV-1 disease progression. Identified protective HLA-class I alleles could be used to present viral epitopes to either NK cells via KIRs or CTLs and enhance immune activation which may promote resistance to HIV-1 infection.

CHAPTER 1

1.1 INTRODUCTION AND LITERATURE REVIEW

Great progress has been made in the development of effective therapeutic strategies for the prevention of Human Immunodeficiency Virus type-1 (HIV-1) mother to child transmission (MTCT). However HIV-MTCT still remains a huge challenge in resource limited settings such as Sub-Saharan Africa. Without intervention, 15-35% of HIV-exposed infants become infected annually and HIV-MTCT accounts for more than 90% of paediatric HIV-1 infections (WHO, 2009; Ahmad, 2011, Winchester et al., 2004, Zhou et al., 2010). Women of child bearing age have the highest prevalence of HIV-1 infection in South Africa (Tiemessen and Kuhn, 2006; Ahmad, 2008). Most HIV-1 infections could be prevented if pregnant mothers can have unlimited access to effective preventative MTCT regimens and important medical interventions in developing countries.

In countries such as United Kingdom, United States, Brazil and Botswana, adherence to highly active antiretroviral therapy (HAART) regimen, elective caeserian section delivery and avoidance of breastfeeding have been shown to reduce HIV-1 MTCT to approximately 1-2% (Tiemessen and Kuhn, 2006; Taha et al., 2009, Zhou et al., 2010). However, 800 000 children world wide per year are still getting infected with HIV-1, even with the use of ARV regimens and 90% of these infections occur in Sub-Saharan Africa (WHO, 2009). The possible explanation for this could be that most women–especially in rural communities do not have access to HAART or do not adhere to self administered drugs, possibly due to the stigma attached to HIV-1 infection (Ahmad, 2011). An alternative explanation could be that selective caeserian section delivery and avoidance of breastfeeding are not safe or affordable in developing countries. If HIV-1 vaccines were available, most HIV-1 MTCT could be

prevented thereby, saving many lives. Human genetic variation may contribute significantly to the heterogeneity in HIV-1 pathogenesis (Joubert et al., 2010). Futhermore, several immune related genes have been shown to reduce HIV-1 transmission and disease progression to AIDS. More specifically, genes that encode receptors such as *Killer Immunoglobulin-like Receptors* (KIR) and their cognate *Human Leukocyte Antigen* (HLA) class I ligands have been shown to provide connection between innate and adaptive immune responses (Gaudieri et al, 2005; Martin et al, 2002; Martin et al, 2007). The interaction between activating and inhibitory polymorphic KIR receptors and their cognate HLA-class I ligands regulate NK cell responses (Rajagopalan and Long, 2000).

If it were possible to identify KIR/HLA compound genotypes that are associated with protection from HIV-1 mother-to-child transmission, this information could be used to design specific recombinant HLA-restricted HIV-1 peptides. We speculate that HIV-1 peptides would be presented by HLA class I molecules (HLA-restricted peptides) that associate with control of HIV. These HLA/peptide complexes would then be recognized and bind to specific activating KIR receptors on the surface of NK cells. This would result in NK cell activation and target cell lysis. A previous study showed that HLA-B Bw4-80I alleles that associate with protection from disease progression were also associated with the presence of KIR3DS1, an activating receptor. The authors presume that engagement of this receptor would lead to activation of NK cell-mediated killing and protection against HIV-1 infection (Martin et al., 2002).

The HLA/peptide complexes would also be recognized and bind to specific inhibitory KIR receptors on the surface of NK cells and result in inhibition of NK cell responses. However, if the individuals express high levels of inhibitory *KIR3DL1 (KIR3DL1*h)*, when the MHC class I is downregulated by HIV-1 *Nef*, the strong *KIR3DL1*-mediated inhibitory signal is

abrogated resulting in strong NK cell activation that may prevent HIV-1 infection. Previous studies showed that highly expressed inhibitory *KIR3DL1*h/*y* in combination with *HLA-B Bw4-80I*, particularly *HLA-B*57* was associated with reduced risk of HIV-1 progression to AIDS (Martin et al., 2007) and lower risk of HIV-1 infection (Boulet et al., 2008). Again, the authors presume that engagement of this receptor would lead to inhibition of NK cell-responses, however, the inhibitory signals will be lost when HIV-1 *Nef* downregulates HLA-B alleles, that would result in activation of NK cell-mediated killing and protection against HIV-1 infection.

HIV-1 Mother-to-child transmission studies offer several unique features in comparison with sexual transmission studies; both mother and infant can be investigated at the same time, it provides accurate timing of the infection and acute HIV-1 infection can be studied (Farquhar and John-Stewart, 2003). These studies also assist in understanding resistance to HIV-1 infection during repeated exposure to the virus and could play a significant role towards development of novel therapeutic and preventative intervention strategies (Winchester et al., 2004; Paximadis et al., 2011). Transmission of HIV-1 from mother to child may occur at one of three stages; pre-partum (during pregnancy), intrapartum (delivery) or postpartum (through breastfeeding) (Joubert et al., 2010).

The underlying mechanisms of transmission and HIV-1 acquisition during these various phases are not well-described. However, recent studies indicate that approximately 50% of infection takes place during labour due to infant's exposure to mother's blood in the birth canal (Ahmad, 2011). Most of these HIV-1 infected infants develop higher viral loads, severe symptoms of infection and progress rapidly to acquired immunodeficiency syndrome (AIDS) during their first month of life (Ahmad, 2011, Little et al., 2007). Infants have well developed innate immune responses, thus a full investigation on NK cell receptors is required to

understand activation and inhibition of neonatal NK cell responses. KIR receptors interact with HLA-class I ligands and results in activation or inhibition of NK cell responses. This study will investigate the role of neonatal natural killer cell surface receptors, KIRs and their cognate HLA ligands in preventing HIV-1 acquisition in HIV-1 exposed infants.

1.1.1 DNA isolation from dried blood spots

Whole blood sample collection from infants is difficult and requires expertise, however dried blood spots (DBS) collection provide a useful alternative. Like whole blood, DBS can also be used to detect biomarkers, however there is no standard procedure for genomic deoxyribose nucleic acid (gDNA) isolation from DBS. Different commercial and non-commercial methods have been identified for genomic DNA extraction from DBS samples; however, the most optimal method for the acquisition of gDNA with sufficient yield and quality to allow single nucleotide polymorphism (SNP) analysis remains undetermined. Previously, dried blood specimens collected on filter papers have been shown to be stable and non-infectious (Fischer et al., 2004, Jacob et al., 2008).

Initially, DBS samples were successfully used for neonatal diagnostic screening of inborn metabolic and inherited diseases in the United States (Guthrie and Susi, 1963, Yang et al., 2010). In low-income countries, DBS have been used for drug resistance testing and early detection of HIV-1 infection in infants by PCR (Bereczky et al., 2005, Fischer et al., 2004, Jacob et al., 2008, Patton et al., 2007, Sherman et al., 2005) and more recently by quantitative HIV RNA assay (Coovadia et al., 2007, Mehta et al., 2009, Newell et al., 2004). Moreover, DBS have shown high sensitivity and specificity in early HIV-1 detection in infants by real time PCR when compared to plasma (Viljoen et al., 2010).

Dried blood spots have also been widely used in programmes of prevention of mother-to-child transmissions because it allows the usage of small blood volumes for gDNA isolation from the infants (Bland et al., 2009, Coovadia et al., 2007). They have been used in adult surveillance and viral load testing (Johannessen et al., 2009, Lira et al., 2009). Dried blood spots have also been largely used in forensic science, more especially for DNA fingerprinting by restriction fragment length polymorphism (Ledray and Netzel, 1997). However, a reliable DNA extraction method from DBS samples has not been established. A standard method used for DNA extraction for DNA extraction for DNA fingerprinting was chelex®-100 (Ledray and Netzel, 1997). If we could identify a robust method for DNA extraction from DBS samples, then DBS samples could provide a viable alternative to whole blood samples for genetic analysis in resource-limited settings, particularly in Sub-Saharan Africa, where sample collection and transport from rural areas are often a limitation.

Fresh ethylene diamine-tetra acetic acid (EDTA) whole blood samples have been used as a source of gDNA, however there are various challenges in using specimens such as a lack of expertise to perform venipuncture on young infants, the specimens require processing within 4 days of collection, storage and transportation between 2-25^{o}C (Nsojo et al., 2010). DBS offer the following advantages over routine whole blood samples; feasible collection with a finger or heel prick, the use of a small volume of blood (50µl) and a phlebotomist is not required (Lira et al., 2009). Air-dried filter papers can be easily stored at room temperature and can be easily transported to laboratories for genotyping. This approach would also allow killer immunoglobulin-like receptors (KIR) and human leukocyte antigen (HLA) analysis of previously collected and stored DBS samples from established cohorts. These cohorts would provide a useful resource for additional host genetic studies, including KIR and HLA analysis. Moreover, DBS reduce the risk of needle stick injury which could result in HIV-1 infections of health professionals (Yang et al., 2010).

Genomic studies enhance our understanding of various diseases, however they require large amounts of good quality genomic DNA (Dean et al., 2002, Barker et al., 2004). Low DNA yield or quality obtained from DBS samples could be a limiting factor to perform reliable KIR/HLA genotyping. Theoretically, a 3.2mm disk produces approximately 60ng total gDNA using a Chelex-100 based method, which is insufficient for most genetic studies (Hannelius et al., 2005; Hollegaard et al., 2009a). However, newer technologies such as whole genome amplification (WGA) may provide practical solutions to these issues. In this study, we assess the feasibility of using WGA to increase the amount of a template for subsequent genetic analysis.

1.1.2 Whole genome amplification

Whole genome amplification (WGA) is an *in vitro* method of DNA amplification to allow further genetic analysis (Bergen et al., 2005). It increases the chances of performing various genetic studies from a limited amount of template (Sjoholm et al., 2007). Previously, two commercially available whole genome amplification methods: multiple displacement amplification (MDA) and Omniplex were compared (Barker et al., 2004, Bergen et al., 2005, Hollegaard et al., 2009a). From these studies, MDA produced the most concordant genotyping results in comparison with unamplified gDNA derived from whole blood of the same individuals. Furthermore, Hollegaard et al (2009 b) obtained reliable SNP genotyping from a single 3.2mm disk of DBS sample stored for approximately 21 years. Multiple displacement amplification was suitable for un-fragmented DNA and produced strands approximately 10kb long, whereas Omniplex was suitable for fragmented DNA and produces libraries of shorter fragments approximately 500bp long (Hollegaard et al., 2009a).

Multiple displacement amplification relied on ϕ29 DNA polymerase and random primers to amplify longer fragments of gDNA (Hollegaard et al., 2009b). Whole genome amplification technologies have been used to amplify limited quantities of DNA to facilitate genetic association studies (Alsmadi et al., 2003, Barker et al., 2004, Berthier-Schaad et al., 2007, Singh and Spector, 2007, Sjoholm et al., 2007, Hollegaard et al., 2009a, Hollegaard et al., 2009b, Chainonthee et al., 2010). These studies used MDA-derived DNA to genotype various SNPs, including KIR and HLA-class I analysis and found high fidelity and good concordance rate between whole genome amplified and genomic DNA. However there was a loss of limited number of loci that failed to amplify (Paynter et al., 2006, Berthier-Schaad et al., 2007, Chainonthee et al., 2010). Using this approach, Chainonthee et al., (2010) demonstrated the feasibility of whole genome amplification from whole blood to perform *KIR* and *HLA-C* genotyping by sequence specific primer (SSP)-PCR. They found discrepancies in KIR typing results for *KIR2DS1*, KIR*2DL1*, *KIR3DS1* and *KIR3DL1* genes. Exon 4 sequence analysis revealed that it was due to cross-priming between *KIR2DL1/KIR2DS1* and *KIR3DL1/KIR3DS1* pairs. Consequently, they re-designed their primers and updated the KIR typing protocol for wgaDNA. Furthermore, they found no difference in the HLA-C epitopes between whole genome amplified (MDA-derived DNA) and native genomic DNA as template for KIR SSP-PCR. However, they used whole blood derived DNA as a template for whole genome amplification and they did not assess other epitopes of HLA-class I. Moreover, Singh and Spector (2007) found 100% concordance in both HLA class I and II alleles between wgaDNA and gDNA derived from dried blood spot samples. Our study extends the findings of these studies by comparing methods of gDNA isolation and evaluating the feasibility of WGA for complete KIR and HLA gene repertoire determination. Unlike previous studies our analysis of KIR and HLA repertoires is comprehensive and includes most genes found among South Africans.

The objective of the study was to assess whether wgaDNA provide a feasible template for KIR and HLA genotyping by standard methods. Previous studies have shown that the reliability of genotyping results when using wgaDNA is proportional to the amount of gDNA template used for WGA but not affected by the age of the DBS sample (Hollegaard et al., 2009b). They found no correlation between the amount of template DNA used for WGA and the amount of DNA output (Hollegaard et al., 2009b). Thus, we predict that concordant KIR and HLA genotyping results will be obtained between whole genome amplified DNA and genomic DNA derived from whole blood. Moreover, whole genome amplified DNA is a true representative of the gDNA therefore the results obtained with both methods will be identical.

1.1.3 Multiple displacement amplification (MDA)

Multiple displacement amplification is a non-PCR based uniform isothermal amplification, it results in uniform amplification and produces large amounts of DNA regardless of the amount of gDNA template used (Shoaib et al., 2008). It was designed to amplify large circular DNA templates for example, plasmids and bacteriophages (Dean et al., 2002). Random exonuclease-resistant primers and high fidelity ϕ29 DNA polymerase isolated from *Bacillus subtilis* bacteriophage are used due to its proof reading and strong strand displacement activity (Lasken, 2009). Firstly, denaturation occurs, followed by annealing of random hexamer primers at several sites of the template DNA strand, this is followed by initiation of DNA synthesis and elongation carried out by a ϕ29 DNA polymerase (Fig 1.1). This reaction occurs at an isothermal condition of 30^{o} C and the enzyme has a proof reading-mechanism which prevents accumulation of mutations.

Previous studies have shown that the ϕ29 DNA polymerase adds 70 000 nucleotides each time it binds to the primer template (Lasken, 2009). The following step is strand displacement, ϕ29 DNA polymerase removes a newly formed single stranded DNA and allows new primers to anneal to the new template strand (Fig 1.1). This is followed by elongation of primers on the newly formed strand and formation of double stranded DNA. Random primers are linked by thiophosphate bonds at the 3' end of the nucleotide and they are resistant to all 3'-5' exonuclease, particularly, ϕ29 DNA polymerase thus to avoid degradation of primers during gDNA synthesis (Lasken, 2009). Whole genome amplified DNA can be used in various applications such as genetic analysis of single nucleotide polymorphism. This study is unique in its application of cutting edge technologies to address the need for reliable gDNA isolation methods and characterising genotypes from stored dried blood samples. Many clinical trials have archived DBS samples, however, the methods for obtaining optimal and reliable genetic information from these samples have not been well established.

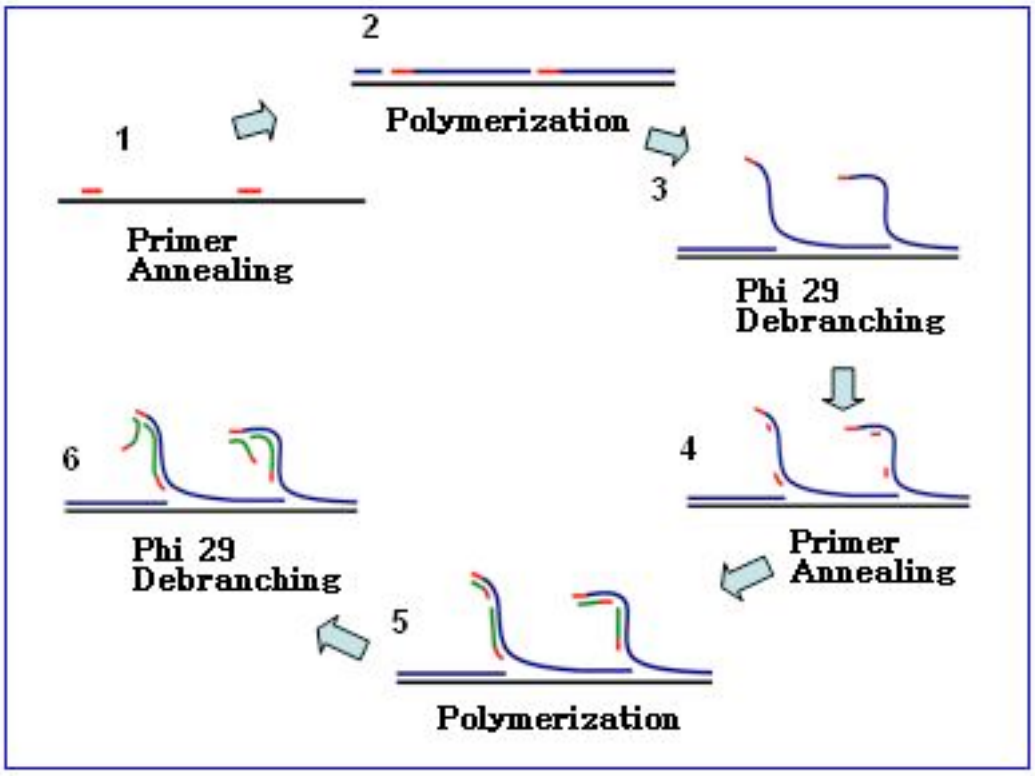


Figure 1.1: Schematic diagram of whole genome amplification by polymerase based-multiple displacement amplification. (http://en.wikipedia.org/wiki/File:MDA_reaction_1.JPG; 21 June 2011; 14:02).

1.1.4 The role of natural killer cells in HIV-1 infection

Natural Killer (NK) cells are critical components of innate immunity and they provide the first line of defence against viral infections (reviewed by Biron et al., 1999). They are large granular lymphocytes mainly derived from bone marrow and they comprise approximately 10% of the total blood lymphocytes in healthy individuals (Ward and Barker, 2008; Cooper et al., 2001). NK cells were first described as null cells due to the lack of T or B cell receptors, however over the past few decades it has been recognized that they are more

complex than simple killers (Alter et al., 2009, Tiemessen et al., 2009). NK cells recognize and directly kill tumour and virally infected cells early before viral replication occurs, thereby preventing viral replication (Biasin et al., 2010, Alter et al., 2004, Cooper et al., 2001, Iannello et al., 2008).

Unlike T cells, NK cells lack antigen-specific receptors such as the T-cell receptor. However they can recognize and preferentially kill aberrant cells that lack expression of MHC-class I molecules through a process referred to as the missing self hypothesis (Ljunggren and Karre, 1991; Karre, 1986). The missing self hypothesis, which was first proposed by Klaus Karre (Karre 1986, Karre 2002), states that the absence of MHC-class I molecules on a normal hematopoietic cell is enough to make it susceptible to NK-mediated killing. Under normal conditions, the healthy cells express MHC-class I molecules that interact with dominant inhibitory KIRs on the surface of NK cells and result in inhibition of NK cell-mediated killing of the normal cells (Fig 1.2B). Sometimes, inhibitory KIRs may interact with ligands apart from MHC-class I molecules and result in inhibition of NK cell responses and this is known to protect the cells that normally lack MHC such as erythrocytes (Fig 1.5A) (Lanier, 2005). Inhibitory KIRs monitor the cells that lack MHC expression "missing self" as a result of downregulation of MHC-class I molecules, which occurs frequently in HIV-1 infected, or malignant cells. In the absence of MHC-class I molecules, activating receptors interact with stress inducible ligands and results in activation of NK cell-mediated killing of the target cell (Fig 1.2-C). When the target cell expresses both inhibitory and activating ligands, NK cell responses are determined by the balance between activating and inhibitory signals (Fig 1.2D).

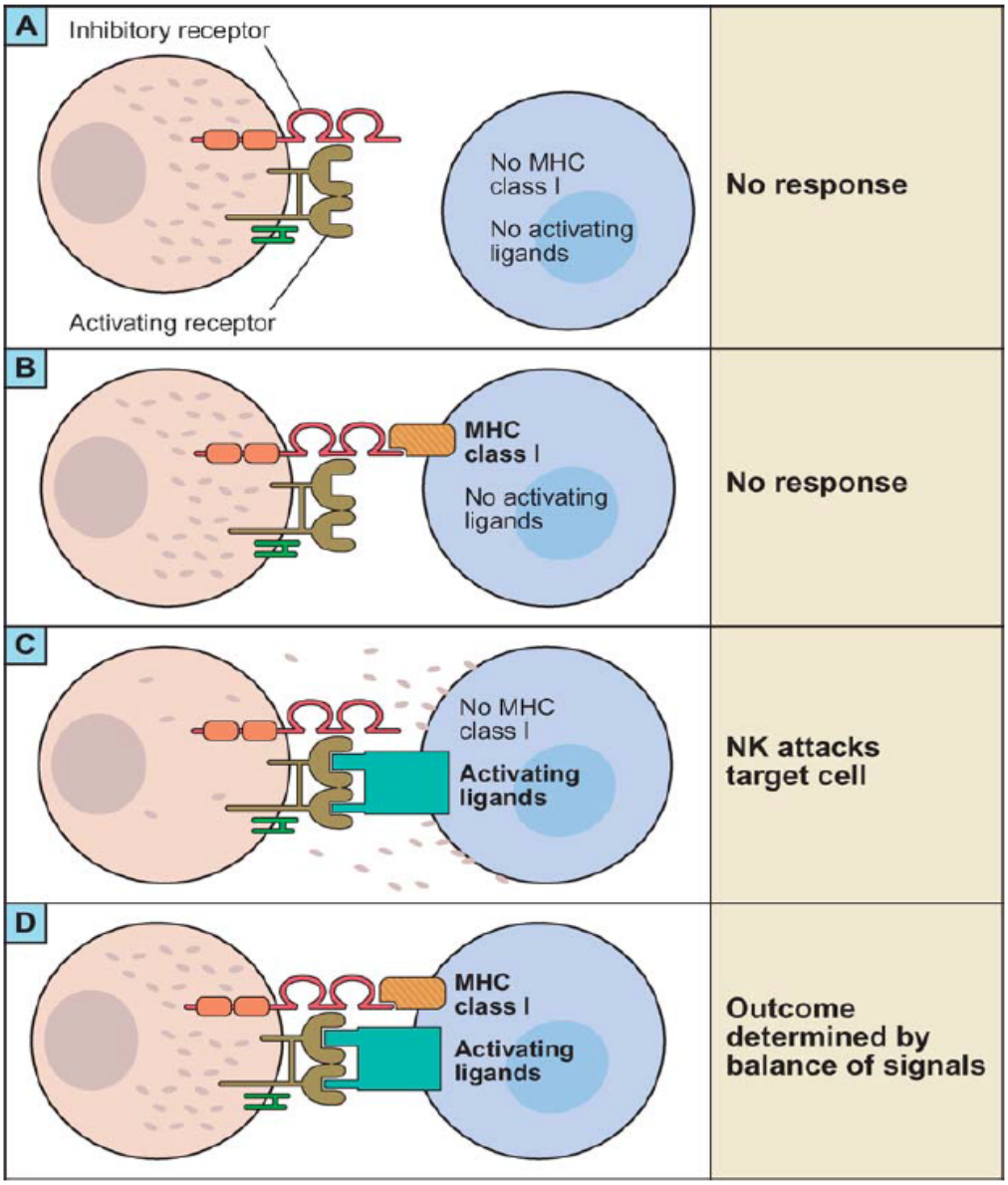


Figure 1.2 A-D: The missing self hypothesis. **A:** Under normal conditions, Inhibitory receptors may recognize other ligands, apart from MHC and inhibit NK cell killing to protect erythocytes and other normal cells that lack MHC class I molecules.

B: Inhibitory KIR receptors bind to their cognate HLA-class I ligands and inhibit NK cell responses to protect healthy cells from NK cell-mediated killing. **C:** In the absence of MHC-class I alleles, activating alleles may interact with stress inducible ligands and results in activation of NK –cell responses. **D:** When the target cell expresses both inhibitory and activating ligands, NK cell responses are determined by the balance between activating and inhibitory signals (Lanier, 2005; Annu. Rev. Immunol 23:225-274).

Previous research revealed that HIV-1 has an ability to evade the host immune responses. Specifically, HIV-1 *nef* encoded protein downregulates the expression of HLA-A and B molecules on the surface of infected cells and initiates viral escape from cytotoxic T lymphocyte recognition (Cohen et al., 1999; Williams et al., 2002; Collins et al., 2005). Theoretically these HIV-1 infected cells would be susceptible to NK cell-mediated killing due to downmodulation of the MHC-class I molecules. However, HLA-C remain on the surface of HIV infected cells, it interacts with inhibitory KIR receptors such as *KIR2DL1* and *KIR2DL2* and lead into inhibition of NK cell-mediated lysis and this is known as the 'double escape mechanism' (Cohen et al., 1999).

Previous research has shown that NK cells are the first cytotoxic cells to be activated and increased during acute HIV-1 infection, but they decline drastically during chronic HIV-1 disease, even though their activity remains enhanced (Fig 1.3) (Alter et al., 2004, Alter et al., 2007, Ravet et al., 2007). In response to HIV-1 infection, activated NK cells kill infected cells by natural cytotoxicity: they secrete perforins, granzyme B and granulosysin to initiate the target cell lysis (Ravet et al., 2007, Iannello et al., 2008). They also produce high levels of Th1 immunoregulatory cytokines such as IFN-γ, TNF-α and β-chemokines (MIP-1α, MIP-1β and RANTES) (Fig 1.3) reviewed by Alter and Altfeld, (2009). NK cell-mediated production of IFN-γ and NK-dendritic cell cross talk provide a major link between innate and adaptive immunity (Fig 1.3) (Ravet et al., 2007), leading into activation of effector functions of adaptive immunity (Iannello et al., 2008, Alter et al., 2009). However, a previous study on HIV-1 mother to child transmission found no difference in NK cell production of IFN-γ and TNF-α between exposed uninfected (EU) and HIV-1 infected children (Ballan et al., 2007).

NK cell activity has been associated with protection from HIV-1 infection among exposed, uninfected individuals (EU) (Scott-Algara et al., 2003, Ravet et al., 2007, Ballan et al., 2007, Boulet et al., 2008a, Boulet et al., 2008b, Iannello et al., 2008). Vietnamese exposed, uninfected intravenous drug users had higher cytolytic activity in vitro and higher production of cytokines such as INF-γ, TNF-α, α and β-chemokines compared to HIV-1 infected and healthy uninfected groups (Scott-Algara et al., 2003). Previous research on EU adults suggested a protective role of NK cells towards HIV-1 infection (Scott-Algara et al., 2003, Ravet et al., 2007).

A recent study on exposed, uninfected infants has shown that higher levels of $CD3^-$ lymphocyte responses to HIV-1 peptides were associated with protection against mother-to-child transmission or acquisition of HIV-1 (Tiemessen et al., 2009). They showed that NK cells respond with high specificity and great extent to HIV-1 peptides from Env and Regulatory proteins, however, heat-inactivated serum had no effect on HIV peptides, therefore they suggested that complement did not play a role in activation of NK cell-responses (Tiemessen et al., 2009). In contrast, other studies reported an antibody-dependant HIV-1 peptide-specific IFN-γ expression by NK cells towards Env, Pol and Vpu (Stratov et al., 2008; Chung et al., 2009). More recently, Chung et al (2011) also found antibody-dependant cytotoxicity complex (ADCC) responses to either gp-140 Env protein or HIV-1 peptides in NK cells from HIV-1 positive subjects, however there responses did not correlate with markers of HIV disease progression. This study indicates the role of antibody-mediated NK cell activation during HIV-1 infection. Several studies conducted in mice suggest that NK cells may respond to specific viral antigens and may generate antigenic recall responses (memory) (Arase et al., 2002; Kielczewska et al., 2009; Cooper et al., 2009). Taken together,

these studies support hypothesis of a "memory" response in NK cells. However, antigen-specific response by human NK cells have not been identified and biomarkers of NK cell memory in human have not been established. This is quite controversial as NK cells have been previously shown to lack memory function (Lanier, 2005).

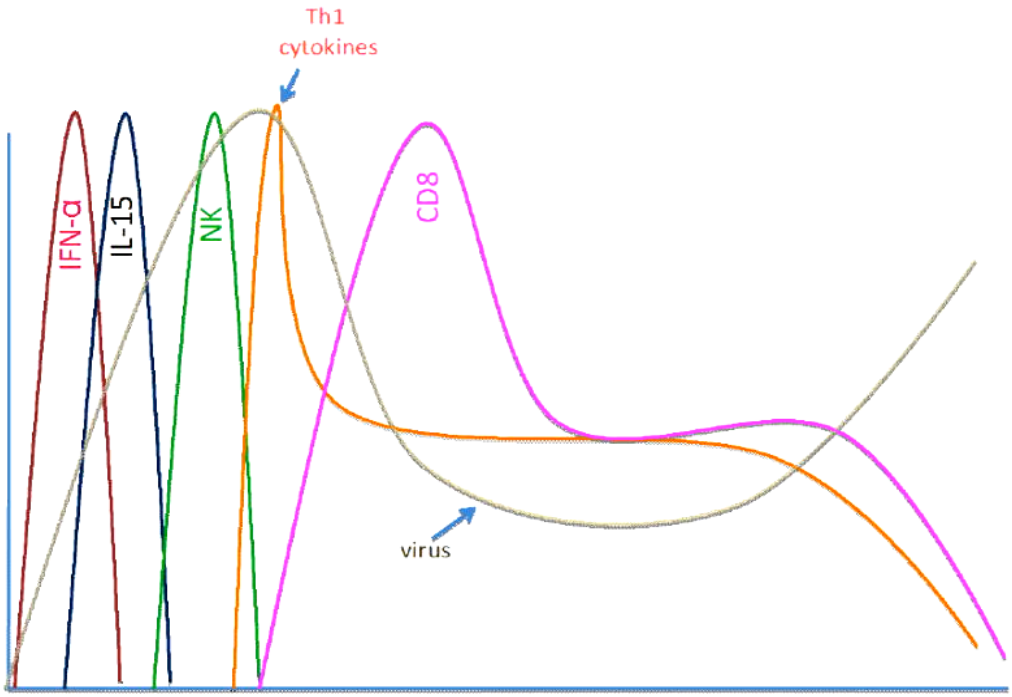


Figure 1.3: Innate and adaptive immune response to acute HIV-1 infection. During acute HIV-1 infection type 1 interferons are released, followed by secretion of interleukin 15 which results in rapid expansion of NK cells that leads into regulation of HIV-1 replication, production of Th1 chemokines and cytokines that link innate to adaptive immunity by recruiting CD8 T cell immune response (Alter and Altfeld, 2009. *Journal of Internal Medicine* ***265****: 29-42*).

1.1.4.1 Killer Immunoglobulin-like receptors

There are 15 KIR polymorphic genes and 2 pseudogenes located on chromosome 19q13.4 and encoded within 100-200kb region of the leukocyte receptor complex. They are type I intergral membrane glycoproteins expressed on NK cells and CD8 T cells indicating their role in both innate and adaptive immunity (Lebedeva et al., 2007, McQueen et al., 2007, Martin et al., 2007, Gardiner, 2008, Merino et al., 2011, Paximadis et al., 2011). Killer immunoglobulin-like genes encode cell-surface proteins that regulate activation and inhibition of NK cell responses through their recognition of HLA class I molecules on potential target cells; and thus, they may impact on susceptibility to HIV-1 infection (Martin et al., 2002, Carrington et al., 2008).

Classification of KIR genes depend on the length of their cytoplasmic tails and number of immunoglobulin-like domains; they either have short "S" or long "L" cytoplasmic tails and two or three Ig-like extracellular domains (2D/ 3D) which determine ligand-binding specificity (Marsh et al., 2003, Jennes et al., 2006, McQueen et al., 2007) (Table 1.1). Inhibitory receptors have two immuno-receptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tail. These motifs interact with Src homology proteins that function as tyrosine phosphatases (Uhrberg et al., 1997, Christensen and Geisler, 2000, Lebedeva et al., 2007, Vivier et al., 2004, Gardiner, 2008, Yu et al., 2008). In comparison, activating receptors have short (DS) cytoplasmic tails with lysine residue in their trans-membrane domain which interacts with tyrosine-based activation motif (ITAM) containing adapter molecules upon NK cell activation (Marsh et al., 2003, McQueen et al., 2007, Lebedeva et al., 2007, Merino et al., 2011). An exception is the *KIR2DL4* receptor which can both activate and inhibit NK cell responses (Faure and Long, 2002).

Previous studies have found that Africans have higher frequencies of *KIR3DL1, KIR2DS2, KIR2DS4* and lowest frequencies of *KIR2DS5, KIR3DS1, KIR2DL5* and *KIR2DS1* compared to South Asians (Norman et al., 2002). Inhibitory KIR proteins with two Ig-domains (2D) recognise HLA-C allotypes (Uhrberg et al., 1997; Long, 2008). *KIR2DL1* recognise and bind C2 allotypes (HLA-Cw2, -Cw4, -Cw5, -Cw6, -Cw15, -Cw17 and -Cw18) which carry asparagine at position 77 and lysine at position 80 of of α-1 helix (Table 1.1) (Khakoo et al., 2004; Middleton et al., 2002; Ravet et al., 2007). Whereas both *KIR2DL2* and *KIR2DL3* recognise and bind HLA-C1 allotypes (HLA-Cw1, -Cw3, -Cw7, -Cw8 and -Cw12, -Cw14 and Cw16) which carry serine at position 77 and asparagine at position 80 of the α-1 helix (Ravet et al., 2007; Moretta et al., 1993; McQueen et al., 2007; Norman et al., 2002; Long, 2008). *KIR3DL2* recognise and bind HLA-A3 and HLA-A11 allotypes (Table 1.1) (Ravet et al., 2007; Jennes et al., 2006). Even though ligands for activating KIR genes have not been fully defined, previous studies reported a weak affinity of *KIR2DS1* for C2 (Biassoni et al., 1997) and *KIR2DS2* for C1 (Wagtmann et al., 1995a) (Table 1.1). It has been shown previously that activating KIR receptors use DAP12 adaptor protein to facilitate activation of NK cells (Carr et al., 2007). Inhibitory KIR genes with three Ig-domains (3D), particularly *KIR3DL1* recognise HLA-Bw4 epitopes that carry isoleucine at position 80 (HLA-Bw4-I80) (Uhrberg et al., 1997; Gumperz et al., 1995; Martin et al., 2007).

In healthy individuals, inhibitory KIRs recognise and bind their cognate HLA-class I ligands on the surface of target cells and protect the cells from NK cell-mediated destruction (Bryceson and Ljunggren, 2008; Yu et al., 2008). For example, *KIR2DL1* binds to HLA-C2 ligand on the target cell and results in activation of Src family kinases which in turn lead into phosphorylation of tyrosine residues of the ITIMS (Bryceson and Ljunggren, 2008, Yu et al., 2008). Phosphorylated ITIMS recruit and activate tyrosine phosphatases (SHP1 and SHP2) to bind to the inhibitory KIR through SH2 domains (Long, 2008). A previous study has shown

that β-arrestin 2 interacts with *KIR2DL1* to further initiate binding of SHP to the inhibitory receptor (Yu et al., 2008). Binding prevents phosphorylation of tyrosine residues on activating receptors such as Vav 1 and results in inhibition of NK cell effector functions (Bryceson and Ljunggren, 2008). These mechanisms will help us to understand how NK cell responses get activated or inhibited by KIR receptor and HLA-class I allotype interaction. We predict that HIV-1 exposed, uninfected infants have higher number of activating KIR interactions with cognate HLA ligands that enhance phosphorylated ITAMS to stimulate NK cell activation.

KIR gene	Signalling	Ligand	Antibody
2DL1	Inhibitory	HLA-C2	EB6, HP-MA4
2DL3 (including 2 DL2)	Inhibitory	HLA-C1	GL183, DX27
2DL4	Activatory	HLA-G	mAb 33
2DL5	Inhibitory	not known	UP-R1
3DL1	Inhibitory	HLA-Bw4	DX9, Z27, 5.133
3DL2	Inhibitory	HLA-A3, A11	DX31, 5.133, Q66
2DS1	Activatory	HLA-C2	EB6
2DS2	Activatory	HLA-C1	GL183, DX27
2DS3	Activatory	not known	none
2DS4	Activatory	not known	5.133
2DS5	Activatory	not known	none
3DS1	Activatory	HLA-Bw4?	Z27
3DL3	Not known	not known	CH21
2DP1, 3DP1	Not expressed		

Table 1.1: Killer Immunoglobulin-like receptors with their cognate HLA-ligands and corresponding antibody binding. HLA-C2 and C1 refer to HLA-C ligands that have lysine and asparagine at position 80 respectively. HLA-Bw4 refers to alleles with isoleucine or threonine at position 80 (Gardiner, 2008. *International Journal of Immunogenetics,* **35**:1-8).

1.1.4.2 The Role of KIRs in HIV-1 infection

The KIR repertoire determines whether NK cells are activated or inhibited by the recognition of HLA-class I molecules on potential target cells; and thus, it may impact on susceptibility to HIV-1 infection (Jennes et al., 2006, Yawata et al., 2006, Ballan et al., 2007, Carrington et al., 2008). In this manner, the susceptibility or resistance to HIV-MTCT may be determined by particular KIR/HLA compound genotypes in the mother or infant.

A previous study revealed that resistance to HIV-1 infection among African HIV-1 exposed, uninfected female sex workers (FSWs) is associated with inhibitory receptor *KIR2DL2/KIR2DL3* heterozygosity in the absence of its cognate ligand HLA-C1 and likewise, *KIR3DL1* receptor in the absence of HLA-Bw4 (Jennes et al., 2006). Furthermore, exposed, uninfected female sex workers had significantly higher frequencies of inhibitory receptors, particularly, *KIR2DL2, KIR2DL5* and heterozygous *AB* haplotype compared seropositive FSWs (Jennes et al., 2006). Another study in Zambian serodiscordant heterosexual couples found that in the absence of *HLA-Cw*04, KIR2DS4*001* was associated with higher risk of HIV-1 transmission and relatively higher viral load (Merino et al., 2011). The receptor, *KIR2DS4*001* in combination with its cognate ligand *HLA-Cw*04* was associated with increased rate of HIV-1 transmission in discordant couples, whereas *HLA-Cw*04* alone was not associated with the risk of HIV-1 transmission (Merino et al., 2011).

Previous research has also revealed that *KIR3DS1* receptor in combination with the *HLA-B Bw4-80I* allotype was associated with slower HIV-1 disease progression to AIDS (Martin et al., 2002). In contrast, in the absence of *KIR3DS1* receptor, HLA-Bw4-80I was not associated with HIV-1 clinical outcome, however, in the absence of *HLA-Bw4-80I, KIR3DS*1 receptor

was associated with rapid progression of HIV-1 to AIDS defining illness (Martin et al., 2002). Taken together, these findings suggest that KIR and HLA genotypes function alone or synergistically in causing resistance to HIV-1 transmission and disease progression to AIDS in both adults and infants.

Prior research on HIV-1 exposed uninfected infants found higher levels of a degranulation marker, CD107, lower expression of inhibitory *KIR2DL1, KIR3DL1, KIR2DL3* and *NKG2C* in HIV-1 EU compared to HIV-1 infected infants (Ballan et al., 2007). Furthermore, they found a negative correlation between NKp46 and the frequencies of $CD4^+$ T cells. In contrast, a recent study in Chinese HIV-1 infected children found a higher expression of *KIR2DL1/KIR2DS1, KIR3DL1, NKG2D* and lower cytolytic activity in uninfected children compared to HIV-1 infected children (Fu et al., 2010). The difference in cytotoxicity could be caused by the difference in composition of the cohorts. One study was conducted in HIV-1 perinatally exposed infected infants (Fu et al., 2010). While the other study included prenatally and postnatally HIV-1 exposed, infected children (Ballan et al., 2007). Another possible explanation could be the difference in ethnicities. One study was performed among African Americans, whereas the other among indigenous Chinese respectively. KIR and HLA repertoires are different between the two ethnical groups which could lead to differences in the genes involved in protection or susceptibility to HIV-1 infection.

A study in South African maternal-infant HIV-1 transmission revealed that intrapartum transmitting mothers (IP) had decreased frequencies of *KIR2DL2* and *KIR2DL3* genes. Transmitting mothers had higher homozygosity for *KIR2DL3* receptor in combination with HLA-C allotype heterozygosity (C1/C2) compared to non-transmitting mothers (Paximadis et al., 2011). On the other hand, HIV-1 infected infants had lower frequencies of *KIR2DL3* receptor in combination with its ligand HLA-C1 as well as lower frequencies of *KIR2DL3* receptor homozygosity in combination with -*C1/C2* in comparison with EU infants (Paximadis et al., 2011). This study suggests that *KIR2DL2/KIR2DL3* receptors together with their ligands *HLA-C1/C2* are associated with lower risk of HIV-1 infant's acquisition in South African population.

KIR3DL1 receptor in combination with its cognate ligand HLA-Bw4 has been associated with lower risk of HIV-1 transmission in exposed uninfected individuals (Boulet et al., 2008a) and slower disease progression to AIDS (Martin et al., 2007). There is a variation in binding affinity of *KIR3DL1* towards *HLA-Bw4* and *KIR3DL1* can be divided into three groups. *(i) KIR3DL1* high expression allotypes (*KIR3DL1*h)*, *(ii)* low expression allotypes (*KIR3DL1*l)* and *(iii)* intercellular allotypes (*KIR3DL1*004* (*y*) (Martin et al., 2007, Boulet et al., 2008a). *KIR3DL1*h* allotypes have a higher binding affinity for *HLA-Bw4I80* and the strongest protective effect towards HIV-1 disease progression (Martin et al., 2007) and HIV-1 infection (Boulet et al., 2008a). It was established that, the combination of *HLA-Bw480I* and *KIR3DL1h/y* was protective against AIDS progression (Martin et al., 2007). *KIR3DL1* shares 97% homology on the sequence with *KIR3DS1*, therefore HLA-Bw4-I80 is a possible ligand for *KIR3DS1*. The interaction between *KIR3DS1* receptor and its ligand, which has not been identified yet, leads to activation of NK cell responses and the interaction between *HLA-*

Bw4 and *KIR3DL*1 leads to inhibition of NK cell responses. A previous study by Boulet et al., (2008b) revealed that exposed, uninfected individuals had increased *KIR3DS1* homozygosity compared to HIV infected patients and that could contribute towards NK cell activation and resistance to HIV acquisition among African Americans.

These genetic studies specifically identified KIR/HLA genotypes that are associated with HIV-1 transmission and acquisition. Collectively, they suggest that NK cells play a crucial role in protection from HIV acquisition. However, most of these studies were performed on adults and little associations are known between HIV-1 transmission and mother-to-child pairs in Sub-Saharan Africa. In this study, we aim to identify KIR genes that may be associated with protection or susceptibility to HIV-1 MTCT acquisition to understand the role of KIR genes in infant HIV-1 acquisition.

1.1.4.3 KIR Haplotypes

Based on genetic content, KIR gene repertiores can be divided into haplotype A and haplotype B. Both KIR haplotype A and B have *KIR3DL3* on their centromeric end, *KIR3DL2* on their telomeric end, central *KIR2DL4* and *KIR3DP1* which are known as "framework genes" (Fig 1.4) (Norman et al., 2002, McQueen et al., 2007, Gardiner, 2008). Haplotype A is defined by a restricted number of genes: a single gene encoding an activating receptor, *KIR2DS4* and 4 genes encoding inhibitory receptors; *KIR3DL2, KIR2DL1, KIR2DL3* and *KIR3DL1* (Marsh et al., 2003, Gardiner, 2008). Conversely, haplotype B is defined by 2 inhibitory KIR gene; *KIR2DL2, KIR2DL5* and a variable number of activating KIR genes; *KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5* and *KIR3DS1* (Marsh et al., 2003, Yawata et al., 2006). In summary, haplotype B has many genes encoding activating KIRs

whereas haplotype A has more genes encoding inhibitory KIRs. Previous research found that Caucasians and Japanese populations have higher frequencies of KIR haplotype A, whereas African population have an equal distribution of haplotype A and B frequencies (Norman et al., 2002). It has been shown previously that haplotype B is associated with faster CD4 T-cell decline and rapid progression to AIDS, however haplotype B had no effect on markers of disease progression (Jennes et al., 2011; Gaudieri et al., 2005). Previous research on HIV-1 mother-to-chid transmission has shown that there was no difference in the haplotypes A and B between EU and HIV-1 infected children (Ballan et al., 2007; Paximadis et al., 2011). However, these findings must be validated with larger cohorts, we extended their findings by including HIV-1 unexposed healthy infants to represent the general population of Sub-Saharan African infants. Thus we aim to identify KIR haplotypes that could be associated with protection or susceptibility to HIV-1 mother-to-child acquisition. Based on these studies, we predict that haplotype B is associated with resistance towards HIV-1 acquisition.

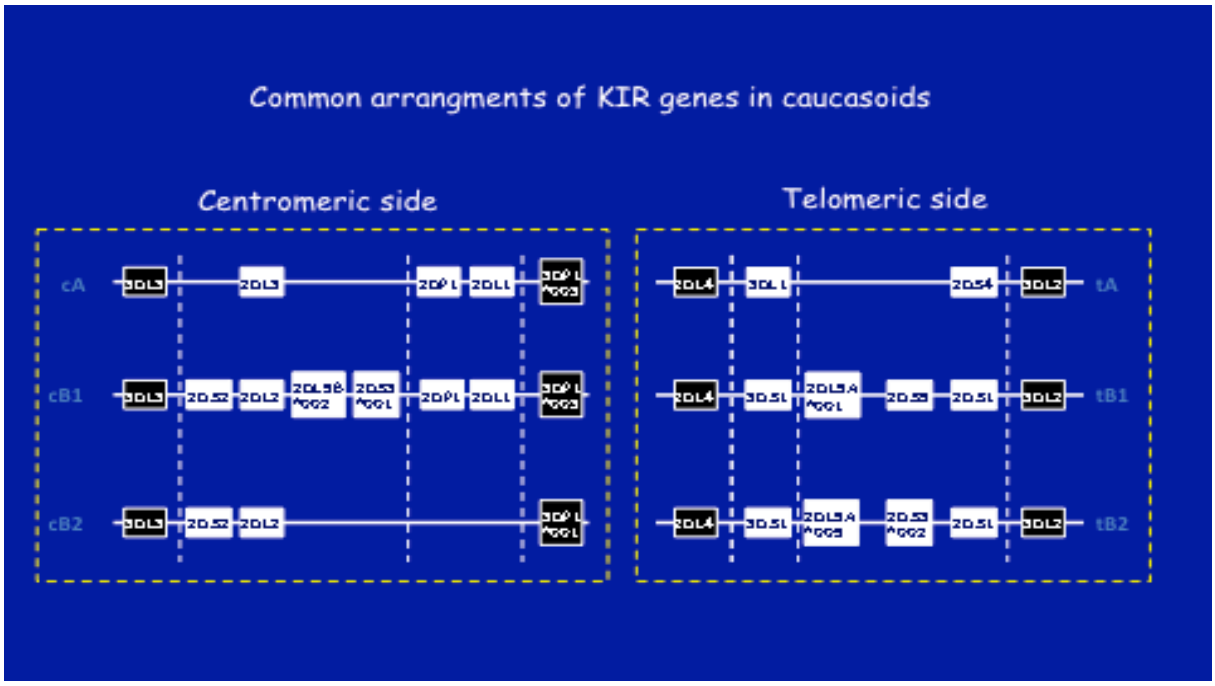


Figure 1.4: Sketchical diagram showing the centromeric, central and the telomeric ends of KIR Haplotypes in Caucasians (Ordonez et al., 2008. *Genes and Immunity* 9:431-437).

1.1.5 Human Leukocyte Antigen (HLA)-Class I in HIV-1 Transmission

Human leukocyte antigen (HLA) commonly known as the major histocompatibility complex (MHC)-class I molecule is the most polymorphic loci in the human population genome and it is located at chromosome 6p21.3 (Beck et al., 1999). There are two important functions of HLA-class I molecules in mitigating HIV-1 pathogenesis. They process and present HIV-1 derived epitopes on the surface of infected cells to CD8 cells and results in activation of the cytotoxic T cell response (Welzel et al., 2007, Thomas et al., 2009). They also act as ligands to natural killer cell surface receptors, killer immunoglobulin-like receptors (KIR) to allow activation or inhibition of NK cell responses (Jennes et al., 2006, Welzel et al., 2007, Thomas et al., 2009). Previous studies have shown that HLA-class I may influence susceptibility or protection from HIV-1 infection (Fabio et al., 1992; Macdonald et al., 1998; Polycarpou et al., 2002; Winchester et al., 2004; Ballan et al., 2007, Mackelprang et al., 2008, Paximadis et al., 2011).

Specifically in mother-to-child transmission (MTCT), viral load has been shown to be the strong risk factor for both intra-partum and *in utero* HIV-1 transmission, whereas low CD4 T lymphocyte count was only associated with intrapartum transmission (Mock et al., 1998). However, previous research also revealed that genetic variation may protect or predispose exposed individuals to HIV-1 infection (Winchester et al., 2004; Paximadis et al., 2011). We speculate that despite viral load, KIR and HLA genotypes influence HIV-1 mother-to-child acquisition. Previous research revealed that mother-to-child HLA concordance was associated with higher risk of HIV-1 mother-to-child transmission (Polycarpou et al., 2002; Mackelprand et al., 2008).

On the other hand, maternal HLA-class I homozygosity was also associated with increased susceptibility to perinatal HIV-1 mother-to-child transmission *in utero*, during labour and via breastfeeding (Macdonald et al., 1998; Mackelprang et al., 2008). These studies suggest that increased risk of HIV-1 mother-to-child transmission in homozygous HLA-class I mothers is probably due to decreased alloimmunity and less diverse immune response towards maternal HIV-1 virus. Whereas on the contrary, recent studies did not find any association between mother-child HLA allele concordance or maternal HLA homozygosity and mother-to-child HIV-1 transmission in South African mother-infant pairs (Paximadis et al., 2011). This could be due to geographical differences between the two populations, most of these studies were performed in West African (Kenyan) population which has a higher epidemic of malaria and this could influence susceptibility to HIV-1 infection, however, follow up studies are required to validate these findings in different African mother-infant cohorts. A recent study found that HLA-C had no effect in HIV-1 MTCT or acquisition, however, HLA-B was the only locus that influenced HIV-1 mother-to-child transmission (Paximadis et al., 2011).

A previous study evaluated associations between HLA-class I and the risk of HIV-1 mother-to-child transmission and discovered that *HLA-B*67* and *-B*58* among infants were associated with higher risk while *-B*12* was associated with protection from HIV-1 acquisition (Polycarpou et al., 2002). Another study by Farquhar et al (2004) found that *HLA-B*18* was associated with lower risk of HIV-1 early and breast milk acquisition in Kenyan mother-infant pairs (Farquhar et al., 2004). It has been shown that when the infant carries protective HLA alleles such as *-B*57* or *-B*27*, these alleles do not confer protection from HIV-1 infection if the maternally transmitted virus has an escape mutation in their Gag epitopes (Goulder et al., 2001).

Previous research on Women and Infants Transmission Study (WITS) has shown that almost half (48%) of mothers who transmitted HIV-1 disease to their infants with lower viral loads had higher frequencies of *HLA-B*13:02*, *-B*35:01*, *-B*44:02* and *-B*50:01* alleles compared to 8% of non-transmitting mothers (Winchester et al., 2004). Whereas, 25% of HIV-1 non-transmitting mothers, despite high viral loads, had higher frequencies of *HLA-B*49:01* and *HLA-B*53:01* compared to 5% of HIV-1 transmitting mothers (Winchester et al., 2004). This suggests that *HLA-B*53:01* and *B*49:01* are associated with protection from HIV-1 MTCT. These two alleles only differ at position 77-83 of the α-helix from *HLA-B*35:01* and *-B*50:01* which have been previously associated with high risk of infection (Winchester et al., 2004). Position 77-83 is known to be the site of *KIR3DL1* interaction (Gumperz et al., 1995). Protective *HLA-B*53:01* and *-B*49:01* have Asn, Ile, Ala, Leu and Arg at position 77-81 respectively. While the high risk *HLA-B*35:01* and *-B*50:01* have Ser, Asn, Leu, Arg and Gly at position 77-81 respectively (Winchester et al., 2004). The residues at position 77-81 affect the pocket that binds the C-terminus of the antigenic peptide (Winchester et al., 2004). Residues at position 82-83 encode for HLA-Bw4 epitopes in protective *-B*53:01* and *-B*49:01* which bind to KIR receptor results in NK cell activation (Winchester et al., 2004). However, they found no associations between HLA-B alleles and the risk of HIV-1 mother to child acquisition (Winchester et al, 2004).

This was in line with adult studies which have shown that men who are homozygous or heterozygous for HLA-Bw4 were less likely to transmit HIV-1 virus to their sexual partners (Welzel et al., 2007). Previously, homozygosity for *HLA-B*35* has been associated with increased susceptibility to HIV-1 infection and reduced NK cell activity against HIV-1 (Gao et al., 2005). Fabio et al (1992) evaluated associations between HLA-class I and the risk of HIV-1 transmission among sexually exposed, uninfected drug abusers, they discovered that *HLA-B*44* and *-B*52* were associated with protection while *-B*51* was associated with

susceptibility to HIV-1 infection (Fabio et al., 1992). Taken together these findings suggest that it is possible that HLA-B alleles have a primary protective role towards HIV-1 transmission.

However, HLA-A and HLA-C have also been shown to influence HIV-1 transmission. *HLA-A*2/68:02* supertype such as *-A*02:02, -A*02:05, -A*02:14* and *-A*68:02* were associated with lower risk of HIV-1 infection, whereas, *HLA-A*23:01* was significantly associated with higher risk of HIV-1 seroconversion in highly exposed Kenyan female sex workers (MacDonald et al., 2000). Similar findings were obtained in a study of vertical transmission in which *HLA-A*2/68:02* supertype was associated with lower risk of perinatal HIV-1 mother-to-child transmission (MacDonald et al., 2001). On the other hand, *HLA-Cw*04* was associated with higher risk of HIV-1 transmission in adults (Gao et al., 2005). Another study on European Americans indicates that high HLA-C levels were correlated with protection from acute HIV-1 infection which is believed to be extended to chronic HIV-1 infection (Thomas et al., 2009). Together, these findings suggest that the mechanisms of protection are likely determined by the interactions between KIR receptors expressed on natural killer (NK) cells and HLA-class I ligands expressed on potential target cells. Based on these studies, we will validate these observations in a South African mother-infant cohort and determine HLA genotypes that are associated with the risk of HIV-1 mother-to-child acquisition.

1.1.5.1 HLA in HIV-1 Disease Progression

Previous research indicate that genetic variation at HLA-class I locus has a huge impact in HIV-1 disease progression as in comparison to any other identified genes (Carrington and O'Brien, 2003, Gao et al., 2005). Moreover, *KIR* and *HLA* genes function independently and synergistically to mitigate HIV-1 disease progression (Gaudieri et al., 2005, Lopez-Vazquez et al., 2005, Martin et al., 2002, Merino et al., 2011). Mother-to-child HLA concordance and HLA homozygosity have been associated with faster disease progression to AIDS or death (Thobakgale et al., 2009; Kuhn et al., 2004) (Table 1.2). Futhermore, protective alleles such as *HLA-B*27* and *-B*57* were beneficial in HIV-1 perinatally infected infants if the acquired virus was not pre-exposed to these protective alleles (Fenney et al., 2004) or if the protective HLA allele was inherited paternally (Kuhn et al., 2004).

Among chronically HIV-infected infants, previous research revealed that *HLA-B*58:02* and *HLA-Cw*06:02* were associated with faster HIV-1 disease progression to AIDS (Paximadis et al., 2009). *HLA-B*45:01* and *-B*42:01* were associated with a trend towards faster HIV-1 disease progression and they found no associations between the C1, C2 and HIV-1 clinical disease outcomes (Paximadis et al., 2009). However, unlike adult studies which suggested the protective effect of HLA-Bw4 homozygosity towards HIV-1 disease progression (Flores-Villaneuva et al., 2001). A previous study by Paximadis et al (2009) found that HLA-Bw4 homozygosity was associated with faster disease progression in HIV-1 infected infants. *HLA-B*27* and *HLA-Cw2* were both associated with slower HIV-1 disease progression, whereas *HLA-B*27* and *-A*24* were associated with rapid CNS impairment in chronically HIV-1 infected infants (Singh et al., 2011). Another study on mother-to-child pairs found that slow progression of infants was associated with unshared protective *HLA-B* alleles such as

*-B*57:02, -B*57:03, -B*58:01* and *-B*81:01* in either the mother or the infant (Thobakgale et al., 2009). *HLA-B*18* and *HLA-B*58:02* were associated with faster progression to AIDS in HIV-1 infected infants (Thobakgale et al., 2009).

Prior adult studies revealed that particular KIR/HLA compound genotypes were associated with a change in disease progression (Martin et al., 2002, Martin et al., 2007) (Table 1.2). HLA-class I heterozygosity was associated with delayed HIV-1 disease progression to AIDS (Carrington et al., 1999). Furthermore, Fellay et al (2009) revealed that genetic variants located at HLA-B and HLA-C loci are the strongest determinants of HIV-1 control as compared to others correlations.

Gene	Genotype	Effect
HLA	*HLA-A, HLA-B, HLA-C* homozygosity	Accelerate disease progression
	*B*35Px*	Accelerate disease progression
	*B*57, B*27, Bw4*	Slower disease progression
	A1-B8-DR3	Accelerate disease progression
	*G*0105N*	Protection against infection
	*G*010108*	Increased risk of infection
	*E*0103*	Protection against infection
	HLA-C-35 (35 kb upstream of *HLA-C*)	Lower viral load
KIR	*2DS2 2DL2*	Faster rate of CD4 T cell decline
	3DS1	Slower rate of CD4 T cell decline
	3DS1 homozygosity	Protect against infection
KIR+ HLA	*3DS1+ Bw4–80I*	Slower disease progression Slower progression to opportunistic infections
	*3DL1+ B*57s*	Slower disease progression
	*3DL1*h + Bw4–80I*	Slower disease progression, lower viral load
	Absence of cognate *HLA* ligand for inhibitory *KIR*	Protect against infection

Table 1.2: KIR and cognate HLA-class I ligands that have an effect in HIV-1 transmission and HIV-1 disease progression to AIDS (Carrington et al., 2008. *Trends in Microbiology* **16***:* 620-627).

1.1.5.2 The Role of HLA-B locus in HIV-1 disease progression

HLA-B alleles have been associated with different HIV-1 disease outcomes and they have been shown to have a crucial role in determining HIV outcome after infection (Flores-Villanueva et al., 2001, Martin et al., 2002, Martin et al., 2007, Welzel et al., 2007). Based on serologically defined epitope, HLA-B alleles were divided into two groups, known as HLA-Bw4 and HLA-Bw6 (Flores-Villanueva et al., 2001, Welzel et al., 2007). HLA-Bw4 forms 40% while HLA-Bw6 forms 60% of the HLA-B alleles in North America and Thailand (Gumperz et al., 1995). Gumperz et al., (1995) revealed that the difference between HLA-Bw4 and HLA-Bw6 epitopes was only detected at position 77-83 on the carboxyl end of the α1-helix of HLA-class 1 molecules. HLA-Bw4 epitopes are ligands of inhibitory *KIR3DL1* and result in inhibition of NK cell responses (Gumperz et al., 1995). Moreover, *KIR3DL1* has a stronger affinity for HLA-Bw4 epitopes that carry isoleucine at position 80 (HLA-Bw4-I80) particularly, particularly *HLA-B*57* (Cella et al., 2004). This particular HLAs have been associated with lower viral load and slower HIV-1 disease progression to AIDS in Causacians and African Americans (Flores-Villanueva et al., 2001, Norman and Parham, 2005, Martin et al., 2007, Lopez-Vazquez et al., 2005). However, HLA-Bw4 alleles with threonine at position 80 had no effect on HIV-1 clinical outcomes (Martin et al., 2002, Lopez-Vazquez et al., 2005). HLA-Bw4 homozygosity was shown to be beneficial for maintaining normal CD4 cell count and delaying HIV-1 disease progression to AIDS compared to heterozygosity for HLA-Bw4 or to homozygosity for HLA-Bw6 (Flores-Villanueva et al., 2001). Together, these findings suggest a synergistic effect between *KIR3DL1* and HLA-Bw4 I80 epitopes in controlling HIV-1 disease outcome.

In contrast, HLA-B with Bw6 epitopes such as *HLA-B*08* and *-B*35-Px* have been associated with faster HIV-1 disease progression to AIDS (Brettle et al., 1996; Carrington and O'Brien, 2003, Gao et al., 2005, Flores-Villanueva et al., 2001). *HLA-B*35-Px* subtypes such as *HLA-B*35:02*, *-B*35:03, -B*35:04* and *HLA-B*53:01* have been associated with faster HIV-1 disease progression (Gao et al., 2001). In contrast, no association was found between *HLA-B*35-Py* and HIV-1 disease outcome (Gao et al., 2001, Huang et al., 2009). Gao et al (2005) showed that *HLA-B*57* mediated protection occurs early during HIV-1 infection therefore is associated with slower decline of CD4 T cell count and that the protective effect of *-B*57* become inactive when the CD4 cell count is <200 cells/μl. In contrast, *HLA-B*27* had no protection against CD4 cell decline as compared to *HLA-B*57*, but it was shown to delay HIV-1 progression to AIDS –defining illness after the CD4 cell count has dropped (Gao et al., 2005). Even in Sub-Saharan Africa, *HLA-B*57* alleles such as *HLA-B*57:01*, *-B*57:02*, *-B*57:03* and *-B*58:01* have been shown to be associated with slower HIV-1 disease progression in Zambian population and Rwandan women (Lopez-Vazquez et al., 2005).

HLA-B alleles that are protective against HIV-1 disease progression have been shown to have more Gag specific epitopes which lead into selection of Gag-specific escape mutations (Matthews et al., 2008). All together these studies suggest that HLA-B alleles alone or in combination with specific KIR receptor may be protective or pre-dispose individuals to HIV-1 infection. However, most of these studies were performed in adults, little is known on genetic factors that limit HIV-mother-to child infections. Thus, we aim to determine specific KIR/HLA interactions that may be associated with slower or faster HIV-1 disease progression in HIV-1 infected infants.

1.1.5.3 The role of HLA-C in HIV-1 disease progression

Structurally, HLA-C appears the same as both HLA-A and HLA-B on the surface of nucleated cells, however HLA-C is expressed approximately l0 fold less than HLA-A and B alleles (Snary et al., 1977). Like HLA-A and B, HLA-C also presents viral epitopes to the CD8 T cells or interacts with KIR receptors and activate or inhibit NK cell responses resulting in inhibition of natural cytotoxicity and cytokine production (Goulder et al., 1997, Adnan et al., 2006). Previous research revealed that HLA-C surface expression is significantly increased in antigen presenting cells, macrophages (Schaefer et al., 2008).

During HIV-1 infection, both HLA-A and B are downregulated by Nef to escape recognition of infected cells by CTLs, however the surface expression of the HLA-C alleles remain unchanged (Cohen et al., 1999). Theoretically, this approach is supposed to be beneficial to the virus because it escapes CTL-mediated killing and HLA-C is a strong ligand of inhibitory *KIR2DL1* and *KIR2DL2* (Cohen et al., 1999). However, HLA-C has the ability to bind activating KIR receptors such as *KIR2DS1* and *KIR2DS2* and activate NK cell-mediated killing of the infected cells. HLA-C may also present viral peptides to CTLs which results in destruction of HIV infected cells by the CTLs (Goulder et al., 1997).

A previous whole genome association study identified several polymorphisms that could explain the role of HLA-B and -C variation in HIV-1 disease progression to AIDS (Fellay et al., 2007). A single nucleotide polymorphism (SNP) HLA complex P5 (HCP5) (*rs2395029*), located 100kb from HLA-B was associated with lower viral load (Fellay et al., 2007). Furthermore, T>C SNP -35kb away from the transcription initiation site of HLA-C,

rs9264942 was also associated with lower viral load (Fellay et al., 2007; Thomas et al., 2009). The three different alleles of -35 genotypes are *CC, CT* and *TT* based on higher or lower gene expression respectively. Furthermore, Thomas et al (2009) reported a variation in surface expression of HLA-C across -35 genotype. They showed higher expression of HLA-C alleles that are in positive linkage disequilibrium with *-35C* genotypes and lower surface expression of HLA-C alleles that are in LD with *-35T*. They also reported that individuals with high-expressing HLA-C alleles progress slowly to AIDS compared to individuals with low-expressing HLA-C alleles during acute and chronic phase of HIV-1 infection (Thomas et al., 2009). More recently, Kulkarni et al (2011) showed that the variation within *3' UTR* of HLA-C regulates binding of micro-RNA, has *Mir-148* to its target site. The binding of this micro-RNA reduces surface expression of HLA-C. High expressing HLA-C alleles escape this post-translational regulation. Taken together, these studies suggest that protection towards HIV-1 disease progression to AIDS is caused by high-expressing HLA-C alleles and might enhance innate or adaptive immunity.

In contrast, another study reported that HLA-C was associated with increased infectivity of HIV-1 virus through interactions with gp120, which increase fusion efficiency of both CCR5 and CXCR4 tropic viruses (Matucci et al., 2008). In addition, *HLA-Cw*04* has been shown to reduce NK cell activity against HIV-1 thereby assisting the virus to escape the immune response (Gao et al., 2005, Carrington and O'Brien, 2003). This suggests that HLA-C could be important in controlling HIV-1 replication, however, some alleles of the HLA-C could be detrimental to the host.

Furthermore, another study by Thananchai et al (2009) assessed recognition of HIV-1 HLA-Cw4 restricted gp120 epitopes such as SL9 and SF9 by CTLs and NK cells. They found that CTLs only recognized SF9 peptides, on the contrary, SL9 peptides escaped CTL-mediated immune recognition (Thananchai et al., 2009). However, HLA-Cw4-restricted SL9 bound to the cognate ligand KIR2DL1 and led to inhibition of NK cell responses which results in "double escape", a term used for escaping both NK and CTL immune responses (Thananchai et al., 2009*)*. This study indicates that HIV-1 is capable of acquiring enough mutations to escape both CTL and NK cell recognition. However, Most of these studies were done on HIV-1 infected adults, little is known about the role of HLA-C in HIV-1 infected infants, we propose to perform HLA-C genotyping to determine the role of HLA-C in perinatally HIV-1 infected infants.

1.1.6 NK cell responses during HIV-1 infection

HIV-1 evades host immune responses by down-regulation of MHC molecules on the surface of infected cells. Previous studies have shown that HIV-1 regulatory protein *Nef*, down-modulates HLA-class I molecules by increasing the extent of endocytosis or lysosomal degradation thereby reducing expression of HLA-class I on the surface of infected cells (Fig 1.5) (Schwartz et al., 1996, Cohen et al., 1999, Specht et al., 2010). This downmodulation of HLA-class I molecules should allow the virus to escape recognition and lysis by the CTL cells and pre-dispose the HIV-1 virus to NK-cell mediated immune response (Alter et al., 2009, Collins et al., 1998). However, the virus manages to escape both CTL and NK cell mediated immunity by down modulating only highly expressed HLA-A and HLA-B, leaving HLA-C and HLA-E (inhibitory ligands) on the surface of infected cell. Upon binding to inhibitory KIR receptors, NK cell responses are inhibited and that leads to increased HIV-1

replication (Cohen et al., 1999). The negative effects of *Nef* were demonstrated when the individuals that were infected with aberrant HIV-1 strain with deletion of *Nef* progressed slowly to AIDS (Dyer et al., 1997). This study suggests that *Nef* enhance HIV-1 disease progression to AIDS

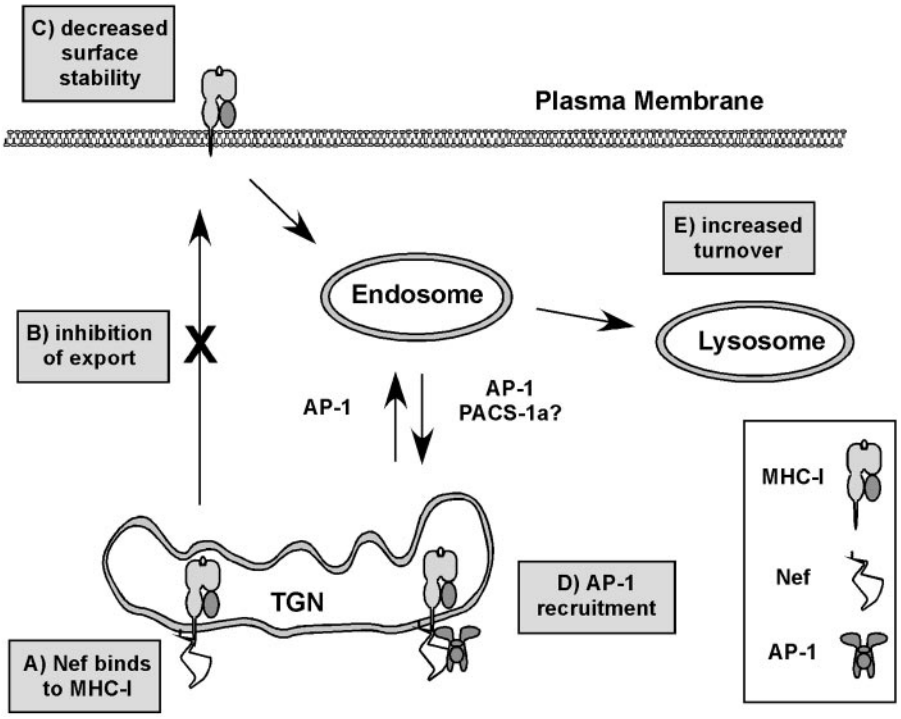


Figure 1.5: Nef-mediated downmodulation of MHC-class I molecules, A= Nef binds to MHC class I molecules and B= inhibit transportation of newly formed MHC-class I molecules to the surface of the infected cell, C= Lower surface expression of MHC-class I leads into unstable plasma membrane. The Nef-MHC-molecules interaction recruits API-1 and result into lysosomal degradation of the MHC-class I molecules (Roeth and Collins, 2006. *Microbiology and Molecular Biology reviews*, **70 (2)**: 548-563)

Nef has been shown to interfere with signal transduction in the T-cell receptors which leads into activation of CD4 T lymphocytes and increase their susceptibility to HIV-1 infection (Baur et al., 1994). *Nef* also inhibits apoptosis of newly infected cells by the disruption of the apoptotic pathways such as FasL and p53 molecules (Xu et al., 1999, Greenway et al., 2002). It has been associated with higher viral loads, lower CD4 lymphocyte count, lower expression of CD28 and CXCR4 co-receptors (Specht et al., 2010).

It has been shown that *nef* also downregulate NKG2D stress inducible ligands, such as MIC-A, ULBP 1 and 2 which are upregulated during HIV-1 infection and upon ligand interaction with NKG2D they lead into NK cell activation, therefore downmodulation of these ligands leads into loss of NK activation (Cerboni et al., 2007). A recent study on HIV-1 exposed, uninfected infants found that NK cell function is reduced in viraemic HIV-1 infected infants due to *Nef*-mediated downmodulation of HLA-A and HLA-B (Ballan et al., 2007). HIV-1 cause dysregulation of cytokine production which stimulates NK cell functions. Some viral peptides directly inhibit expression of NK cell surface proteins resulting in changes in expression of NK cell receptors. We predict that slow progressors (infants with lower viral load and higher CD4 T cell count) have higher expression of HLA-C alleles that protect them against HIV-1 disease progression to AIDS. The purpose of the study was to identify KIR/HLA compound genotypes that are associated with protection or susceptibility to HIV-1 acquisition in HIV-1 exposed infants.

CHAPTER 2

METHODS DEVELOPMENT

2.1 BACKGROUND

Analysis of KIR and HLA gene repertoires may yield new insights in understanding HIV-1 mother-to-child transmissions. Previous studies have shown that inhibitory KIR genes, in particular, *KIR2DL1, KIR2DL3* and *KIR3DL1* are associated with susceptibility to HIV-1 acquisition in exposed, uninfected infants (Ballan et al., 2007). Furthermore, a recent study by Paximadis et al (2011) found that homozygosity for *KIR2DL3* plus heterozygous HLA-C1/C2 ligands were lower in frequency among HIV-1 infected infants compared to exposed uninfected South African infants. However, the standard methods of clinical sample collection and preparation are not feasible for analyzing large cohorts in a resource limited setting or using stored samples. Standard KIR/HLA genotyping protocols require fresh blood samples that have been collected by venipuncture into EDTA tubes. These methods are expensive, time consuming, technically difficult and not feasible for analysis of archived samples. Therefore, DNA preparation from DBS samples to perform KIR genotyping is an easy, useful, convenient, inexpensive and much more acceptable alternative to whole blood to reduce blood volume required for genotyping techniques (Wijnen et al., 2008).

Most biobank repositories have stored dried blood spot samples for research purposes or from HIV screening of infants born to HIV-infected mothers, however, a robust method for gDNA isolation from stored DBS has not been established (Fischer et al., 2004; Hollegaard et al., 2009a).

Previously, stored DBS samples have produced insufficient yield or poor quality nucleic acids for most genotyping techniques due to fragmentation during storage (Hollegaard et al., 2009b). These stored DBS samples cohorts would provide a useful resource for ancillary host genetic studies, including complicated genetic loci analysis such as KIR and HLA. However, low DNA yield and quality may also be a limiting factor to perform reliable KIR/HLA genotyping (Sjoholm et al., 2007). Thus, KIR genotyping from whole genome amplified DNA may provide a useful alternative. Although previous investigators have evaluated the use of whole genome amplified DNA for KIR and HLA genotyping, their analysis has been limited to selected KIR or HLA epitopes. A recent study by Chainonthee et al (2010) revealed the feasibility of wgaDNA to perform selected KIR and HLA-C genotyping by SSP-PCR. However, they did not perform full KIR and HLA typing, they used KIR defining epitopes specifically for HLA-C1 and C2. We extended their findings by genotyping the full KIR and HLA-class I profile, we also used wgaDNA derived from DBS samples to perform KIR/HLA genotyping, while they used whole blood derived wgaDNA which may not be a true representative of DBS samples. Singh and Spector (2007) found 100% concordance in HLA-class I genotyping between wgaDNA and gDNA. One limitation in the interpretation of their findings is the lack of a gold standard for comparison. We extended their findings by including a gold standard, our study will have important implications in designing genetic studies using DBS samples.

2.2 STUDY AIMS

The aims of the study were:

1) To evaluate 3 different commercially available kits: QIAamp DNA mini (Qiagen), Nuclisens miniMAG (Biomerieux) and *prep*-GEM (Zygem) for genomic DNA extraction from a single punch of DBS. From this aim, we expected to learn which method could produce sufficient DNA yield and quality for IL-10, KIR and HLA-class I genotyping. Thus, we would use that robust method to isolate gDNA suitable for KIR and HLA genotyping on archived DBS samples in the vertical HIV-1 mother to chid transmission cohort.

2) To evaluate whole genome amplification for subsequent *KIR*, and *HLA* genetic analyses. From this aim, we expected to learn whether whole genome amplified DNA derived from DBS would be suitable for *KIR* and *HLA*-class I genotyping using sequence specific primer (SSP)-PCR. The findings would help to design genetic analysis studies appropriately.

2.3 HYPOTHESES

We hypothesized that:

1) An approach that produced the highest gDNA yield with good quality (260/280 ratio between 1.8-2.00) is the best method of gDNA extraction from DBS samples. We speculate that sufficient DNA yield and quality would be suitable to perform reliable *IL-10* SNP, *KIR* and *HLA* genotyping.

2) Concordant KIR and HLA genotyping results will be obtained between whole genome amplified DNA and genomic DNA derived from whole blood. We speculate that whole genome amplified DNA would be a true representative of the gDNA therefore the results obtained with both methods will be identical.

2.4 MATERIALS AND METHODS

2.4.1 Study Design

For this crossectional pilot study, we used archived samples from 21 South African adults who had matching whole blood (gold standard) and dried blood spots. We compared DNA isolation using three different commercially available kits such as QiaAmp® DNA mini kit (Qiagen), Nuclisens mini-MAG™ (Biomeriuex) and prep-GEM (Zygem). We compared the DNA yield and quality as determined by the average absorbance at 260/280 and 260/230 wavelengths. We also compared the ability to perform PCR amplification of the reference gene, HLA-DRB1 with and without whole genome amplification. We compared the ability to genotype IL-10 SNP -592 and -1082 polymorphism on the gDNA isolated from DBS samples and whole blood. We then determined the feasibility of using wgaDNA as a template for KIR and HLA genotyping by comparing wgaDNA to gDNA isolated from whole blood as a gold standard (Fig 2.1).

2.4.2 Study Population

As a sub-study of a completed cohort that was used previously to investigate mother-to-child pairs (Bland et al., 2009; Coovadia et al., 2007; Rollins et al., 2007), we selected archived DBS samples from 21 South African adult women aged 16 years or older from 1000 samples stored for approximately 8 years (Table 2.1). Specific samples were selected based on sample availability, similar storage conditions and duration of storage. DBS were taken previously in the field by spotting whole blood droplets onto filter papers and air-drying (FTA paper card, Whatmann Bio-Science). Women were enrolled during their antenatal care visits at the clinics in Umkhanyakude and outside Durban, KwaZulu-Natal, South Africa. Written informed consent was obtained from all study participants and the study ethical approval was obtained through a research protocol T050/01 approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal, Durban, South Africa, and the Internal Review Board of Massachusetts General Hospital.

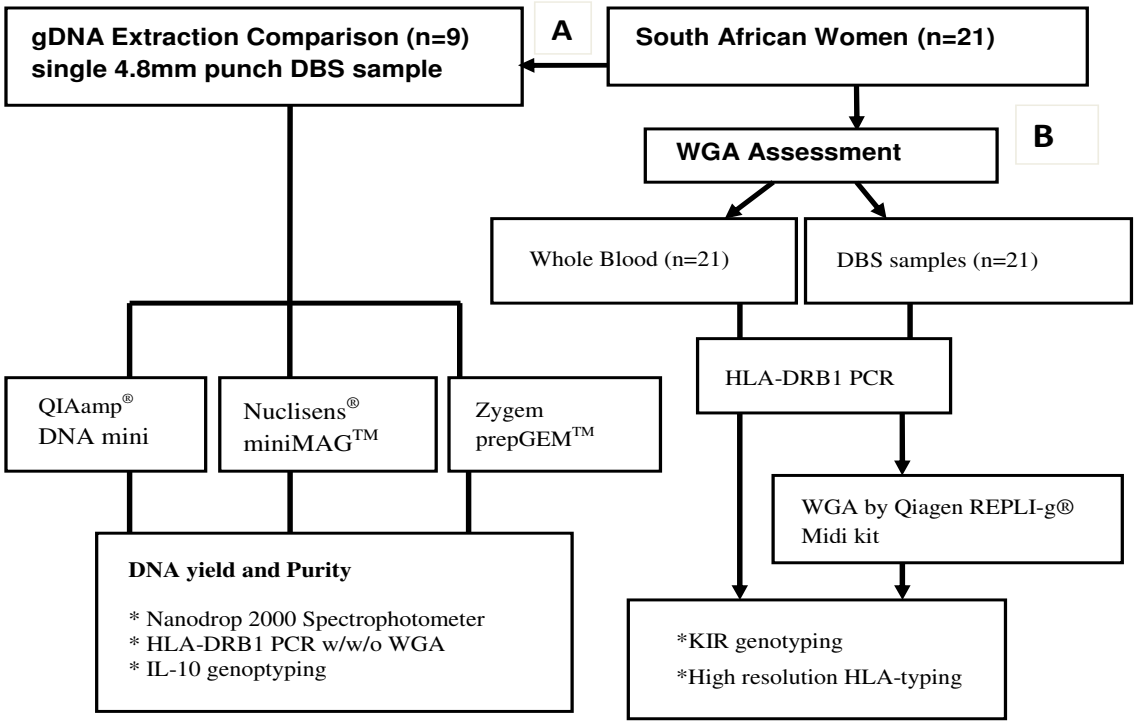


Figure 2.1: Workflow plan on the method development; 2.1-A: The determination of the most optimal gDNA extraction method from a 4.8mm disk of stored dried blood spot (DBS) samples (n=9 of 21). IL-10 genotyping was done in two replicates with (w) and without (w/o) whole genome amplification (WGA). Whole blood gDNA derived by the QIAamp blood mini kit was used as the gold standard for each individual. **2.1-B:** Determination of KIR genotypes from gDNA isolated from matched whole blood and wgaDNA from DBS (n=21). Whole genome amplification was done prior to KIR genotyping by sequence specific primer (SSP)-PCR.

2.4.3 Statistical Analysis

Graph Pad Prism 4 and SPSS version 14 were used for statistical analysis. Wilcoxon signed rank test for pairwise comparisons was used to compare the median DNA yield and quality (260/280 and 260/230 ratios) between the three different gDNA isolation methods

(Wilcoxon, 1945). We used paired Wilcoxon test because the same samples were used to isolate gDNA using different methods. Non-parametric Chi-square's McNemar's test was used to analyse paired binary data of *HLA-DRB1* amplification and *IL-10* polymorphism determination because the samples were paired. We also used McNemar's test to compare the proportions between HLA genotyping results from gDNA isolated from whole blood and wgaDNA template from DBS samples from the same individuals. KIR genotyping was performed once or under one specific conditions, we calculated the sensitivity and specificity of wgaDNA for each KIR gene using whole blood as a gold standard. P values were two-sided and adjusted for multiple comparisons, p values were multiplied by the number of comparisons we made using Holm's method (Holm, 1979) and a p-value<0.05 was considered significant.

2.4.4 Experimental Methods

2.4.4.1 Genomic DNA Extraction

To identify the most optimal method of DNA isolation from dried blood spots, we conducted a pilot study with samples randomly selected from 9 of the 21 individuals based on sample availability. Genomic DNA was isolated from these samples to compare three commercially available kits: QIAamp DNA mini kit, PrepGEM^{TM} Storage card blood kit, and the Nuclisens miniMAG magnetic extraction kit (Table 2.1). We hypothesized that the most optimal method would produce sufficient gDNA yield and quality (260/280 and 260/230 ratios) compared to the other gDNA isolation methods.

Extraction kit	Company	Supplier	Catalogue no	Country of manufacture
QIAamp® DNA mini	Qiagen	Southern Cross	51306	USA
QIAamp® DNA Blood mini	Qiagen	Southern Cross	51106	USA
NucliSENS® miniMAG®	Biomerieux	Molecular Diagnostics	N/A	Netherlands
PrepGEM™ Storage card blood	ZyGEM	N/A	PSB0050	New Zealand
Repli-g® midi	Qiagen	Southern Cross	150090	USA

Table 2.1: gDNA extraction kit information, QIAamp DNA mini kit, Nuclisens miniMAG and prep-GEM methods were used for gDNA isolation from DBS samples while QIAamp blood mini kit was used to isolate gDNA from whole blood.

2.4.4.1.1 gDNA extraction using QIAamp® DNA mini kit

Genomic DNA was isolated according to the manufacturer's instructions with few modifications. The DBS filter papers were punched with a 4.8mm punch and 1 disk was used per extraction method with two replicates. Blank filter cards were punched in between the samples to prevent cross contamination. gDNA was isolated from each 4.8 mm disk. The DBS disk (4.8 mm) was placed in a labelled 1.5ml microcentrifuge tube, lysis buffer ATL (180µl) was added and then the samples were vortexed and incubated in water bath at 85^{o}C for 10 min. Following incubation, the tubes were briefly centrifuged to remove drops from inside the lid, Proteinase K (20µL) was added, the mixture was vortexed and incubated in water bath at 56^{o} C for 1 hour. Following incubation, the tubes were centrifuged briefly to remove drops from inside the lid and Lysis buffer AL (200µL) was added to the sample. The samples were vortexed thoroughly to ensure efficient lysis and then incubated in water bath at 70^{o}C for another 10 minutes. Following incubation, the samples were centrifuged to

remove drops from inside the lid. Absolute ethanol (100%, 200µL) was added to the sample mixed thoroughly by vortexing and centrifuged briefly. Without wetting the rim, the sample mixture was applied into labelled QIAamp mini spin column in a 2ml collection tube. The cap was closed and the mixture was allowed to bind to the column and then centrifuged at 8000 rpm for 1 min. The QIAamp mini spin column was transferred into a new collection tube, without wetting the rim, the column was washed with wash buffer 1 (AW1) (500µL) to remove excess ethanol and the mixture was centrifuged at full speed 8000 rpm for 1 min. The filtrate was discarded and the samples were transferred to the new collection tubes. The column was then washed with wash buffer 2 (AW2) (500µL) and the sample mixture was centrifuged at 14000 rpm or full speed for 3 minutes. The filtrate was discarded and the spin columns were transferred into a new collection tubes and they were centrifuged at full speed for 1 min to eliminate possible buffer carryover. The QIAamp DNA mini spin columns were placed on clean 1.5ml eppendorf tubes and the spin-column bound gDNA was eluted with elution buffer AE (150µl) and incubated at room temperature for 5 minutes. The gDNA was centrifuged at 8000 rpm for 1 minute and the gDNA was kept at -20^{o}C.

2.4.4.1.2 gDNA extraction using prep-GEM^{TM} storage card blood kit

The DBS disk (4.8mm) was placed in a thin walled PCR tube, washed with Nuclease free water (100µl), the sample mixture was incubated at room temperature for about 15 minutes. Following incubation, water was aspirated and discarded. Buffer Magenta (10X, 5µl), DNAase free water (44µl) and prep-GEM^{TM} were added to the DBS sample. The samples were then incubated in the thermal cycler at 75^{o}C for 15 minutes and 95^{o}C for 5 minutes, following incubation the samples were taken out and centrifuged at 13000 rpm and the supernatant (gDNA) was transferred to a new fresh labelled tube.

2.4.4.1.3 gDNA extraction using NucliSens® miniMAG™ nucleic acid purification system

Lysis and Binding: gDNA was isolated according to the manufacture's instructions with few modifications. The dried blood spot disk (4.8 mm) was placed in a labelled 15ml falcon tube and Lysis buffer (9ml) was added. The sample mixture was vortexed and incubated on a shaker at room temperature for an hour. Following incubation, the solution was centrifuged at 1500 rpm for 2 minutes, the supernatant was transferred into another 15ml falcon tube and the used filter paper was discarded. Next, silica (50µL) was added and vortexed immediately and the samples were incubated at room temperature for 10 minutes without mixing. Following incubation the samples were centrifuged at 1500rpm for 2 minutes and the supernatant was discarded.

Washing: The pellet was washed twice with wash buffer 1 (400µl) following each washing step the tubes were incubated on the MiniMag magnetic extraction rack for 30 seconds and the supernatant was aspirated. The MiniMag magnetic extraction rack was turned off and the sample was washed twice with wash buffer 2 (400µl) and incubated on the Minimag magnetic extraction rack for 30 seconds, following incubation, the supernatant was removed carefully. The sample was then washed with wash buffer 3 and incubated on the MiniMag Magnetic extraction rack for 15 seconds, wash buffer 3 was aspirated.

Elution: The pellet was resuspended in elution buffer (25µL) and incubated in a thermoshaker at 60^{o}C at the speed of 1400rpm for 5 minutes. Following incubation, the tubes were placed on the MiniMag magnetic rack, DNA samples were transferred to clean labelled tubes for storage and gDNA was stored at -20^{o}C.

2.4.4.2 gDNA extraction from whole blood (gold standard) using QIAamp Blood mini kit

Whole blood (200μL) was added in a 1.5 microcentrifuge tubes, proteinase K solution (20μl) was added. Buffer AL (200μl) was added to the sample and the sample mixture was vortexed for 15 seconds. The solution was incubated in the water bath at 56^{o} for 10 minutes. The tubes were briefly centrifuged to remove drops from inside the lid. Absolute ethanol (200μL) was added to the sample mixture and the mixed thoroughly by vortexing briefly, the sample mixture was centrifuged briefly. ***Please refer to section 2.4.4.1.1 for the rest of the protocol.***

2.4.4.3 gDNA yield and quality assessment

Genomic DNA was quantified using a micro-volume NanoDrop 2000 spectrophotometer (Thermo Scientific) and elution buffers were used to blank the Nanodrop. Spectrophotometer determines DNA yield by detecting UV light absorbance at wavelength 260 nm and protein concentration at wavelength 280nm. Genomic DNA quality was measured by the ratio of absorptions at 260/280nm, phenolate and thiocynates contamination were detected by ratio of absorptions at 260/230nm.

gDNA quality was also assessed by the ability to PCR amplify a reference gene, *HLA-DRB1*. *HLA-DRB1* (796 bp) was amplified using forward and reverse primers; 5' TGC CAA GTG GAG CAC CCA 3' and 5' GCA TCT TGC TCT GTG CAG AT 3' respectively as previously described by Martin and Carrington et al (2008). The results were compared between all three gDNA isolation methods from DBS samples and whole blood samples were used as a gold standard. The samples were amplified in a programmable thermal cycle using the following parameters: 3 min at 94^{o}C, 5cycles of 15s at 94^{o}C, 15s at 65^{o}C, 30s at 72^{o}C; 21 cycles of 15s

at 94°C, 15s at 60°C, 30s at 72°C; 4 cycles of 15s at 94°C, 1 min at 55°C, 2 min at 72°C with a final extension step at 72°C.

2.4.4.4 IL-10 Genotyping

To further assess gDNA quality, we performed IL-10 genotyping by TaqMan®human single nucleotide polymorphisms (SNP) Genotyping Assay using MGB primers and probes for IL-10 detection at position 592 and 1082 (Applied Biosystems, Foster city, USA) (Fig 2.2). SNP genotyping primers (40X) with forward and reverse sequence specific primers and Taqman probes (VIC® dye which detects allele1 and FAM™ which detects allele 2) dye mix were diluted into a working stock of 20X by addition of TE buffer, the mixture was vortexed, centrifuged briefly and stored at -20°C. Taqman Universal master mix (2X without Emperase® UNG, AmpliTaq Gold® DNA polymerase) was swirled gently and 12.5μL per sample was added into a sterile microcentrifuge tube. SNP genotyping primers and probes dye mix (20X) were vortexed and centrifuged briefly and 1.25μL was added into the tubes, the tubes were capped and inverted several times. The sample tubes were centrifuged to remove air bubbles.

Purified genomic DNA (10ng) was prepared using a wet preparation method (gDNA diluted with nuclease free water) and was loaded in a 96-well plate and cross contamination was avoided. Three known Genomic DNA control samples previously genotyped for IL-10 were used as internal standards for both SNP -592 *(rs1800872)* and -1082 *(rs1800896*) (Ladhani et al., 2010). PCR cocktail (Universal PRC master mix and SNP genotyping assay primer and probe dye mix) (13.75μL) was added into gDNA (11.25μL) making the final volume of 25μL. The wells were inspected for uniformity of volumes and the plate was sealed, vortexed and centrifuged.

The samples were amplified using the following parameters: 10 min at 95°C; 40 cycles of 15 sec at 92°C and 1 min at 60°C. Following PCR amplification, allelic discrimination plate read was performed using Applied Biosystems RT-PCR system. Allelic discrimination data was analysed using the ABI Prism® Sequence Detection system 7000 software. Alleles were converted to genotypes as follows: For IL-10 SNP-592, CC represented wild type, AA= mutation and AC= heterozygous. Whereas for SNP -1082: AA represented wild type, GG= mutation and AG= heterozygous.

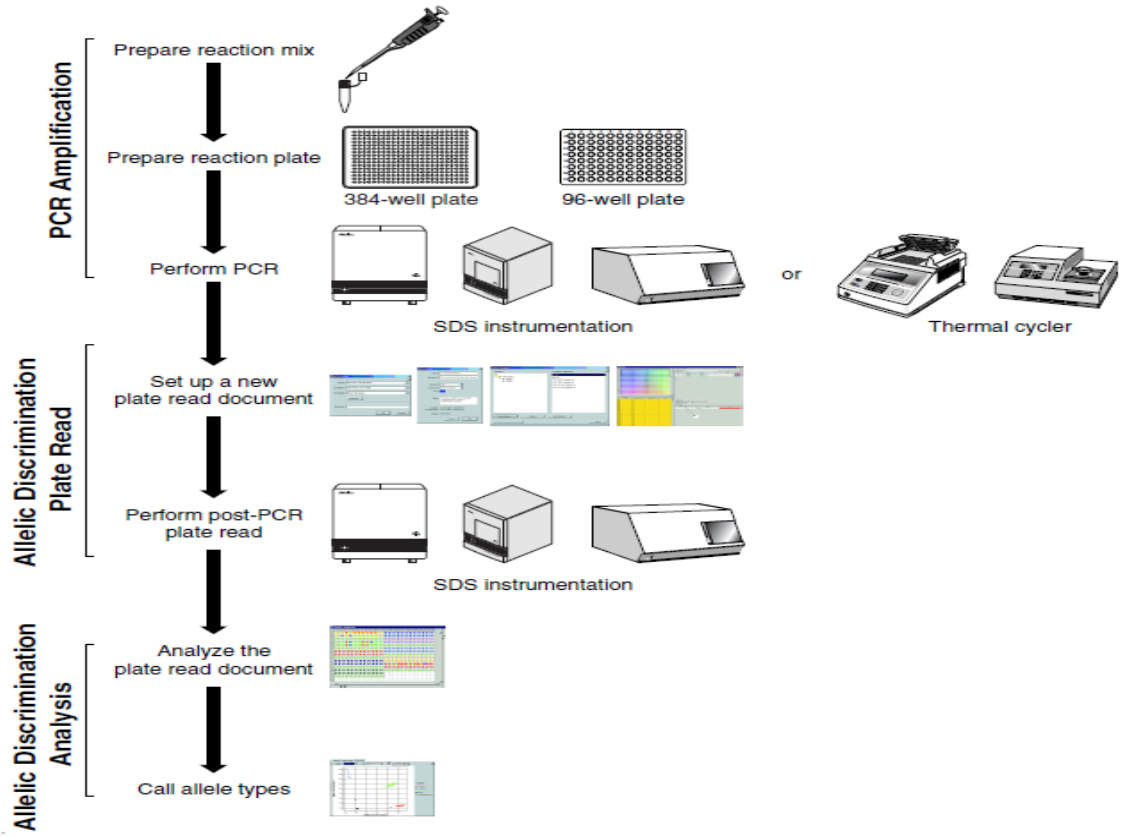


Figure 2.2: A sketchical representation of IL-10 Taqman SNP genotyping assay using primers and Taqman MGB probes (VIC® dye) which detects allele1 and FAM™ which detects 2 primers.

(http://www3.appliedbiosystems.com/sup/URLRedirect/index.htm?xDoD=4332856; 07 July 2011; 14:55)

2.4.4.5 Whole genome amplification by Multiple Displacement amplification

For whole genome amplification, purified gDNA extracted by Qiagen QIAamp DNA mini kit was amplified using REPLI-g® Midi kit (Qiagen; Germany) according to manufacturer's instructions with few modifications. In brief, DNA template (2,5μL) was placed in a thin walled PCR tube, theoretically DNA template should be 10 ng or more. However, most of our DBS-derived DNA was less than 10 ng. Denaturation buffer (2,5μL) was added and the solution was mixed by vortexing and centrifuged briefly. The samples were incubated at room temperature for 3 minutes. Following incubation, neutralising buffer (5μL) was added and the samples were mixed by vortexing and centrifuging briefly. REPLI-g midi DNA polymerase was thawn on ice and all other components were thawn at room temperature. The reagents were vortexed and centrifuged briefly. Master mix was prepared on ice by mixing nuclease free water (10μL), REPLI-g Midi reaction buffer (29μL) and REPLI-g Midi DNA polymerase (1μL) per sample, the mixture was vortexed and briefly centrifuged. Immediately, master mix (40μL) was added to denature DNA sample and the sample was incubated in a thermal cycler at 30^{o}C for 16 hours or overnight. Following incubation, REPLI-g Midi DNA polymerase was inactivated by heating the sample at 65^{o}C for 3 minutes. Amplified gDNA samples were stored at 4^{o}for 1 week and -20^{o}C for months.

2.4.4.6 KIR and HLA genotyping by Sequence Specific Primer (SSP)-PCR

KIR genotyping was performed by sequence specific primer (SSP)-PCR mixes containing forward and reverse primers (5μM) specific for each KIR gene and internal control which uses two primer sequences to determine the presence or the absence of each gene.

The protocol of gene specific PCR using sequence specific primers previously established by Martin and Carrington (2008) was followed without deviation. Specific oligonucleotide primer mix (1μL) containing forward and reverse primers was dispensed into separate wells in a 384 well plate, each sample took 30 wells, two vertical raws thus allowing genotyping of 12 samples per plate. A PCR master mix was prepared by adding 10X PCR buffer (16.5μL, 200mM Tris-HCL, pH 8.4, 500mM KCL) (Invitrogen, Carlsbad CA, USA), 50mM $MgCl_2$ (4.95μL) (Invitrogen, Carlsbad CA, USA), 25mM dNTP (1.32μL; equal mix of 100mM each dATP, dTTP, dGTP and dCTP) (Fermentas), Platinum Taq polymersae (0.825μL) (Invitrogen Carlsbad CA, USA). Nuclease free water (109.5μL) (Invitrogen Carlsbad CA, USA) and DNA template (200 ng). Master mix (4μL) was added into the wells containing special primers making a total PCR volume of 5μL. The plates were covered with acetate film and centrifuged briefly. The samples were amplified in a programmable thermal cycle using the following parameters. 3 min at 94^{o}C, 5cycles of 15s at 94^{o}C, 15s at 65^{o}C, 30s at 72^{o}C, ; 21 cycles of 15s at 94^{o}C, 15s at 60^{o}C, 30s at 72^{o}C; 4 cycles of 15s at 94^{o}C, 1 minute at 55^{o}C, 2 min at 72^{o}C with a final extension step at 72^{o}C.

Following PCR amplification 3% agarose gel was prepared by adding 4.5g of agarose powder to 1X TE buffer (150mL) per gel and the solution was heated until it dissolved completely. The gel was allowed to cool down and then 10mg/ml ethidium bromide (15μl) was added. The gel was casted in a gel casting tray and the combs were inserted, the gel was allowed to set for 30 minutes. The combs were removed and the gel was submerged in the gel tank containing 800ml of TE buffer. 100bp DNA ladder (2μl) was loaded, orange gel loading dye (5μL; 0.5% Orange G, 20% Ficoll, 100mM acetic acid , 10mM EDTA) was addded in the PCR products which were mixed thoroughly and using a multi channel pipettor, 5μL of

the product was loaded in the wells. The remaining PCR product was saved for *KIR2DS4* subtyping. The samples were electrophoresed for 45 minutes at 100V or until the dye reached the edge of the gel. The gel was visualised using an ultraviolet light source and the gel photograph was taken with a digital camera for a permanent record. The wells containing *KIR2DS4* were run in a 3% gel for 2 hours. We used half (100µl) of the samples to do high resolution HLA-genotyping at the laboratory of Dr. Mary Carrington (National Cancer Institute, Frederick, MD, USA) to perform high resolution HLA-genotyping.

2.4.4.7 Determination of KIR haplotypes

Haplotypes were determined based on the guidelines available on the IPD-KIR Sequence database (http://www.ebi.ac.uk/ipd/kir/haplotype.html, 01 July 2011; 11:22 am). Haplotype B was determined by the presence of one or more of the following genes, inhibitory *KIR2DL2, KIR2DL5* and activating *KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5* and *KIR3DS1* (Marsh et al., 2003; Yawata et al., 2005; McQueen et al., 2007). Conversely, haplotype A was determined by the absence of the above mentioned receptors (http://www.ebi.ac.uk/ipd/kir/haplotype.html, 01 July 2011; 11:22 am). Previous studies have also shown that haplotype A is also determined by the presence of one or more of the following genes, activating *KIR2DS4* and inhibitory *KIR3DL2, KIR2DL1, KIR2DL3* and *KIR3DL1* (Gardiner, 2007, Marsh et al., 2003).

2.5 RESULTS

Our objective was to evaluate the feasibility of using whole genome amplification to generate sufficient template for KIR, HLA-class I and IL-10 SNP genotyping. However, before conducting this analysis we first needed to identify the most robust method for gDNA isolation from dried blood spots. gDNA extracted from the three different methods was assessed. To assess gDNA quality, protein contamination was measured by the ratio of absorbance at 260 and 280nm, and chemical contamination was measured by the ratio of absorbance at 260 and 230nm (Fig 2.3).

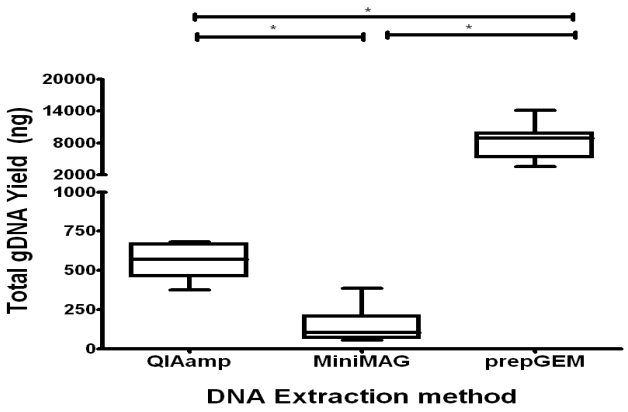
2.5.1 Evaluation of gDNA yield and quality

Although prep-GEM yielded higher amounts of gDNA on average, the quality of gDNA isolated by the QIAamp DNA mini approach was lower than the optimal range but significantly higher as measured by the 260/280 and 260/230 ratios ($p<0.001$) (Fig 2.3 A-C). Furthermore, gDNA yield was significantly higher in samples isolated by QIAamp DNA mini kit in comparison with Nuclisens minimag ($p<0.01$) (Fig 2.3 A). Although the 260/280 absorbance ratio of gDNA between the QIAamp DNA mini kit and Nuclisens minimag method was not significantly different, the average ratio of absorbance at 260/230 nm of the samples isolated by QIAamp DNA mini approach was significantly higher than that using the Nuclisens miniMAG approach (Fig 2.3 C) ($p<0.001$). The gDNA yield and quality measured by 260/230 was significantly higher in samples isolated by PrepGEM as compared to Nuclisens mimimag approach ($p<0.001$) (Fig 2.3-A, C). Whereas the quality measured by 260/280 ratio was significantly higher in samples isolated by Nuclisens miminag compared to prepGEM approach ($p<0.01$) (Fig 2.3 B). Previous studies have shown that standard pure gDNA is determined by the 260/280 absorbance ratio of 1.8-2.00, whereas the 260/230

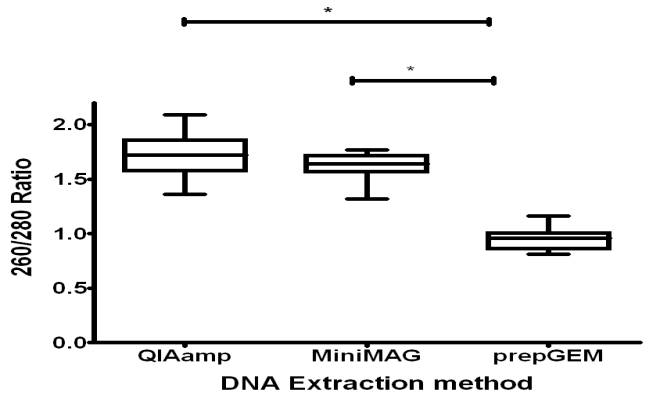
absorbance ratio less than 2.00 may indicate residual phenol or residual guanidine from column based extractions (Psifidi et al., 2010). Thus, these results indicate that QIAamp DNA mini kit produced sufficient gDNA yield and quality compared to both Nuclisens Minimag and prep-GEM kits, however, the gDNA isolated from dried blood spots using all three methods had low 260/230 ratio which could indicate residual phenol or guanidine carryover as explained above.

To further assess gDNA quality, we used two independent approaches. Firstly, we measured the ability to amplify a reference gene *HLA-DRB1*; and secondly, we genotyped two *IL-10* using a TaqMan® SNP Genotyping Assay. QIAamp DNA mini kit continued to be the best method of sample preparation for *HLA-DRB1* and *IL-10* SNP detection. QIAamp yielded the most optimal results for PCR-based detection of *HLA-DRB1* compared to the Nuclisens minimag method with consistently higher number of successful detection ($p<0.001$) (Table 2.2). Similarly, in comparison to the Prepgem approach, gDNA samples isolated with the QIAamp DNA mini kit had consistently higher number of successful *HLA-DRB1* detection (Table 2.2) ($p<0.001$). Similarly, *IL-10 SNP* determination results obtained from gDNA samples isolated using the QIAamp DNA mini kit were completely concordant with results obtained using gDNA from whole blood. Moreover, QIAamp had the highest number of successful *IL-10-592* and *-1082* determination compared to the other methods ($p<0.001$) (Table 2.2).

A



B



C

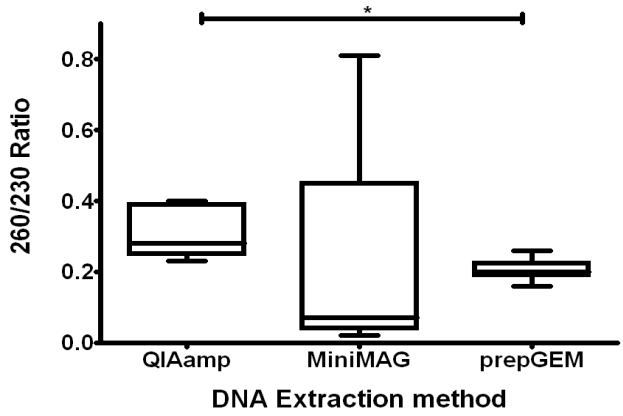


Figure 2.3 A-C: Qiagen QIAamp gDNA extraction kit yields the most optimal quality and quantity of gDNA from a 4.8mm disk of DBS samples. A) A comparison of total gDNA yield, B) A comparison of gDNA quality measured by the 260/280 ratio and C) A comparison of gDNA quality measured by the 260/280 ratio. Statistical comparisons between groups were made using paired Wilcoxon signed rank test and adjusted for multiple comparisons. * denotes p-values $p<0.05$.

Table 2.2: Qiagen QIAamp gDNA extraction kit yielded the most optimal results for PCR-based detection of *HLA-DBR1* and *IL-10 SNP-592* and *-1082* determination. A Pairwise comparison of the average of two experiments of the frequency of successful *HLA-DRB1* PCR amplification and IL-10 SNP determination with (w) and without (w/o) whole genome amplification. Statistical comparisons between the groups were made using McNemar's statistical test and adjusted for multiple comparisons, significant p value was $p<0.05$.

Genotyping status	Methods			P-value
	QIAamp DNA mini	Nuclisens Minimag	Prep-GEM	
***HLA-DRB1* W/O WGA**				P< 0.001
Yes	9	0	0	
No	0	9	9	
***HLA-DRB1* With WGA**				P<0.001
Yes	9	1	0	
No	0	8	9	
IL-10 SNP -592				P<0.001
Yes	9	0	1	
No	0	9	8	
IL-10 SNP -1082				P< 0.001
Yes	7	0	1	
No	2	9	8	

2.5.2 Whole genome amplification (wgaDNA)

Since the gDNA yield from DBS samples is typically low, we next evaluated the feasibility of using whole genome amplification (WGA) to obtain higher gDNA yields to perform subsequent KIR and HLA genetic analysis. We performed WGA using the Qiagen REPLI-g midi kit to compare the suitability of DBS gDNA samples isolated by the QIAamp DNA mini kit, Nuclisens Minimag, and *prep*-GEM methods. We compared the quality of the amplified products by assessing the ability to PCR amplify a housekeeping gene, *HLA-DRB1*. We found that either with or without whole genome amplification, the frequency of successful *HLA-DRB1* amplification was significantly higher for samples isolated using the QIAamp DNA mini kit compared to the other isolation methods ($p<0.001$; McNemar's test) (Table 2.2). The frequency of successful HLA-DRB1 amplification using Nuclisens miniMAG approach marginally increased by 11.1% after whole genome amplification. For both Nuclisens minimag and prepGEM gDNA isolation methods there was no difference in successful HLA-DRB1 amplification before and after WGA whereas WGA marginally increased the frequencies of successful HLA-DRB1 amplification from samples isolated by Nuclisens minimag. Based on these findings, we suggest that QIAamp DNA mini kit is a robust method for DNA isolation from the DBS samples.

2.5.3 KIR genotyping of wgaDNA

Overall, we observed that the frequencies of *KIR* genes in our cohort were consistent with previously described *KIR* frequencies for an African population (Norman et al., 2002). Notably, *KIR3DS1* was absent and *KIR2DS1* was present at a low frequency (Fig 2.4). Likewise the distribution of *KIR* haplotypes in our cohort was consistent with previously

described *KIR* haplotypes among Africans (Norman et al., 2002; Ashouri et al., 2009). We found that 76% of our cohort consisted primarily of the Bx *KIR* haplotype (Fig 2.4).

To address the question of applying this technology more broadly to assess highly polymorphic genetic loci we next evaluated WGA for generating templates for KIR repertoire determination. For KIR genotyping by SSP-PCR we used gDNA isolated from stored DBS samples from a cohort of 21 South African adults. In our analysis we compared KIR repertiores determined using unamplified gDNA from DBS samples and gDNA from whole blood and we found 100% concordance (Fig 2.4). We also compared KIR repertoires determined following WGA of DBS derived gDNA (wgaDNA) to a gold standard of KIR repertoires determined from whole blood derived gDNA isolated from the same individuals. We obtained concordant genotyping results between whole genome amplified gDNA and unamplified gDNA for a majority (i.e, 11 of the 16) of the genes tested (Fig 2.5). However, we found discordant results with *KIR2DL1*, *KIR2DL3*, *KIR2DL5* and *KIR2DS1*. These genes were present using unamplified, whole blood derived gDNA, but absent using wgaDNA. Thus, the sensitivity for detecting these genes was lower using wgaDNA (McNemar's test; $p<0.01$) and ranged from 50% to 94% (Fig 2.5). The specificity was 100% in most of the KIR genes. However, the specificity was not applicable in genes that were present in all samples such as *KIR2DL1, KIR2DL4, KIR2DS4, KIR3DL1, KIR3DL2, KIR3DL3* and *KIR2DP1* because no false positives results were detected.

PID	2DL1	2DL2	2DL3	2DL4	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DS1	3DL3	2DL5	2DP1	2DS1	DRB1	KIR Haplotype
1	■	■	□	■	■	■	■	■	■	■	□	■	■	■	□	■	B
2	■	■	■	■	■	■	■	□	■	■	□	■	■	■	□	■	B
3	■	■	■	■	□	□	■	■	■	■	□	■	■	■	□	■	B
4	■	■	■	■	■	■	■	□	■	■	□	■	■	■	□	■	B
5	■	■	■	■	■	□	■	■	■	■	□	■	■	■	■	■	B
6	■	□	■	■	□	□	■	□	■	■	□	■	□	■	□	■	A
7	■	■	□	■	■	■	■	■	■	■	□	■	■	■	□	■	B
8	■	■	□	■	■	□	■	■	■	■	□	■	■	■	□	■	B
9	■	■	■	■	■	□	■	■	■	■	□	■	■	■	□	■	B
10	■	□	■	■	□	□	■	□	■	■	□	■	□	■	□	■	A
11	■	■	□	■	■	■	■	□	■	■	□	■	■	■	□	■	B
12	■	□	■	■	□	□	■	□	■	■	□	■	□	■	□	■	A
13	■	□	■	■	□	□	■	□	■	■	□	■	□	■	□	■	A
14	■	■	□	■	■	□	■	■	■	■	□	■	■	■	□	■	B
15	■	■	□	■	■	□	■	■	■	■	□	■	■	■	■	■	B
16	■	■	□	■	■	■	■	■	■	■	□	■	■	■	□	■	B
17	■	■	■	■	□	□	■	■	■	■	□	■	■	■	□	■	B
18	■	□	■	■	□	□	■	□	■	■	□	■	□	■	□	■	A
19	■	■	□	■	■	□	■	■	■	■	□	■	■	■	□	■	B
20	■	■	■	■	■	□	■	□	■	■	□	■	□	■	□	■	B
21	■	■	□	■	■	□	■	■	■	■	□	■	■	■	□	■	B
Sens	100	100	100	100	100	100	100	100	100	100	N/A	100	100	100	100	100	
Spec	N/A	100	100	N/A	100	100	N/A	100	N/A	N/A	100	N/A	100	N/A	100	N/A	
Freq	1	0.76	0.571	1	0.67	0.29	1	0.571	1	1	0	1	0.71	1	0.1	1	

Figure 2.4: Killer-Immunoglobulin like receptor (KIR) genotyping results from unamplified gDNA derived from DBS compared to unamplified gDNA derived from whole blood (the gold standard) for 21 adult South Africans. KIR genotyping results were completely concordant between gDNA from whole blood and gDNA from DBS. Grey squares represent concordant presence, open squares represent concordant absence between wgaDNA and gDNA template from whole blood. Detection of the HLA-DRB1 gene has been included as a positive control and the experiments were done in duplicates. The average sensitivity and specificity of each KIR gene on gDNA from DBS samples relative to gDNA from whole blood and KIR haplotype of each participant.

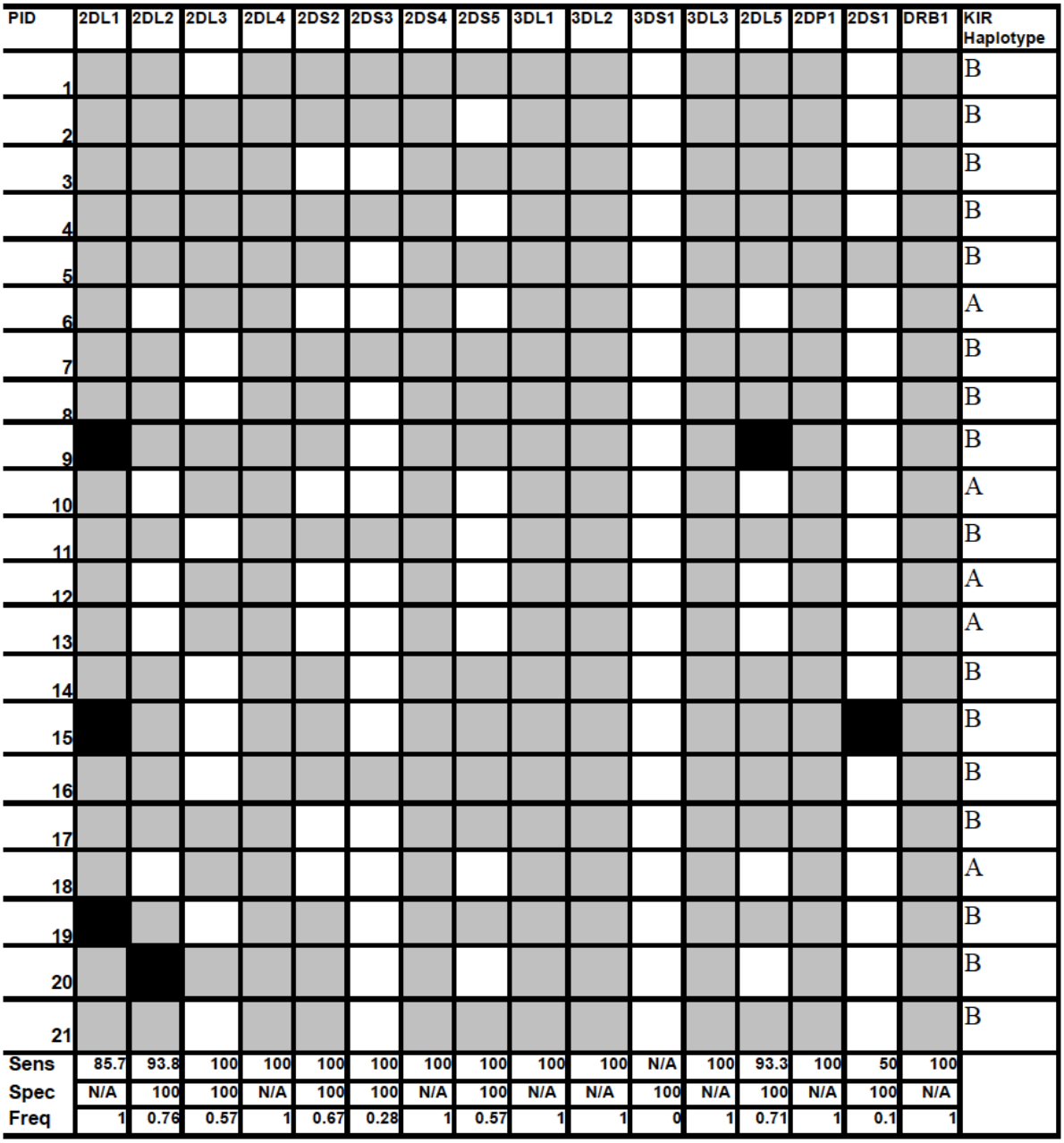


Figure 2.5: Killer-Immunoglobulin like receptor (KIR) genotyping results from whole genome amplified gDNA derived from DBS compared to unamplified gDNA derived from whole blood (the gold standard) for 21 adult South Africans. Discordant results were found between wgaDNA and gDNA. Grey squares represent concordant presence, open squares represent concordant absence and black squares represent discordant results between wgaDNA and gDNA template from whole blood (i.e., presence of a gene in the gold standard but not the experimental sample). Here detection of the HLA-DRB1 gene has been included as a positive control and the experiments were done in duplicates. The average sensitivity and specificity of each KIR gene on wgaDNA relative to gDNA from whole blood and KIR haplotype of each participant.

2.5.4 HLA Genotyping of wga DNA

To further evaluate the suitability of wgaDNA for quantifying highly complex polymorphic genes, we performed high resolution (four digit) sequence based HLA-genotyping. In these assays we used the same samples that had been used for KIR repertoire determination to characterize HLA class I (HLA-A, -B, and -C) loci. As done previously, we based our comparisons on the gold standard of unamplified whole blood derived gDNA. wgaDNA template increased homozygosity for each of the loci due to allelic dropouts (i.e., concordant for only one allele) and an increased frequency of failed reactions ($p<0.01$) (Fig 2.6). In particular, the *HLA-C* locus had the highest frequency of failed reactions compared to *HLA-A* and *HLA-B* ($P<0.001$). Likewise, the *HLA-C* locus had the lowest frequency of concordant results compared to either *HLA-A* or *HLA-B* loci ($p<0.01$; McNemar's test). Although we observed the lowest frequency of failed reactions with the *HLA-A* locus, we found that this locus had the highest frequency of homozygosity that was associated with the second allele ($p<0.01$; Mcnemar's test). In contrast, the *HLA-B* locus had the most reliable results with the lowest frequency of homozygosity and a higher percentage of concordant results compared to either HLA-A or C ($p<0.01$; McNemar's test).

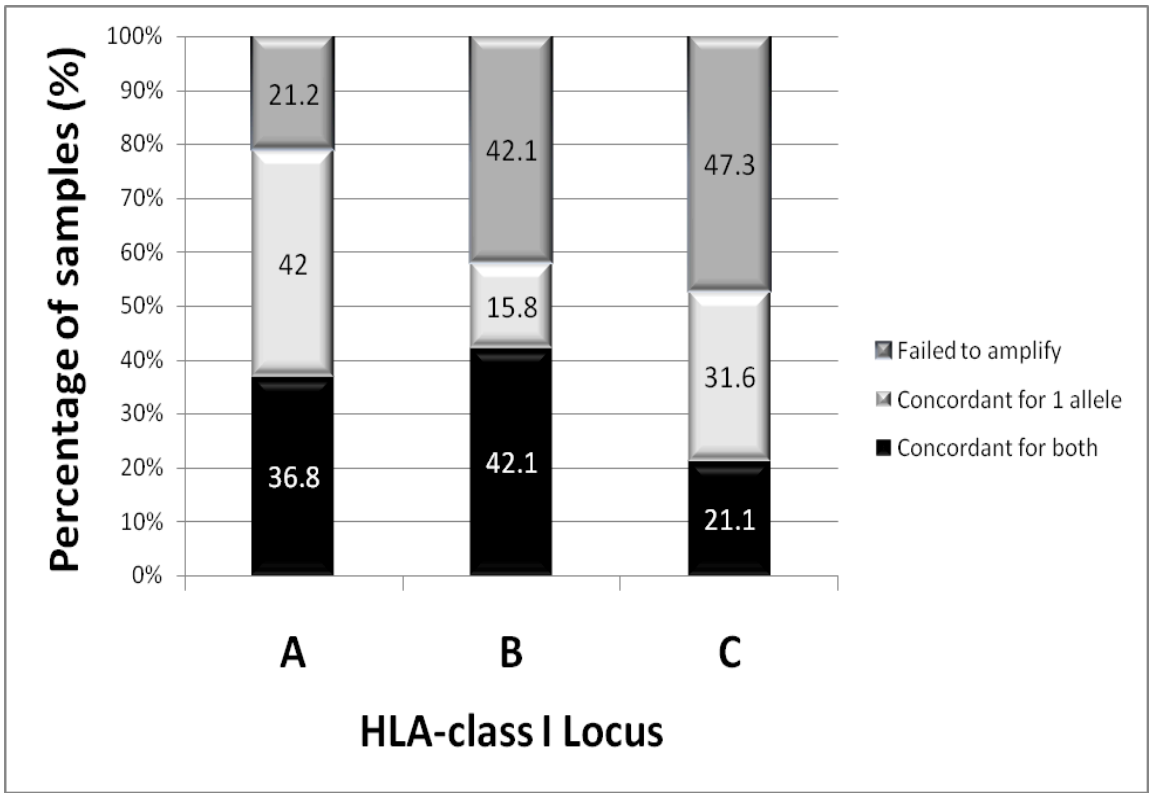


Figure 2.6: HLA-class I genotyping was completely concordant between gDNA derived from DBS and whole blood samples. However, some samples were concordant for both alleles others were concordant for one allele, discordant alleles and some samples failed to amplify in wgaDNA relative to whole blood (gold standard).

2.6 DISCUSSION

Initially, among the isolation methods that we evaluated, QIAamp DNA mini kit was the best method, it produced sufficient gDNA yield and quality as measured by the ratio of absorbance at 260nm and 280nm. These two spectrophotometric measurements, the 260/280 and the 260/230 ratios, have been frequently used to assess gDNA quality (Psifidi et al., 2010; Kong et al., 2006). Previous studies have shown that a 260/280 ratio of approximately 1.8-2.00 indicates pure DNA, while the 260/230 ratio lower than 2 indicates chemical contamination possibly due to formation of phenolates or orthocyanates (Psifidi et al., 2010; Kong et al., 2006). Previously, Martin and Carrington (2009) found that 200ng of good quality (260/280 = 1.8-2:00) gDNA was required for reliable KIR genotyping by SSP-PCR and our results have confirmed their findings. Based on the differences in 260/230 ratios among the methods we tested, we propose lower gDNA quality produced by the Nuclisens miniMAG approach may be attributed to residual guanidine, phenolates and thiocynates carry over during the extraction process. From our data we conclude that the QIAamp DNA mini kit yielded gDNA of sufficient quantity and highest purity compared to the other methods evaluated here.

These findings are consistent with previous research, using both Picogreen and Real-time PCR for DNA yield quantification, Sjoholm et al (2007) also found that the Qiagen QIAamp DNA mini kit produced the highest DNA yield compared to EZNA, Chelex 100 and alkaline lysis methods. Thus, this method consistently performs well for gDNA isolation. Furthermore, we found no significant difference in the gDNA yield when one or two 4.8mm punches, or the whole DBS spot were used (Appendix 7, Supplementary table 7.4). These results are consistent with a previous study by Hollegaard et al., 2009a. The lack of higher

gDNA yields in QIAamp DNA mini kit may be attributed to limited binding capacity of the silica membranes used in the fast spin-column procedure, in which gDNA binds to a silica membrane allowing inhibitors or contaminants to pass through (Hollegaard et al., 2009 a).

Our results revealed that QIAamp continued to yield the most optimal results for PCR-based detection of *HLA-DRB1* and *IL-10* SNP detection before and after whole genome amplification. The significantly lower frequencies of *HLA-DRB1* amplification with the other methods suggest that the presumed protein or chemical contaminants may have been interfering with PCR amplification (Psifidi et al., 2010). These results were not consistent with the previous findings of Pachot et al (2007), who reported a complete concordance in *HLA-DRB1* oligotyping between DBS-minimag and phenol chloroform extraction methods. One possible explanation for the difference in outcomes is the age of the DBS samples used for analysis. Here we used DBS samples that were approximately 8 years old whereas they used freshly prepared DBS samples. Thus, most of our DBS derived gDNA could be fragmented during storage. Although they found that few samples produced insufficient DNA template for WGA, regardless of the age, the extent of fragmentation of gDNA isolated from archival DBS increased with increasing age of the specimens.

Following WGA, the frequencies of success in *HLA-DRB1* amplification remained significantly lower for Nuclisens minimag and prep-GEM than that of samples isolated by QIAamp DNA mini kit. From this we concluded that we found no difference in *HLA-DRB1* amplification with and without WGA. Similarly, previous research have previously shown that WGA by multiple displacement amplification significantly increases DNA yield to allow limited KIR and HLA-class I SNP analysis (Chainonthee et al., 2010; Shao et al., 2004; Singh and Spector, 2007). However, the feasibility of applying this technology to assess the

use of a single punch of dried blood spot to a complete range of highly polymorphic HLA class I and KIR genes remained unresolved.

We obtained concordant genotyping results between whole genome amplified gDNA and unamplified gDNA for a majority except *KIR2DL1,KIR2DL3, KIR2DL5* and *KIR2DS1*. Thus the sensitivity for detecting these genes was lower in wgaDNA and ranged from 65% to 92% (Fig 2.5). This difference in sensitivity was not attributed to differences in the source of the gDNA (whole blood vs. DBS) as we found no difference between whole blood derived gDNA and unamplified DBS derived gDNA. We also found similar discrepant results between wgaDNA and unamplified gDNA in an independent analysis of 38 additional stored DBS samples from the same cohort (Table 2.7, appendix 7.6).

Taken together, our data suggest that whole genome amplification is not a robust method for generating template for the detection of *KIR2DL1*, *KIR2DL3*, *KIR2DL5* and *KIR2DS1;* however it provides a robust method for the analysis of other *KIR* genes. The most likely explanation for failure to detect some of the KIRs is unequal gDNA template amplification by the method of WGA that we used; however, we cannot exclude the possibility of inadequate primers for detection by WGA. Chainonthee et al (2010) also found discrepant results for the detection of *KIR2DS1*, *KIR2DL1*, *KIR3DS1* and *KIR3DL1* until they redesigned their primers. It is possible that some of our primers were complimentary for regions that are underrepresented in wgaDNA. Further sequence analysis of wgaDNA is required to resolve this matter. Taken together these findings imply either unreliability of wgaDNA for highly polymorphic loci such as KIR or that some KIR genotyping primers may

need to be designed and optimized specifically for using wgaDNA templates, however, further research to validate these findings is required.

To further evaluate the suitability of wgaDNA for highly complex polymorphic genes, we performed high resolution HLA-typing. Our results imply that wgaDNA is not reliable for high resolution HLA genotype determination by sequence based typing. Sequence based typing yielded an unacceptably high frequency of failed reactions and increased homozygosity. In contrast, Singh and Spector (2007) assessed the fidelity of whole-genome amplification of DBS derived DNA for HLA typing and found 100% concordance between wgaDNA and gDNA from DBS samples; however, they did not extend their analysis to other immune-related genes with equivalent polymorphic variation. The most likely explanation for our opposing findings is the difference in approaches used for HLA genotype determination. Here we used sequence based typing whereas they used an automated DNA typing system utilizing the Luminex Luminex® 100 platform probes to detect HLA-class I alleles which could be more specific than sequence specific primers. During whole genome amplification, gDNA regions are not amplified equally and Oligonucleotide probes are more sensitive than primers, they were able to detect the regions that were not amplified well, while we failed to detect those regions using primers.

We speculate that sequence-specific probes are more robust in their recognition of wgaDNA compared to sequence based approaches because probes are more specific and could detect the regions that were not amplified well. However comparisons between HLA-class I genotyping on wgaDNA using high resolution primer-based typing and sequence-specific oligonucleotide probes remain to be tested directly.

Another possible explanation is the difference in the number of DBS samples used for DNA isolation. Singh and Spector (2007) used 3-6 punches while we used a single punch for each sample. Futhermore, they used more gDNA template (10-20ng) while we used less (5-8ng) gDNA for whole genome amplification. Previously, it has been shown that the amount of gDNA input in WGA reaction is critical in determining success of downstream applications and that low gDNA input can result in loss of heterozygosity and increased number of failed reactions (Bergen et al., 2005). Another possible explanation in support of this conclusion, it has been previously shown that the method of genotyping impacts the outcome of PCR-based typing from wgaDNA (Shao et al., 2004). Consistent with our results, Shao et al (2004) found that genotyping using sequenced-based typing from wgaDNA for *HLA-C* locus determination yielded proportionally more failed reactions than either HLA-A or –B loci. One limitation in the interpretation of their findings is the lack of a gold standard for comparison. We extended their findings by including a gold standard and discovered increased homozygosity in the determination of HLA genotypes by sequence-based typing. Taken together, our findings suggest that the method of HLA genotyping may be critical for the reliable determination of high resolution HLA genotypes from whole genome amplified DNA.

2.7 CONCLUSION

Although previous investigators have evaluated the use of whole genome amplified DNA for KIR and HLA genotyping, their analysis has been limited to selected KIR genes, or HLA epitopes for KIR (Chainonthee et al., 2010). Here we have extended the findings of these previous studies by evaluating the feasibility of using wgaDNA derived from DBS to characterize complete repertoires of both KIR and HLA class I in an African cohort. We accepted our first hypothesis, QIAamp DNA mini kit produced sifficient gDNA yield with

good quality from DBS, suitable to perform reliable subsequent genetic analysis of *KIR*, *HLA* class I and *IL-10* genes. Although we found that WGA produced a template suitable for *KIR* genotyping of most *KIR* genes, it was not suitable for high resolution HLA determination by sequence based typing therefore, we rejected our second hypothesis. These findings suggest that wgaDNA can be used for KIR genotyping of 11 genes, except for *KIR2DL1*, *KIR2DL3*, *KIR2DL5*, *KIR2DS1* and HLA-class I genotyping due to increased false homozygosity. Our data suggest that WGA should be used with great caution for subsequent genetic analysis and that downstream applications may determine the methods selected for genotyping. These findings have important implications for the design of genetic studies using stored DBS samples.

CHAPTER 3

HIV-1 MOTHER-TO-CHILD ACQUISITION

3.1 BACKGROUND

HIV-1 mother to child transmission (MTCT) remains a significant public health problem -especially in resource-limited settings, particularly sub-Saharan Africa, where women of childbearing age have the highest prevalence of HIV-1 infection. Transmission of HIV-1 from mother to child may occur in three stages; during pre-partum (pregnancy), intrapartum (delivery) or postpartum (through breastfeeding) (Joubert et al., 2010). In developed countries such as United Kingdom and United States adherence to highly active antiretroviral therapy (HAART) regimen, selective ceaserian section delivery and avoidance of breastfeeding have been shown to reduce HIV-1 MTCT to approximately 1-2% (Tiemessen and Kuhn, 2006; Taha et al., 2009, Zhou et al., 2010). However, despite the use of ARV regimen, approximately 500 000 infants are born with HIV-1 per year worldwide and 90% of these infections occur in Sub-Saharan Africa.

The possible explanation for this high mother-to-child transmission of HIV-1 is that most women–especially in rural communities in Sub-Saharan Africa do not have access to HAART or do not adhere to self-administered drugs like HAART, possibly due to the stigma attached to HIV-1 infection (Ahmad, 2011). Other alternative explanation could be that selective ceaserian section delivery and avoidance of breastfeeding are not safe or affordable in developing countries. If effective preventative HIV-1 mother-to-child acquisition vaccines for mothers or infants were available, most HIV-1 MTCT could be prevented thereby, saving many lives from death caused by HIV-1 related illnesses.

Most of these HIV-1 infected infants develop higher viral loads, severe symptoms of infection and progress rapidly to AIDS during their first year of life (Ahmad, 2011, Little et al., 2007). Previous research revealed that mother-to-child transmission of HIV-1 is multifactorial, however high systemic maternal viral load is the strongest risk factor for HIV-1 perinatal mother-to-child transmission (Mock et al., 1998; Bryson et al., 1996). Other maternal immunological markers, particularly lower birth weight was associated with high transmission *in utero*, while lower CD4 T cell count and natural killer cell percentage were also associated with increased risk of intrapartum HIV-1 MTCT (Mock et al., 1998).

On the other hand, human genetic variation may also contribute significantly to the heterogeneity in HIV-1 pathogenesis. Furthermore, several immune related genes have been shown to reduce HIV-1 transmission and disease progression to AIDS. More specifically, genes that encode receptors such as *Killer Immunoglobulin-like Receptors* (KIR) and their cognate *Human Leukocyte Antigen* (HLA) class I ligands have been shown to link innate to adaptive immune responses (Gaudieri et al, 2005; Martin et al, 2002; Martin et al, 2007). KIR receptors and their HLA class I ligands have been shown to influence HIV-1 transmission and disease progression to AIDS in adults (Gaudieri et al, 2005; Martin et al, 2002; Martin et al, 2007). The interaction between activating and inhibitory polymorphic KIR receptors and their cognate HLA-class I ligands regulate NK cell responses (Rajapalagon and Long, 2000). Recently, KIR haplotype B was associated with lower CD4 T cell counts and faster HIV-1 disease progression (Jennes et al., 2011). However, the role of KIR/HLA genotypes in HIV-1 mother-to-child acquisition remains unknown, thus our study will determine the role of KIR/HLA in HIV-1 mother-to-child acquisition.

Previous research has demonstrated the role of HLA-class I in susceptibility or protection from HIV-1 infection (Fabio et al., 1992; Macdonald et al., 1998; Polycarpou et al., 2002; Winchester et al., 2004; Ballan et al., 2007, Mackelprang et al., 2008, Paximadis et al., 2011). Mother-to-child HLA concordance was associated with higher risk of HIV-1 mother-to-child transmission (Polycarpou et al., 2002; Mackelprang et al., 2008) and faster disease progression to AIDS or death (Thobakgale et al., 2009; Kuhn et al., 2004). On the other hand, maternal HLA-class I homozygosity was also associated with increased susceptibility to perinatal HIV-1 mother-to-child transmission *in utero*, during labour and via breastfeeding (Macdonald et al., 1998; Mackelprang et al., 2008). Prior research revealed that higher risk of HIV-1 transmission has been associated with *HLA-B*18, -B*13:02, -B*35:01, -B*35:03, -B*44:02*, *-B*50:01* and *-B*58:02* in transmitting mothers (Winchester et al., 2004; Kipiela et al., 2004). In contrast, protection from HIV-1 MTCT has been associated with *HLA-B*49:01, -B*53:01, -B*57:01, -B*57:03, -B*58:01* and *-B*81:01* in non-transmitting mothers (Kipiela et al., 2004; Winchester et al., 2004). Taken together, these studies suggested an important role of HLA-class I alleles in prevention of HIV-1 mother-to-child transmission.

Previous studies on the role of KIR genes in HIV-1 infection revealed that KIR receptors alone or in combination with their HLA-class I ligands influence HIV-1 acquisition (Paximadis et al., 2011) and disease progression progression in HIV-1 infected infants (Ballan et al., 2007). Previous research on HIV-1 exposed uninfected infants found higher expression of inhibitory *KIR2DL1, KIR3DL1, KIR2DL3* and *NKG2C* in HIV-1 infected infants compared to EU infants (Ballan et al., 2007). In contrast, another study in Chinese HIV-1 infected children found a higher expression of *KIR2DL1/KI2DS1, KIR3DL1, NKG2D*

and lower cytolytic activity in uninfected children compared to HIV-1 infected children (Fu et al., 2010). More recently, Paximadis et al (2011) found lower frequencies of *KIR2DL3* in combination with C1 ligand and lower frequencies of homozygous *KIR2DL3* with heterozygous C1/C2 ligand in HIV-1 infected infants compared to EU infants. A previous study revealed that resistance to HIV-1 infection among African HIV-1 exposed uninfected female sex workers (FSWs) is associated with inhibitory *KIR2DL2/KIR2DL3* heterozygosity in the absence of its cognate ligand, HLA-C1 and likewise, *KIR3DL1* in the absence of HLA-Bw4 (Jennes et al., 2006). The possible explanation for the difference in conclusions could be due to ethnic differences, one study was conducted in Chinese infants, while the other study was conducted in South African infants. The distribution of their KIR genotypes is different, we conducted a study to further determine the KIR genes associated with protection or susceptibility to HIV-1 mother-to-child acquisition in exposed South African infants.

If we could identify KIR/HLA compound genotypes that are associated with protection from HIV-1 mother-to-child transmission, we could use those protective KIR/HLA alleles to design preventative and therapeutic vaccines. We would create recombinant HIV-1 peptides that will bind those protective HLA alleles. HLA-class I alleles will present those viral peptides to either NK cells via KIR receptors or to cytotoxic T cells via recognition by the T cell receptors. These interactions will lead into increased immune activation and since immune activation would be associated with protection, it will protect the infants that carry the protective KIR/HLA alleles from HIV-1 acquisition. However, the infants that do not carry protective HLA or KIR alleles would not be protected using the approach of prophylactic vaccination. Thus, this is a limitation of using HIV-1 peptides that are restricted to “protective” HLA and KIR alleles for a vaccine.

3.2 STUDY AIMS

SPECIFIC AIM 1: To identify KIR/HLA compound genotypes that are associated with acquisition of HIV-1 clade-C infection among mother-infant pairs indigenous to South Africa. From this aim, it is hoped to establish which KIR or HLA genotypes are involved in protection or susceptibility to HIV-1 maternal-infant acquisition. We can use that information in designing recombinant HIV-1 peptides that will be presented by identified HLA-class I alleles to the cytotoxic T cells and activate immune responses for the development of preventative vaccines.

SPECIFIC AIM 2: To determine the associations of accelerated HIV-1 disease with KIR/HLA compound genotypes among HIV-1 exposed infected South African infants. From this aim, we expect to learn which KIR/HLA compound genotypes are associated with HIV-1 disease progression in infants. This information could be used to create HIV-1 recombinant peptides that will be presented by identified HLA alleles that are associated with slower disease progression to AIDS and enhance immune activation. The information could be useful in designing therapeutic vaccines

3.3 HYPOTHESIS

Hypothesis 1

1a) We hypothesized that resistance to HIV-1 acquisition among HIV-1 exposed, uninfected infants is determined by the higher frequencies of activating KIR and/ or KIR B haplotypes.

1b) We also hypothesized that resistance to HIV-1 acquisition among HIV-1 exposed uninfected infants is determined by higher frequencies of protective HLA-B alleles

Hypothesis 2

We hypothesized that HIV-1 rapid progressors have lower frequencies of both activating KIR genes and HLA-B alleles compared to slow progressors.

3.4 MATERIALS AND METHODS

3.4.1 Study Design

A total of 3445 pregnant women were voluntary counselled and tested for HIV-1 at the antenatal clinic, 1769 (51.4%) were HIV-1 positive, 1662 (48.3%) were HIV-1 negative and 12 (0.3%) were indeterminate (Bland et al., 2009). Out of 1769 HIV-1 infected women, 1372 infants were born, however, only 124 of these infants were born with the HIV-1 virus resulting in an infection rate of 9.04% (Coovadia et al., 2007; Bland et al., 2009). These women were enrolled in the case-control vertical transmission (VTS) study, 2704 women from rural Umkhanyakude and 741 women from a clinic in the outskirts of Durban. We individually matched the cases (124) to exposed uninfected infants (120) based on maternal viral loads (Table 3.1). We matched transmitting and non-transmitting mothers by viral load because previous studies revealed that viral load is the strongest risk factor of HIV-1 acquisition (Mock et al., 1998). We tried to eliminate the effect of viral load in HIV-1 mother to child acquisition in order to indicate that the KIR/HLA associations that were found were not due to other risk factors. Healthy uninfected infants were individually matched to the cases based on maternal age to eliminate the effect of maternal age in HIV-1 MTCT. Clinical data, particularly CD4 T cell, viral loads, body weight and height were collected from the infants over the period of 2 years and they were used to track HIV-1 disease progression in HIV-1 infected infants.

We isolated gDNA from DBS samples using optimized QIAamp gDNA isolation method. KIR genotyping by SSP-PCR and HLA genotyping by sequence based high resolution HLA-typing was performed without whole genome amplification (Table 3.1). The frequencies of activating, inhibitory KIR, KIR haplotypes and HLA-class I were compared between the HIV-1 infected infants, HIV-1 exposed uninfected and healthy unexposed infants. To assess associations between KIR/HLA genes with HIV-1 disease progression in HIV-1 infected infants, we grouped the infants that had a particular gene and the infants that did not have the gene and calculated the mean viral load in the two subgroups. We then compared the average CD4 and average viral load between the infants that had a specific KIR/HLA gene with the infants that did not have the gene.

Table 3.1: Case-control study design with assessment of KIR and HLA genotyping on the DNA isolated from corresponding infants. Cases: group of infants that acquired HIV-1 perinatally from their mothers, control 1: group of HIV-1 exposed but not infected infants and control 2: group of healthy unexposed infants.

	CASES HIV-1 Exposed infected infants	**CONTROL 1 HIV-1 exposed uninfected infants**	**CONTROL 2 Healthy Unexposed infants**
KIR genotyping	124	120	126
HLA genotyping	124	120	126
Mode of Delivery			
Vaginal	116	99	110
Ceaserean section	8	21	16
Birthweight			
>3500 g	9	15	20
2500-3500g	82	89	101
<2500 g	24	11	13
Missing	9	5	2
Infant's Death	76	12	0
Average Maternal VL	122834 ±19003	113056 ±18306	N/A
Average Maternal CD4 count	359.6 ±18.50	417.5 ±20.52	Missing

3.4.2 Study population

A total of 2938 infants were born to mothers enrolled to a non-randomised intervention study of mother-to-child HIV-1 transmission study also know as Mamanengane (the Zulu name for the study meaning 'Mother and Child') (Bland et al., 2009). Out of 1372 infants, only 124 infants contracted HIV-1 from their mothers (9.04%). Based on matched antenatal maternal viral load during delivery, we then selected 120 HIV-1 exposed uninfected infants born to HIV-1 infected mothers because those were the only mothers that matched 124 transmitting mothers. Finally, matching unexposed mothers to transmitting mothers by maternal age selected 126 unexposed healthy infants born to unexposed mothers (Fig 3.1). The vertical transmission study (VTS) began in 2001 until September 2006 in the rural Umkhanyakude district, which is located in northern KwaZulu Natal, South Africa, where pregnant women attending rural and semi-urban antenatal clinics in KZN were offered voluntary HIV counselling, testing and enrolled into the study (Bland et al., 2009; Rollins et al., 2007). All HIV-1 infected and some uninfected pregnant women were enrolled during antenatal visit if they were 16 years or older, planning to stay in the study for three months after delivery and provided written informed consent. Single-dose nevirapine was administered to all HIV-1 infected mothers at 28 weeks of gestation and to their infants during labour. DBS samples were obtained by a heel prick on infants within 72 hours post-delivery and DBS samples were stored for approximately 8 years prior to our analysis.

HIV status of the baby was determined by performing quantitative HIV RNA assay at 5 months of age in three replicates (Nuclisens HIV-1 QT, Boxtel, Netherlands) with a sensitivity of 80 copies of HIV RNA per milliliter of blood (Rollins et al., 2007). Changes in feeding practices were made based on the HIV-1 status of the infant at 6 months of age. HIV-1 positive breastfeeding mothers were counseled to stop breastfeeding infants who were

confirmed to be HIV-1 negative at 6 months of age. On the other hand, HIV-1 positive breastfeeding mothers were counseled to continue breastfeeding infants who were confirmed to be HIV-1 positive. Both mothers and infants were followed up clinically when the infant was 6 weeks old, and monthly afterwards until the infant reached 9 months of age then at 3 months intervals until 18 months of age. Clinical data, particularly, CD4 T lymphocyte counts, viral loads, feeding practices growth and development (weight and height), morbidity and mortality rate were monitored monthly from birth to 2 years of age (Bland et al., 2009; Coovadia et al., 2007; Rollins et al., 2007). HIV-1 exposed uninfected infants were selected based on maternal antenatal viral load before birth and they were individually matched with infants from HIV-1 positive mothers with comparable antenatal viral loads. In contrast, HIV-1 unexposed negative infants were selected based on maternal age, their mothers were individually matched to HIV-1 infected mothers. There was no difference in terms of educational qualifications or income between the three groups of women and only perinatal transmissions were included in this cohort (Bland et al., 2009).

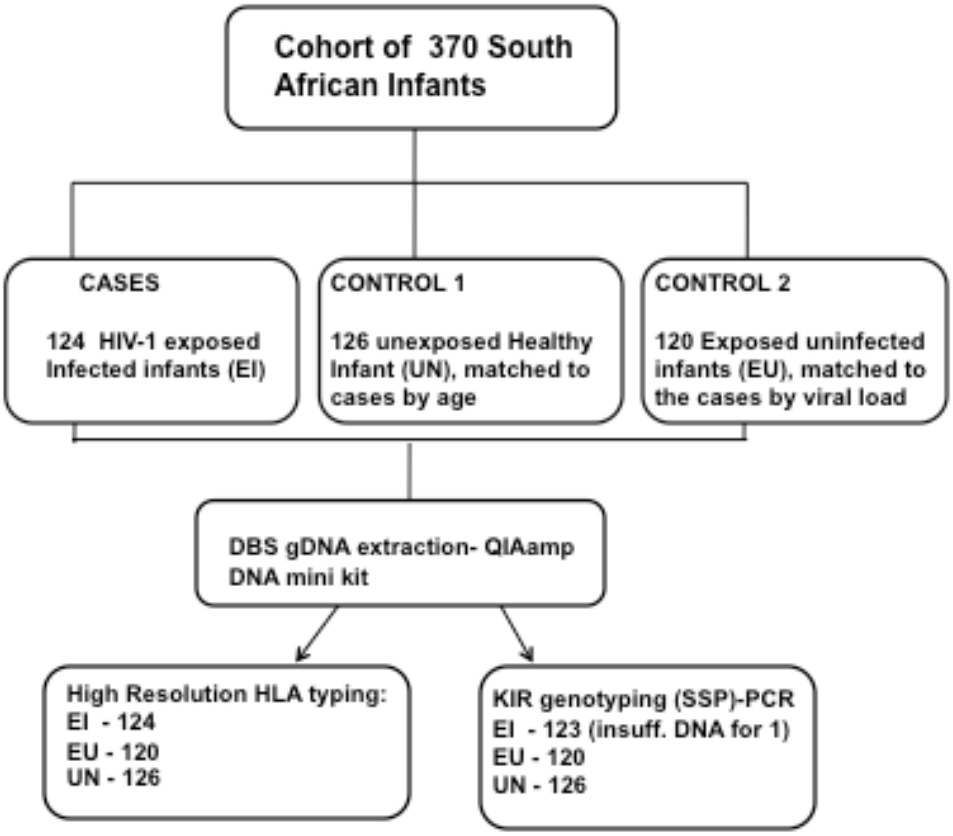


Figure 3.1: Workflow diagram of HIV-1 vertical mother-to-child transmission: KIR and HLA genotyping performed on genomic DNA isolated from dried blood spots of HIV exposed, uninfected infants (EU); exposed infected (EI) and unexposed uninfected (UN) infants.

3.4.3 Statistical Analysis

We used SAS (Statistical Analysis software version 9.1) for statistical analysis and the results are presented with and without correction for multiple comparisons. Using One-way Analysis of Variance (ANOVA), we compared maternal CD4-T-lymphocyte count and viral load (RNA copies per ml) between HIV-1 transmitting and non-transmitting mothers. We used ANOVA to compare continuous variables such as the square root of maternal CD4-T-lymphocyte count and the maternal antenatal log viral load (RNA copies per ml), between HIV-1 transmitting and non-transmitting mothers. The viral load data was normalized by log transformation for this analysis.

Using logistic regression analysis, we tested the associations between each KIR gene, Bx KIR haplotypes, HLA-A, HLA-B, HLA-C allotypes and KIR-HLA interactions with HIV-1 mother-to-child acquisition. To assess the role of Bx KIR haplotypes in HIV-1 mother-to-child acquisition, we grouped KIR repeitores into haplotype A and B based on the guidelines available on the IPD-KIR Sequence database (http://www.ebi.ac.uk/ipd/kir/haplotype.html, 01 July 2011; 11:22am). **See section 2.4.4.7** Logistic regression analysis was also used to determine association between *KIR2DS4* subtypes *197* and *219* or *HLA-C 3'UTR HH* vs *LL*, *HLA-Bw4-80T, HLA-Bw4-80I*, group C1 and C2 of the HLA-C alleles and HIV-1 acquisition. We used logistic regression because these are all categorical data. We compared the presence or absence of particular KIR/HLA genes and then fitted this data into a linear logistic curve. Mixed effects model and linkage disequilibrium were not taken into consideration. Survival analysis was performed using Cox proportional hazards logistic regression model. The 95% Confidence Intervals (CI) and odds ratios (OR) were calculated and used to determine the risk of acquiring HIV-1 infection, if the infant carries a specific KIR/HLA genes. Here the

dichotomous outcome is HIV-1 acquisition and the dichotomous explanatory variable is the presence or the absence of a particular KIR/HLA compound genotype.

One-way ANOVA was used to perform a cross-sectional analysis in order to determine the associations of KIR/HLA genotypes to CD4 T lymphocyte count and viral load in HIV-1 infected infants because these are continuous variables. We did not compare CD4 T cell count or viral load at different time points due to insufficient data. For the methods development section (Chapter 2), we adjusted for multiple comparisons using Holm's method (Holm, 1979). The p value was multiplied by the number of comparisons made and they were two-sided. For the mother-to-child KIR/HLA association study, Bonferroni adjustments were used to correct for multiple comparisons. We multiplied the p values by the total number of comparisons 180 which was the number of HLA alleles that were detected in at least one cohort (60) times the number of comparisons made (3) (60x 3= 180) and a p-value<0.05 was considered significant. The same multiplication factor was used across the HLA-class I alleles

3.4.4 KIR genotyping

KIR genotyping using sequence specific primer (SSP)-PCR was performed on genomic DNA isolated from dried blood spots without whole genome amplification using the protocol described on **section 2.4.4.6**

3.4.5 *KIR2DS4* Subtyping

The remaining PCR product from KIR typing was used to determine the *KIR2DS4* subtypes using an automated electrophoresis instrument, Caliper LabChip Gx 90 DNA Assay that analyzes the quality, size and concentration of the DNA. LabChip components were thawn and the Chip was allowed to equilibrate to room temperature for about 30-45 minutes. The Chip and all active wells were cleaned twice with molecular biology grade water (1.2ml) and waste water was discarded using a vacuum. The active wells were rinsed with DNA storage buffer and the Chip was primed for 10 minutes for HT DNA IK. DNA storage buffer was aspirated with the vacuum; the chip was wiped with a sterile cotton swab dampened with 70% ethanol.

Using reverse pipetting technique, Gel loading-dye was loaded into well 3, 7, 8 and 10 of the Labchip and the DNA marker (140 μL) in well 4. The LabChip was cleaned on both sides with a damp cloth and placed on the designated space on the Chip. DNA ladder (110 μL) was added in the provided 0,2 mL tube and 1X DNA sample buffer (750μL) was added to the provided 2mL tube. Using a 16 multichannel pipettor, nuclease free water (10μL) was added into each well that contained *KIR2DS4*. The plate was inserted in the LabChip instrument and the gel was run automatically. The gels were analyzed using a LabChip GxP software (Caliper Life Sciences).

3.4.6 HLA-class I genotyping by high resolution sequence-based typing

We did a four-digit high-resolution HLA-class I (*HLA-A, -B and –C*) sequence based typing (PCR-SBT) at National Cancer Institute, Frederick MD, USA using Qiagen, SBT excellerator HLA kits. Ambiguities were resolved with a group of specific sequencing primers (GSSPs). Genomic DNA from DBS without WGA was used for genotyping.

PCR cocktail for HLA-A was prepared by adding nuclease free water (5.15µL), 5X Buffer A (2µL), 10mM DNTP (0.2µL), 25mM MgCl2 (0.2µL), 10µM Primer 1 (5A.1) and 10µM primer 2 (3A.1) (0.2µL), KAPA Robust (0.05µL) (KAPA Biosystems) and DNA template (2.5 µL) for each sample (see Appendix 7.10, supplementary Table 7.10.2).

PCR cocktail for HLA-B was prepared by adding nuclease free water (4.65µL), 5X Buffer A (2µL), 10mM DNTP (0.2µL), 25mM MgCl2 (0.2µL), 10µM Primer 1 (5B.1) and 10µM Primer 2 (3B.1) (0.2µL), DMSO (0.5µL), KAPA Robust (0.05µL) and DNA template (2.3 µL) for each sample (see Appendix 7.10, supplementary Table 7.10.2).

PCR cocktail for HLA-C was prepared by adding nuclease free water (5.15µL), 5X Buffer A (2µL), 10mM DNTP (0.2µL), 25mM MgCl2 (0.2µL), 10µM Primer 1 (5C.1) and 10µM Primer 2 (3C.1) (0.2µL), KAPA Robust (0.05µL) and DNA template (2.5 µL) for each sample (see Appendix 7.10, sup Table 7.10.2).

Using an automatic pipette, PCR cocktail (8µL) was transferred into a 96 well plate and 2,5 µl DNA template was added. The PCR plate was centrifuged briefly and the samples were incubated in a thermocycler, HLA-A and –B were amplified with a step down PCR using the following parameters 3 minutes at 95^{o}C; 5 cycles of 15 sec at 95^{o}C, 15 sec at 62^{o}C, 30 sec at 72^{o}C; 26 cycles of 15 sec at 95^{o}C, 15 sec at 58^{o}C, 30 sec at 72^{o}C; 4 cycles of 15 sec at 95^{o}C,

1 min at 55°C, 30 sec at 72°C; 7 min at 72°C and at 4°C infinite. HLA-C alleles were amplified with regular PCR using the following parameters, 2 minutes at 95°C; 40 cycles of 15 sec at 95°C, 15 sec at 70°C, 30 sec at 72°C; 7 minutes at 72°C and 4°C indefinitely. The PCR products were run on a 2% gel and if PCR was successful the samples proceeded to gene sequencing.

3.4.6.1 PCR Product clean up

The PCR product was cleaned with Ampure solution using an automatic robot instrument. Tips (250μL) were placed on the space provided on the robot makings sure that it was stable. Provided deionised water and 70% ethanol were poured on two different lids of the tips box and placed in the space provided. The ampure solution was shaken and poured on the square container and placed on the rack provided and the 96 well plate containing amplified PCR products was also placed on the rack. The amplified DNA samples bind to the magnetic beads, the sample was washed three times with water and ethanol then elution was performed with deionised water. Clean PCR products were kept at -20°C.

3.4.6.2 HLA-class I sequencing

Following PCR, we did high-resolution HLA-class I (HLA-A, -B and –C) polymerase chain reaction sequence based typing (PCR-SBT) (four digit) as described previously by Kulkarni et al (2011). This protocol for HLA class I genotyping was based on recommendations of the International Histocompatibility Working group (www.ihwg.org). In brief, for *HLA-A, -B* and *–C* genes, we amplified and sequenced exons 2 and 3 using specific primers (Appendix 7.10, supplementary Table 7.10.2) and optimised PCR conditions (Appendix 7.10, supplementary Table 7.10.3). We sequenced using a BigDye Terminator v1.1 Cycle Sequencing kit and an

ABI PRISM 3700 DNA Analyzer (Applied Biosystems) with conditions establised previously (Appendix 7.10, supplementary Table 7.10.3).

HLA-A PCR products were sequenced using exon 2 and exon 3, two sequencing cocktails were prepared one containing **INT2R** and another one with **5AE3.2** primers per sample. Nuclease free water (5.675μL), 5X Big Dye Buffer (1.875μL), Big Dye (2,5μL) (KAPPABiosystems), sequencing primers (2μL) were added into 1.5ml tube, the cocktail was vortexed and transferred to the 96 well plates using an automatic pipette. Clean DNA (4μL) was added to the PCR cocktail and the plate was covered with optical covers, centrifuged briefly and incubated in the thermocycler.

HLA-B PCR products were sequenced using exon 2 and exon 3, two sequencing cocktails were prepared one containing **INT2R** and another one with **3BE3.2** primers per sample. Nuclease free water (5.675μL), 5X Big Dye Buffer (1.875μL), Big Dye (2,5μL), sequencing primers (2μL) were added into 1.5ml tube, the cocktail was vortexed and transferred to the 96 well plates using an automatic pipette. Clean DNA (4μL) was added to the PCR cocktail and the plate was covered with optical covers, centrifuged briefly and incubated in the thermocycler.

HLA-C PCR products were sequenced using exon 2 and exon 3, two sequencing cocktails were prepared one containing **INT2C** and another one with **BC3N1B3** primers per sample. Nuclease free water (5.675μL), 5X Big Dye Buffer (1.875μL), Big Dye (2,5μL), sequencing primers (2μL) were added into 1.5ml tube, the cocktail was vortexed and transferred to the 96 well plates using an automatic pipette. Clean DNA (4μL) was added to the PCR cocktail and the plate was covered with optical covers and centrifuged briefly. The following Big Dye sequence parameters were used to sequence HLA-class I alleles; 1 minute at 95^{o}C; 25 cycles of 10 sec at 95^{o}C, 0.5 sec at 50^{o}C, 4 min at 60^{o}C; 4^{o}C infinite.

3.5 RESULTS

3.5.1 There was no difference in maternal plasma viral load between HIV-1 transmitters and non-transmitters

To identify KIR/HLA compound genotypes associated with mother-to-child acquisition of clade-C HIV-1 among South African mother-infant pairs, 124 HIV-exposed infected (EI), 120 Exposed, uninfected (EU) and 126 HIV-1 healthy unexposed infants were included in the study. In order to validate the selection criteria of controls to match the cases and to eliminate confounding factors for the case-control study, we first compared maternal viral load and CD4 T lymphocyte count between HIV-1 transmitting and non-transmitting mothers (Table 3.2). No significant differences were noted in maternal plasma viral load between transmitting and non-transmitting mothers ($p= 0.77$, ANOVA). However maternal CD4 T lymphocytes count was significantly lower in transmitting mothers compared to non-transmitting mothers ($p = 0.03$, ANOVA).

Table 3.2: Transmitting and non-transmitting mothers were matched on viral load. There was no difference in maternal plasma viral load between HIV-1 transmitters and non-transmitters. A: Maternal plasma viral load was not significantly different between HIV-1 transmitting and non-transmitting mothers (p=0.77, ANOVA). **B:** CD4 T cell count was significantly lower in transmitting mothers compared to non-transmitting mothers (p=0.03, ANOVA).

ANOVA on mother's CD4/VL data							
	N	**Mean**	**SE**	**P**	**Difference**	95% CI	
A: Mother's log VL							
Transmitting mothers	118	4.64	0.06	0.77	0.03	-0.15	0.20
Non-transmitting mothers	118	4.61	0.06				
B: Mother's CD4 count							
Transmitting mothers	117	18.16	0.52	**0.03**	-1.62	-3.05	-0.19
Non-transmitting mothers	118	19.78	0.51				

3.5.1.1 The frequency of *HLA-Cw*04:01* tended to be higher in HIV-1 exposed infected infants compared to exposed, uninfected infants

To determine HLA-class I alleles associated with resistance or susceptibility to acquisition of HIV-1, we compared HLA alleles between HIV-1 exposed infected (EI) (n=124), exposed uninfected (EU) (n=120) and unexposed uninfected UN (n=126) infants. Analysis of all HLA genes was done, however, we only show here alleles that were significantly or had a tendency to be higher or lower in HIV-infected infants (Fig 3.2A). The frequency of *HLA-Cw*04:01* was significantly higher in HIV-1 EI compared to EU infants before adjustment for multiple

comparison (p =0.001; Logistic regression). However, after statistical adjustment for multiple comparison, *HLA-Cw*04:01* tended to be significantly higher in EI than EU infants (adjusted p= 0.18). The frequencies of *HLA-Cw*02:10, -A*02:05* and *-A*03:01* were significantly higher in EU compared to EI infants before adjusting for multiple comparisons (p=0.03, p=0.03 and p=0.04 respectively), however, the significance was lost after adjusting for multiple comparisons (adjusted p>0.05; Bonferroni).

3.5.1.2 The frequency of *HLA-Cw*04:01* was slightly higher in HIV-1 exposed infected infants compared to unexposed, healthy infants.

To further assess the role of HLA-class I in acquisition of HIV-1, we compared HLA-class I alleles between 124 HIV-1 exposed infected (EI) and 126 unexposed healthy infants (UN) to represent the general population of South African infants. Analysis of all HLA genes was done, however, we only show here alleles that were significantly or had a tendency to be higher or lower in HIV-infected infants (Fig 3.2B).

Similarly, *HLA-Cw*04:01* was found to be significantly higher in HIV-1 exposed infected (EI) than unexposed healthy (UN) infants before adjustment for multiple comparisons (p=0.02, Logistic regression). However these findings were non-significant after adjustment for multiple comparisons (adjusted p>0.05, Bonferroni). No significant differences were found in *HLA-A*02:05*, *-A*03:01* and *-C*02:10* between HIV-1 infected and HIV-1 unexposed healthy infants (p>0.05).

3.5.1.3 The frequency of *HLA-Cw*04:01* was not significantly different between HIV-1 exposed, uninfected (EU) and unexposed healthy (UN) infants

To further assess the role of HLA-class I in acquisition of HIV-1, we compared HLA-class I alleles between 120 HIV-1 exposed, uninfected (EU) and 126 healthy unexposed infants (UN) to represent the general population of South African infants (Fig 3.2C). Analysis of all HLA gene frequencies was done, however we only discuss here allele frequencies that were significantly or had a tendency to be higher or lower in HIV-infected infants. The frequency of *HLA-Cw*04:01* tended to be high among UN infants compared to EU infants, however these findings did not reach statistical significance ($p>0.05$, Logistic Regression). However, the frequency of *HLA-C*02:10* was significantly higher on EU than UN before adjustment for multiple comparisons ($p=0.02$), Logistic Regression), however the significance was lost following adjustment for multiple comparisons (adjusted $p>0.05$). *HLA-A*02:05* and -*A*03:01* frequencies tended to be higher in EU compared to UN infants, but these differences were not statistically significant.

Logistic analysis of HLA-class I									
HLA-gene	**HIV-1 Exposed Infected**		**HIV-1 Exposed Uninfected**		**P**	**Pc**	**OR**	**95% CI**	
	N	**%**	N	%					
HLA-A* 02:05 Present	5	4.24	14	12.17	**0.03**	1.00	0.32	0.11	0.92
Absent	113	95.76	101	87.83					
HLA-A*03:01 Present	8	6.78	18	15.65	**0.04**	1.00	0.39	0.16	0.94
Absent	110	93.22	97	84.35					
HLA-B*08:01 Present	17	14.17	8	7.02	0.08	1.00	2.19	0.90	5.29
Absent	103	85.83	106	92.98					
HLA-B*15:03 Present	23	19.17	26	22.81	0.49	1.00	0.80	0.43	1.50
Absent	97	80.83	88	77.19					
HLA-C*02:10 Present	20	16.39	32	28.07	**0.03**	1.00	0.50	0.27	0.94
Absent	102	83.61	82	71.93					
HLA-C*04:01 Present	43	35.25	18	15.79	**0.001**	0.18	2.90	1.55	5.43
Absent	79	64.75	96	84.21					

Figure 3.2 A: The frequency of *HLA-Cw*04:01* was significantly higher in HIV-1 infected infants compared to exposed, uninfected infants before adjustments for multiple comparisons (p= 0.001). However, the significance was lost after correction for multiple comparisons (p>0.05). The frequency of *HLA-A*02:05*, *A*03:01* and *C*02:10* were significantly higher in EU before adjustments for multiple comparisons (p=0.03, p=0.04, p=0.03 respectively) but the significance was lost after correction for multiple comparisons (p>0.05).

Logistic analysis of HLA-class I									
HLA-gene	**HIV-1 Exposed Infected**		**Unexposed Uninfected**		**P**	**Pc**	**OR**	**95% CI**	
	N	**%**	N	%					
HLA-A* 02:05 Present	5	4.24	10	8.20	0.21	1.00	0.50	0.16	1.50
Absent	113	95.76	112	91.80					
HLA-A*03:01 Present	8	6.78	14	11.48	0.21	1.00	0.56	0.23	1.39
Absent	110	93.22	108	88.52					
HLA-B*08:01 Present	17	14.17	13	11.21	0.50	1.00	1.31	0.60	2.83
Absent	103	85.83	103	88.79					
HLA-B*15:03 Present	23	19.17	14	12.07	0.14	1.00	1.73	0.84	3.55
Absent	97	80.83	102	81.93					
HLA-C*02:10 Present	20	16.39	19	15.32	0.82	1.00	1.08	0.55	2.15
Absent	102	83.61	105	84.68					
HLA-C*04:01 Present	43	35.25	27	21.77	**0.02**	1.00	1.96	1.11	3.44
Absent	79	64.75	97	78.23					

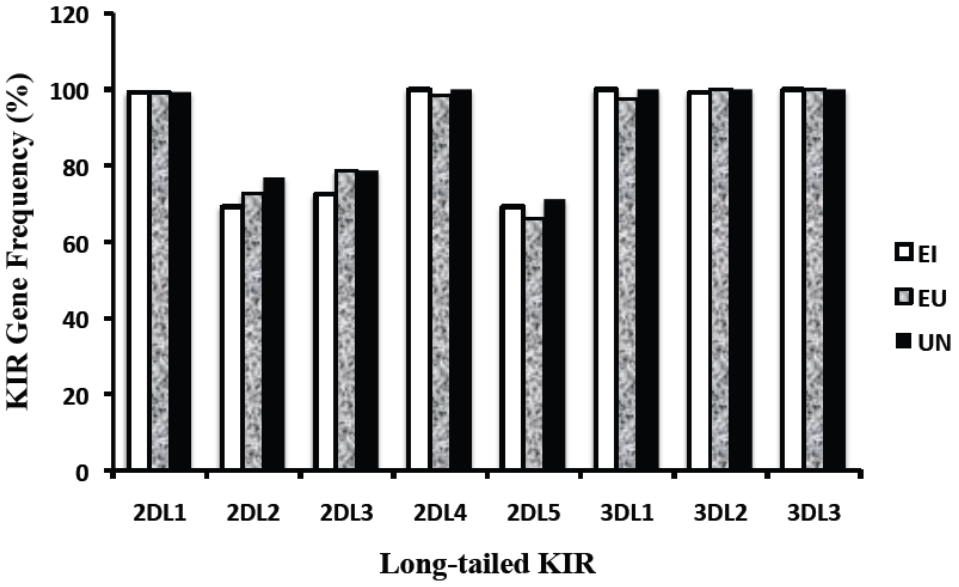
Figure 3.2 B: The frequency of *HLA-Cw*04:01* was significantly higher in HIV-1 exposed infected infants compared to unexposed, healthy infants before adjustment for multiple comparisons (p= 0.02, Logistic regression). However, the significance was lost after adjustment for multiple comparisons (p>0.05, Logistic regression). No significant differences were found in *HLA-A*02:05*, *-A*03:01* and -*C*02:10* between EI and UN infants (p>0.05).

Logistic analysis of HLA-class I									
HLA-gene	**HIV-1 Exposed Uninfected**		**Unexposed Uninfected**		**P**	**Pc**	**OR**	**95% CI**	
	N	**%**	N	%					
HLA-A* 02:05 Present	14	12.17	10	8.20	0.31	1.00	1.55	0.66	3.65
Absent	101	87.83	112	91.80					
HLA-A*03:01 Present	18	15.65	14	11.48	0.35	1.00	1.43	0.68	3.03
Absent	97	84.35	108	88.52					
HLA-B*08:01 Present	8	7.02	13	11.21	0.27	1.00	0.60	0.24	1.50
Absent	106	92.93	103	88.79					
HLA-B*15:03 Present	26	22.81	14	12.07	**0.03**	1.00	2.15	1.06	4.38
Absent	88	77.19	102	87.93					
HLA-C*02:10 Present	32	28.07	19	15.32	**0.02**	1.00	2.16	1.14	4.08
Absent	82	71.93	105	84.68					
HLA-C*04:01 Present	18	15.79	27	21.77	0.24	1.00	0.67	0.35	1.30
Absent	96	84.21	97	78.23					

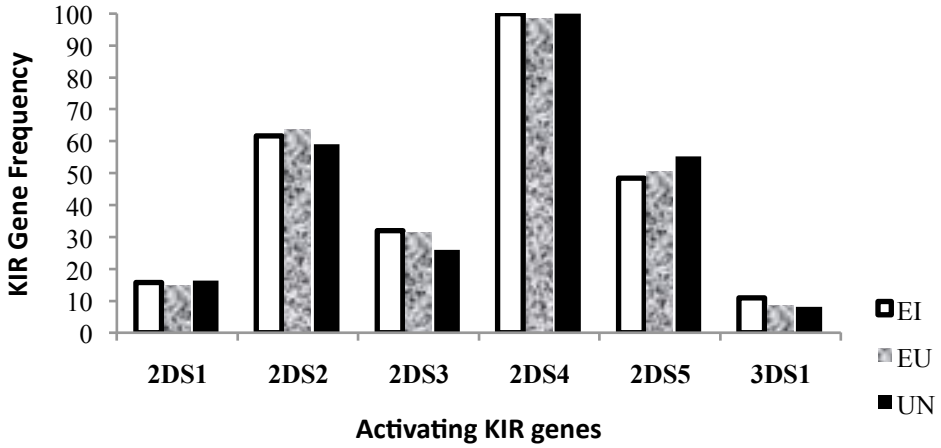
Figure 3.2 C: The frequency of *HLA-Cw*04:01* was not significantly different between HIV-1 in exposed uninfected (EU) and unexposed healthy (UN) infants ($p>0.05$, logistic regression). N: number of individuals in the group, %: percentage of allele, Pc: p value adjusted for multiple comparisons, OR: Odds ratio.

3.5.1.4 There was no difference in KIR gene frequencies among HIV-1 infected and uninfected infants .

In order to identify KIR genotypes that were associated with HIV-1 acquisition among mother-infant pairs, we compared individual KIR frequencies of activating, inhibitory and pseudo-KIR genes between EI (n=124), EU (n=120) and UN (n=126) infants (Fig 3.3). Logistic Regression was used as a statistical test, No significant differences were found in the frequencies of inhibitory KIR between EI, EU and UN (Fig 3.3A). Similarly, we found no significant differences in frequencies of activating KIR genes between the three groups of infants (Fig 3.3B). All individuals irrespective of their HIV-1 status possessed *KIR2DL4*, *KIR2DS4*, *KIR3DL1* and *KIR3DL2*.



A



B

Figure 3.3: There was no difference in KIR gene frequencies among HIV-1 infected and uninfected infants. A: Inhibitory KIR gene frequencies were not significantly different between HIV-1 exposed infected (EI), HIV-1 exposed but uninfected (EU) and unexposed healthy (UN) infants ($p>0.05$). **B:** Activating gene frequencies were not significantlt different between EI, EU and UN ($P>0.05$). KIR frequencies were determined by PROC regression and compared by logistic regression. $p<0.05$ denotes significant difference.

3.5.1.5 There was no difference between the frequencies of KIR haplotypes among HIV-infected and uninfected infants

In order to assess the role of KIR haplotypes in mother-to-child acquisition of HIV-1, we classified KIR genes into either haplotype A or Bx **see section 2.4.4.7** of the methods development. We then compared the frequencies of haplotype A and Bx between EI (n=124), EU (n=120) and UN (n=126) infants (Fig 3.4). We found no significant difference in either KIR haplotype A or haplotype Bx frequencies between HIV-1 infected, EU and UN infants ($p>0.05$, Logistic Regression).

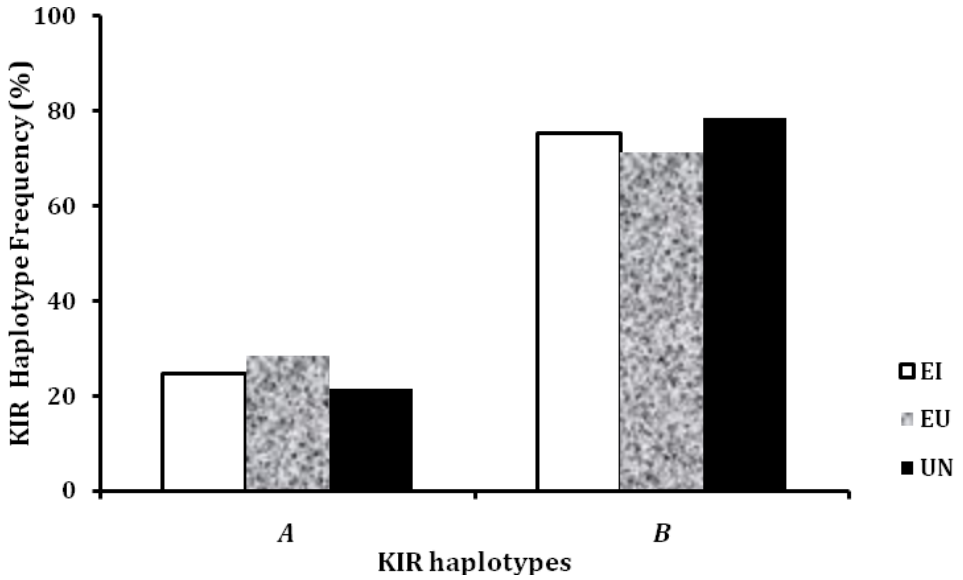


Figure 3.4: There was no difference between the frequencies of KIR haplotypes among HIV-infected and uninfected infants. The frequencies of KIR haplotypes A and Bx were not statistically different between HIV-1 exposed infected (EI), Exposed uninfected (EU) and unexposed healthy infants (UN) ($p>0.05$, Logistic Regression)**.**

3.5.1.6 There was no difference in the frequencies of *KIR2DS4* subtypes among HIV-infected and uninfected infants

To assess the role of *KIR2DS4* subtypes in mother-to-child acquisition of HIV-1, we did KIR subtyping of *KIR2DS4*. We classified *KIR2DS4* subtypes genes into either homozygous *197/197*, heterozygous *197/219* or homozygous *219/219* subtypes. We then compared the presence or absence of *KIR2DS4* homozygous 219, homozygous 197 and heterozygous *219/197* between EI (n=124), EU (n=120) and UN (n=126) infants (Fig 3.5). No significant differences were found in the frequency of homozygous *KIR2DS4 219/219*, heterozygous *219/197* or homozygous *197/197* between EI, EU and UN (p= 0.83, Logistic Regression).

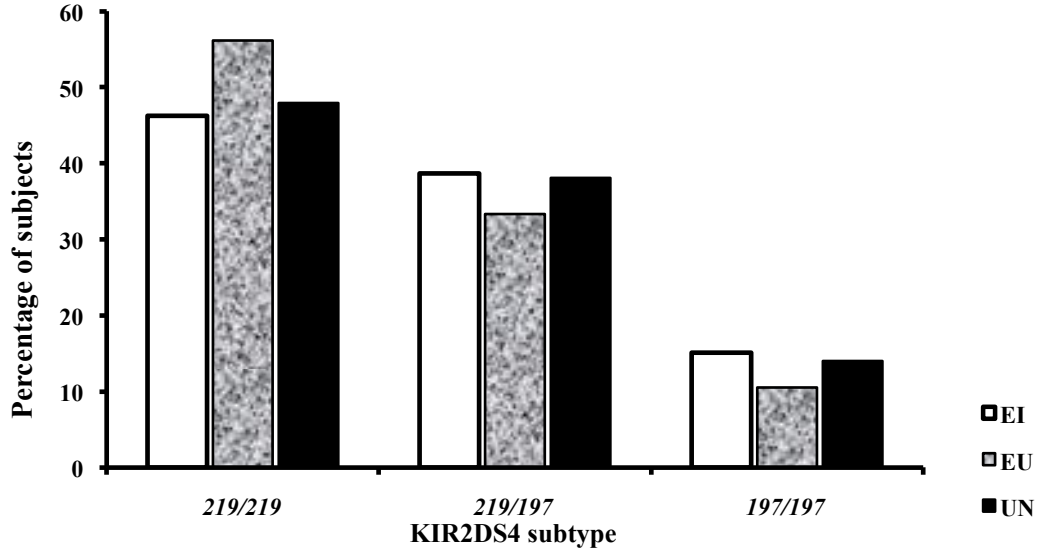


Figure 3.5 There was no difference in the frequencies of *KIR2DS4* subtypes among HIV-infected and uninfected infants. The frequencies of *KIR2DS4* subtypes *197/197, 197/219* and *219/219* were not different between HIV-1 exposed Infected (EI), Exposed uninfected (EU) and unexposed healthy (UN) infants. Comparisons were made using logistic regression analysis with a significant $p < 0.05$

3.5.1.7 The frequencies of KIR ligands, HLA-Bw4 epitopes and C1/C2 alleles were not different between HIV-1 infected (EI) and exposed uninfected (UN) infants

To further evaluate the effect of KIR ligands in acquisition of HIV-1, the frequencies of HLA-Bw4, Bw4-80-I and Bw4-80-T were compared between 124 EI, 120 EU and 126 UN infants. The frequencies of HLA-Bw4, HLA-Bw4-80-I or HLA-Bw4-80-T were not different between HIV-1 infected and exposed, uninfected infants (Table 3.3) ($p>0.05$; Logistic regression) respectively. Next, we analyzed the frequencies of HLA-C1/C2 allotypes among the EI, EU and UN. No significant differences were found in the frequencies of homozygous HLA-C1 or C2 allotypes between EI, EU and UN infants. Although, a trend was noted where EU infants had approximately 12% higher percentage of heterozygous C1/C2 compared to HIV-1 infected infants before adjustment for multiple comparisons (Table 3.3; $p = 0.07$), that trend was lost following adjustment for multiple comparisons ($p>0.05$, Logistic Regression).

Table 3.3: The frequencies of KIR ligands, HLA-Bw4 epitopes and C1/C2 alleles were not different between HIV-1 infected (EI) and exposed uninfected (UN) infants. The frequency of KIR ligands, HLA-Bw4 epitope and C1/C2 alleles were not different between EI, EU and UN infants. Bw4-80-I: HLA-Bw4 with isoleucine at position 80; Bw4-80T: HLA-Bw4 with threonine at position 80, C1: HLA-C group 1 allotype, C2: HLA-C group 2 allotype and Pc: adjusted P value.

Gene	**HIV-1 infected infant**		**HIV-1 exposed uninfected**						
	N	**%**	**N**	**%**	**P**	**Pc**	**OR**	**95% CI**	
-Bw4	67	55.83	64	56.14	0.96	1.00	0.99	0.59	1.66
Absent	53	44.17	50	43.86					
-Bw4-80-I	46	46.46	45	47.37	0.90	1.00	0.96	0.55	1.70
Absent	53	53.54	50	52.63					
-Bw4-80-T	26	32.91	26	34.21	0.86	1.00	0.94	0.48	1.84
Absent	53	67.09	50	65.79					
C1/C2	49	49.00	60	61.86	0.07	1.00	0.59	0.34	1.04
C2/C2	51	51.00	37	38.14					
C1/C1	22	30.14	17	22.08	0.22	1.00	1.58	0.76	3.31
C1/C2	49	69.86	60	77.92					
C1/C1	22	30.14	17	31.48	0.87	1.00	0.94	0.44	2.01
C2C2	51	69.86	37	68.52					

3.5.1.8 The frequency of alleles previously associated with high expression (HH/HL) were over-represented among HIV-1 exposed, infected infants compared to exposed, uninfected infants before adjustments for multiple comparisons

To further evaluate the influence of the number of high and low expression alleles in the risk of HIV-1 transmission, *HLA-C* alleles were classified into higher expression *(HH)* and lower expression (LL) alleles based on their genotype. We then compared number of high and low expression alleles between EU, EI and UN infants. We found that homozygous/ heterozygous high *(HH, HL)* expression alleles of specific HLA-C alleles, particularly *Cw*02:10, -C*08:02, -C*03:04, -C*05:01, -C*07:01, -C*07:02* and *-C*17* were significantly higher among HIV-1 infected compared to EU infants before adjustment for multiple comparisons (Table 3.4, $p<0.02$)). However, after adjustment for multiple displacement, high expression alleles were not associated with the risk of HIV-1 mother-to-child acquisition. In contrast, homozygous low *(LL)* expression of specific alleles such as *-Cw*01:02, -C*02:02, -C*04:01, -C*06:02, -C*08:02, -C*12:03, -C*14:02, -C*16:01* and *-C*18* was significantly elevated in EU compared to EI infants. There were no differences in HLA expression alleles between EI versus UN or EU versus UN ($p= 0.15$, $p=0.31$ respectively). Insertion or deletion at position 263 of *HLA-C 3'UTR* was not associated with the risk of HIV-1 acquisition (Table 3.4). This is the first study to find associations between HLA expression alleles with the risk of HIV-1 mother-to-child acquisition, however these findings should be validated with larger cohorts.

Table 3.4: Specific HLA-C alleles, reported to be associated with higher surface expression are more frequent in HIV-1 infected infants.

Specific HLA-C alleles, reported to be associated with higher surface expression were significantly higher in HIV-infected infants compared to exposed, uninfected infants before adjustment for multiple comparisons (p=0.03), however the significance was lost after adjusting for multiple comparisons (p>0.05; Logistic regression). Insertions or deletions at position 263 of *HLA-C 3'UTR* were not associated with the risk of HIV-1 transmission. homozygous *HH:* high *HLA*-C expression*, HL:* heterozygous high *HLA-C, LL:* homozygous low *HLA-C* expression. DD: deletions, II: insertions, ID: mutations and deletions.

	HIV-1 infected infants		**Exposed uninfected infants**		**Comparisons**					
	N	**%**	**N**	**%**		**P**	**Pc**	**OR**	**95% CI**	
HH	27	50.94	20	33.33	*HH vs LL*	0.06	1.00	2.08	0.97	4.44
LL	26	49.06	40	66.67						
HH	27	30.68	20	30.30	*HH vs HL*	0.96	1.00	1.02	0.51	2.04
HL	61	69.32	46	69.70						
HH/HL	88	77.19	66	62.26	*HH/HL vs HL*	**0.02**	1.00	2.05	1.14	3.69
HL	26	22.81	40	37.74						
HL	61	70.11	46	53.49	*HL vs LL*	**0.03**	1.00	2.04	1.09	3.81
LL	26	29.89	40	46.51						
DD	17	26.15	21	36.21	*DD vs II*	0.23	1.00	0.62	0.29	1.35
II	48	73.85	37	63.79						
DD	17	22.97	21	27.27	*DD vs DI*	0.54	1.00	0.80	0.38	1.66
DI	57	77.03	56	72.73						
DD/DI	74	60.66	77	67.54	*DD/DI vs II*	0.27	1.00	0.74	0.43	1.26
II	48	39.34	37	32.46						

3.5.2 Disease progression among perinatally HIV-1 infected infants

3.5.2.1 HIV-1 infected infants with *HLA-A*23:01* tended to have lower CD4 T lymphocyte counts, whereas infants with *HLA-B*81* tended to have higher CD4 T cell counts.

To determine the role of HLA-class I alleles in HIV-1 disease progression among perinatally HIV-1 exposed infected infants (*n=124*), we did a cross-sectional analysis of the first time point of the CD4 T cell count in HIV-1 infected infants. The CD4 T cell counts were compared in the infants that had a particular gene versus the infants that lacked the gene. We did a cross-sectional analysis because we did not have sufficient CD4 T cell to track the changes over time. For these comparisons, one way-ANOVA was used to evaluate KIR genes, KIR haplotypes A and Bx, HLA-A, -B, -C alleles and lastly, KIR-HLA interactions in mother-to-child acquisition of HIV-1. Our findings showed that infants with *HLA-A*23:01* gene tended to have lower CD4 T lymphocyte counts (Table 3.5, p = 0.01; ANOVA), whereas infants with *HLA-B*81* gene tended to have higher CD4 T cell counts before adjustments for multiple comparisons (p=0.04, ANOVA). However we lost significant results after adjustments for multiple comparisons (p>0.05). We also noted several trends that did not reach statistical significance. Infants with *HLA-A*68:02* tended to have higher CD4 T cell counts (p = 0.06; ANOVA), whereas infants with *HLA-B*35:01* tended to have lower CD4 T cell counts (p = 0.06; ANOVA) before adjustments for multiple comparisons, however, the differences were lost after adjustments for multiple comparisons.

Table 3.5: HIV-1 infected infants with *HLA-A*23:01* tended to have lower CD4 T lymphocyte counts, whereas infants with *HLA-B*81* tended to have higher CD4 T cell counts. The presence of *HLA-A*23:01* could be associated with decreased CD4 T lymphocyte count in HIV-1 infected infants (p=0.01; ANOVA), whereas *HLA-B*81* could be associated with higher CD4 T lymphocyte count (p=0.04, ANOVA). We did a cross-sectional analysis of the first time point to determine the correlations between HLA-class I and the average CD4 T cell count in HIV-1 infected infants.

ANOVA on square root CD4 data								
HLA Gene	**N**	**Mean**	**SE**	**P**	**Pc**	**Difference**	**95% CI**	
HLA-A*23:01 Present	10	27.71	3.22	**0.01**	0.33	-10.29	-17.42	-3.17
Absent	46	38.00	1.50					
HLA-A*68:02 Present	7	43.46	4.00	0.06	1.00	8.33	-0.24	16.91
Absent	49	35.12	1.51					
HLA-B*35:01 Present	1	16.16	10.60	0.06	1.00	-20.19	-41.61	1.24
Absent	56	36.34	1.42					
HLA-B*81 Present	5	45.35	4.71	**0.04**	1.00	10.27	0.39	20.14
Absent	52	35.09	1.46					

3.5.2.2 KIR gene associations with CD4 T cell count

To identify KIR genotypes associated with clinical outcome of HIV-1 disease, the CD4 T cell counts were compared between the infants that had a particular gene and the infants that lacked the gene among 124 HIV-1 infected infants. The frequency of *KIR2DL3* tended to correlate with lower CD4 T lymphocyte count (p=0.08; ANOVA), whereas *KIR2S4 197/219* tended to correlate with higher CD4 T lymphocyte count (p=0.09, ANOVA) in HIV-1 infected infants. However, after correction for multiple comparisons, there was no association between KIR genes and CD4 cell count in HIV-1 infected infants.

Table 3.6: The presence of *KIR2DL3* tended to be associated with lower CD4 counts, while *KIR2DS4 197/219* tended to be associated with higher CD4 counts. *KIR2DL3* tended to be higher in infants with lower CD4 T lymphocyte count (p=0.08; ANOVA), whereas *KIR2S4 197/219* tended to be higher in infants with higher CD4 T lymphocyte count (p=0.09, ANOVA) in HIV-1 infected infants.

ANOVA on square root CD4 data								
KIR Gene	**N**	**Mean**	**SE**	**P**	**Pc**	**Difference**	**95% CI**	
KIR2DL3 Present	40	34.27	1.67	0.08	1.00	-5.32	-11.32	0.68
Absent	18	39.59	2.49					
KIR2DS4 197/219	25	35.54	2.13	0.09	1.00	6.76	-1.12	-14.65
KIR2DS4 197/197	11	40.71	3.21	0.19	1.00	5.17	-2.56	12.90
KIR2DS4 219/219	22	33.95	2.27	0.61	1.00	1.59	-4.65	7.84

3.5.2.3 The presence of *HLA-A*29:11, -B*13:02* and -*C*16:01* tended to be associated with higher viral loads, while *HLA-B*39:10* tended to be associated lower viral loads in HIV-1 infected infants

In order to evaluate the effect of HLA-class I in HIV-1 disease progression, plasma viral load was compared between the infants that had a particular gene and the infants that lacked the gene in 124 HIV-1 infected infants. Here, we are only showing HLA-class I alleles that were associated or tended to associate with differences in viral load. Before adjustments for multiple comparisons, the presence of *HLA-A*29:11*, *-B*13:02* and -*C*16:01* were significantly associated with higher viral loads (p= 0.02, p= 0.03 and p= 0.02, ANOVA) respectively, whereas the presence of -*B*39:10* was significantly associated with lower viral loads in HIV-1 infected infants (Table 3.7, p = 0.04; ANOVA). The presence of *HLA-A*66:02* tended to be higher on infants with higher viral loads (p = 0.08; ANOVA), whereas the presence of *HLA-A*74* tended to be higher on infants with lower viral loads (p = 0.09; ANOVA). No significant associations were found between *HLA-Bw4-80-I/T*, *HLA-C1* or *C2* epitopes and viral load. However, after correction for multiple comparisons, the significant differences were lost (p>0.05).

Table 3.7: The presence of *HLA-A 29:11*, *-B*13:02* and *-C*16:01* tended to be associated with higher viral loads, while *HLA-B*39:10* tended to be associated with lower viral loads in HIV-1 infected infants. Before adjustments for multiple comparisons, the frequency of *HLA-B*39:10* was associated with lower viral load (p= 0.04, ANOVA), whereas the frequencies of *HLA-A*29:11*, *-B*13:02* and *-C*16:01* were associated with higher viral load in HIV-1 infected children (p= 0.02, p= 0.03 and p= 0.02 respectively). However, these significant findings were lost after correction for multiple comparisons (p>0.05). The correlations between HLA-class I alleles and the average viral load of HIV-1 infected infants were assessed by cross-sectional analysis of the average first 3 time points using one-way ANOVA, significant p<0.05

ANOVA on log Viral load data								
HLA Gene	**N**	**Mean**	**SE**	**P**	**Pc**	**Difference**	**95% CI**	
HLA-A*29:11 Present	4	5.99	0.35	**0.02**	1.00	0.86	0.14	1.57
Absent	110	5.13	0.07					
HLA-A*74 Present	10	4.80	0.23	0.09	1.00	-0.40	-0.87	0.07
Absent	104	5.20	0.07					
HLA-B*13:02 Present	1	6.70	0.71	**0.03**	1.00	1.52	0.12	2.93
Absent	115	5.17	0.07					
HLA-B*39:10 Present	5	4.55	0.32	**0.04**	1.00	-0.66	-1.30	-0.02
Absent	111	5.21	0.07					
HLA-C*16:01 Present	12	5.64	0.20	**0.02**	1.00	0.53	0.10	0.95
Absent	106	5.11	0.07					
Bw4-I80	43	5.04	0.11	0.08		-0.26	-0.54	0.03
others								
Bw4-80T	25	5.13	0.15	0.36	1.00	-0.16	-0.52	-0.19
others	53	5.30	0.10					
C1/C1	22	5.08	0.15	0.19	1.00	-0.24	-0.61	0.13
C1/C2	46	5.33	0.10					
C1/C1	22	5.08	0.16	0.89	1.00	0.03	0.36	0.41
C2/C2	50	5.06	0.11					
C1/C2	46	5.33	0.10	0.06		0.27	-0.01	0.55
C2/C2	50	5.06	0.10					

3.5.2.4 The presence of *KIR2DS1* and *KIR2DL5* independantly tended to be associated with higher viral loads among HIV-1 infected infants

To identify KIR genotypes associated with markers of HIV-1 disease progression, plasma viral loads were compared between the infants that had a particular gene and the infants that lacked the gene among 124 HIV-1 infected infants. In Table 3.8, we only show KIR genes that were associated with differences in viral load among HIV-1 infected infants. Before correction for multiple comparisons, the presence of *KIR2DL5* and *KIR2DS1* were associated with higher viral loads (Table 3.8, p = 0.04 and p = 0.02, ANOVA) respectively.

Table 3.8: The presence of *KIR2DS1* and *KIR2DL5* were independantly associated with a trend towards higher viral loads among HIV-1 infected infants. The presence of *KIR2DS1* and *KI2DL5* were associated with higher viral loads in HIV-1 infected infants (p= 0.04 and p= 0.02, ANOVA) respectively. We compared viral loads between the infants that had a specific gene to the infants that lacked the gene and only showed significant findings.

ANOVA on log Viral load data								
KIR Gene	**N**	**Mean**	**SE**	**P**	**Pc**	**Difference**	**95% CI**	
2DL5 Present	80	5.26	0.08	**0.04**	1.00	0.29	0.02	0.56
Absent	36	4.98	0.11					
2DS1 Present	18	5.51	0.16	**0.02**	1.00	0.40	0.06	0.74
Absent	98	5.11	0.07					

CHAPTER 4

DISCUSSION

4.1 MOTHER-TO-CHILD TRANSMISSION OF HIV-1

HIV-MTCT still remains a huge challenge in resource-limited settings such as Sub-Saharan Africa. Without intervention, 15-35% of HIV-exposed infants become infected annually and HIV-MTCT accounts for more than 90% of paediatric HIV-1 infections (Ahmad, 2011, Winchester et al., 2004, Zhou et al., 2010). Growing experimental evidence indicate the role of natural killer cells in regulation of HIV-1 infection. Specifically polymorphisms in KIR/HLA genotypes have been shown to influence HIV-1 acquisition and disease progression to AIDS or death (Martin et al., 2002; Martin et al., 2007; Jennes et al, 2006; Paximadis et al., 2011; Kuhn et al., 2004; Boulet et al., 2008; Merino et al., 2011). However, most KIR/HLA polymorphism studies have been conducted in adult populations. Little is known about KIR/HLA compound genotypes in perinatal HIV-1 acquisition. Thus, the purpose of this study was to identify KIR and HLA compound genotypes that influence mother-to-child acquisition of HIV-1 and disease progression in HIV-1 infected infants. We compared KIR/HLA compound gene frequencies between HIV-1 exposed infected (EI), exposed, uninfected (EU) and healthy unexposed infants.

A previous study by Bryson et al (1996) found that mother-to-child HIV-1 transmission was associated with several risk factors, of which maternal viral load was the strongest risk factor. It was therefore important to establish first if maternal viral loads were different between transmitter and non-transmitters as the controls of non-transmitters were stratified by maternal viral load to eliminate confounding factors.

We found no differences in maternal viral load between HIV-1 transmitters and nontransmitters (Table 3.2, p= 0.77, ANOVA). This is an important finding for the validation of the selection criteria of our controls to match cases and also important to the interpretation of our data. These findings imply that we managed to successfully stratify for maternal viral load between HIV-1 transmitters and non-transmitters. These findings confirm that the influence of viral load in HIV-1 mother-to-child transmission was eliminated. On the contrary, maternal CD4 T cell count was significantly lower in HIV-1 transmitters compared to non-transmitters, however, this finding was incidental. It may be statistically significant, but it is unlikely to be physiologically significant. The mean difference is about 1 CD4/microL of blood, which may not be sufficient to cause significant clinical changes.

Previous studies found that HLA-class I concordance and homozygosity between mothers and infants have been previously associated with higher risk of HIV-1 mother-to-child acquisition (MacDonald et al., 1998; Polycarpou et al., 2002; Mackelprang et al., 2008). A previous study by MacDonald et al (2000) followed a group of Kenyan female sex workers, they found that *A2/68:02* supertype, particularly, *HLA-A*02:02, -A*02:05, -A*02:14* and *-A*68:02* was associated with a decreased rate of HIV-1 seroconversion (Macdonald et al., 2000). Moreover, in a Kenyan mother-to-child transmission cohort, MacDonald et al (2001) revealed that *A2/68:02* supertype among the infants was associated with a 7-fold reduced risk of perinatal HIV-1 transmission among HIV-1 exposed infants (MacDonald et al., 2001). Farquhar et al (2004) investigated the role of HLA-class I in HIV-1 acquisition and found that *HLA-B*18* was associated with protection against early HIV-1 acquisition among Kenyan HIV-1 exposed infants.

Winchester et al (2004) assessed the role of HLA-B alleles in mother-to-child transmission, they found that *HLA-B*13:02, -B*35, -B*44:02* and *-B*50:01* were associated with higher risk of HIV-1 transmission among the transmitters. Whereas *HLA-B*49:01* and *-B*53:01* were associated with protection against HIV-1 transmission among non-transmitting mothers. However, infant's HLA-B alleles were not associated with the risk of HIV-1 acquisition (Winchester et al., 2004). More recently, Paximadis et al (2011) assessed the role of KIR/HLA genotypes in HIV-1 mother-to-child transmission in a South-African mother-infant cohort. They showed that *KIR2DL3* in combination with HLA-C1 allotype among infants was associated with protection against HIV-1 acquisition in HIV-1 exposed uninfected infants (Paximadis et al., 2011).

Our findings indicate that *HLA-Cw*04:01* was consistently associated with a trend towards higher risk of HIV-1 acquisition among infants born to HIV-infected mothers, even after adjustment for both maternal viral load and multiple comparisons. We adjusted for maternal viral load because it has been shown previously to be the primary risk factor for HIV-1 transmission to the infant. This is the first large cohort of mother-infant pairs to find associations between *HLA-Cw*04:01* and HIV-1 acquisition. However, we found no associations between the strong putative ligand of *HLA-Cw*04*, *KIR2DL1* and the risk of HIV-1 acquisition. Consistent with our findings, Tang et al (2008) followed up Zambian sero-discordant couples and found that *A*36-Cw*04-B*53* supertype regardless of the direction of infection was associated with higher risk of HIV-1 transmission. Previously, it has been shown that *HLA-Cw*04* in combination with *HLA-B*35* was associated with faster HIV-1 disease progression to AIDS (Carrington et al., 1999).

More recently, Merino et al (2011) investigated the role of KIR/HLA genotypes in heterosexual HIV-1 transmission among Zambian sero-discordant couples. They revealed that *KIR2DS4*001* in combination with its cognate ligand *HLA-Cw*04* among transmitters was associated with increased rate of HIV-1 transmission in discordant couples (Merino et al., 2011). Whereas, *HLA-Cw*04* alone was not associated with the risk of HIV-1 transmission. Our study showed contrasting findings to Merino et al., (2011) as we found higher frequencies of *HLA-Cw*04* in recipients. Thus one possible explanation for a higher risk of HIV-1 infection is that *HLA-Cw*04:01* ligand binds to *KIR2DL1* receptor, which has an immunoreceptor tyrosine-based inhibitory motif (ITIMs) and lead to a strong inhibition of NK cell responses (Fig 4.1). However, one possible explanation for no associations between inhibitory receptor, *KIR2DL1* and HIV-1 acquisition could be the absence of cognate HLA ligands.

*HLA-Cw*04:01* is a group 2 allotype (C2) of the HLA-C allotypes that contain Lys 80, Arg 145, Asp 83, Lys 146 and Asp 77 in the α1-helix of the protein (Boyington and Sun, 2001). *HLA-Cw*04:01* binds to *KIR2DL1* which has been previously shown to exert the strongest inhibitory signals compared to other HLA-C2 specific receptors such as *KIR2DL2* and *KIR2DL3* (Lanier et al., 2005; Boyington and Sun, 2001; Rajagopalan and Long, 2005). The interaction between *KIR2DL1* and *HLA-Cw:04:01* results in the formation of three salt bridges and 6 hydrogen bonds (Boyington and Sun, 2001). Salt bridges are formed by the the interaction between Asp-135 of *KIR2DL1* and Arg-145 of *HLA-Cw4*, Asp-183 and Lys-146, Glu-187 and Lys-80 (Fig 4.1) (Boyington and Sun, 2001).

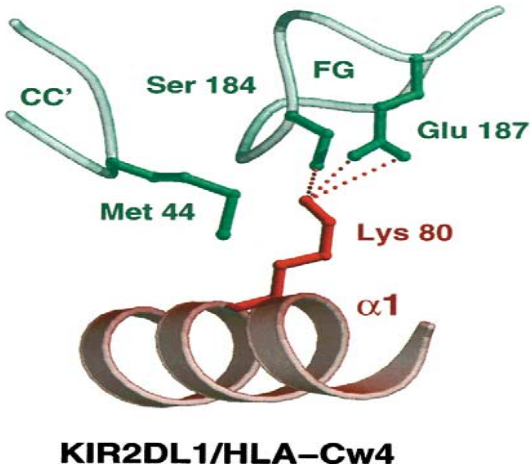


Figure 4.1: Allelic recognition of *HLA-Cw4* (brown) by *KIR2DL1* (green). The interaction is stabilized by three hydrogen bonds and salt bridges (indicated by dotted lines) resulting from the interaction between Asp-135 of *KIR2DL1* and Arg-145 of *HLA-Cw4*, Asp-183 and Lys-146, Glu-187 and Lys-80 respectively (Boyington and Sun, 2001. *Molecular Immunology*; 38).

Consistently, a recent study by Thananchai et al (2009) found that *HLA-Cw4* alleles have been previously shown to enhance the virus to acquire mutations that are capable of escaping from both cytotoxic T cell and NK cell recognition. They investigated recognition of HLA-Cw4-restricted HIV-1 Gp120 epitopes, particularly SF9 and SL9 variants by NK and CTLs. They found a better recognition of SF9 variants by the CTLs, while SL9 variants escaped recognition by the CTLs (Thananchai et al., 2009). In contrast, HLA-Cw4-restricted HIV-1 SL9 epitope formed a better ligand for *KIR2DL1* receptor (Thananchai et al., 2009). They suggested that HLA-Cw4-restricted epitopes, SL9. They also discovered that SF9 provide a selective advantage to the virus to bind *KIR2DL1* and inhibit NK cell responses and also inhibit peptide recognition by the CTLs which is termed as "double escape" (Thananchai et al., 2009).

There are a number of potential mechanisms that lead into increased susceptibility following *KIR2DL1* receptor and HLA-Cw4 interactions. The first potential mechanism is that *KIR2DL1-HLA-Cw4* interaction inhibits NK cell production of CCR5-ligand chemokines (CC-chemokines) such as MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) (Fig 4.2 A). CC-chemokines competitively bind or down modulate the CCR5 co-receptor and thus can inhibit HIV-1 acquisition. However, inhibitions of NK cell production of CC-chemokines lead into increased CCR5 surface expression because less CC-chemokines ligands engage CCR5 receptors. Higher surface expression of CCR5 results in increased fusion efficiency between CCR5 and HIV-1 peptides thereby increasing HIV-1 infant acquisition and replication capacity. Consistent with our findings, several mother-to-child transmission studies revealed that CC-chemokines play a significant role in resistance to HIV-1 acquisition in infants.

Bernstein et al (2004) assessed the ability of neonatal NK cells to produce chemokines, they compared chemokine levels between neonatal and adult NK cells. They found higher levels of CC-chemokines among exposed, uninfected infants compared to adults, which in turn suppressed replication of R5 viruses *in vitro* (Bernstein et al., 2004). However, resistance to HIV-1 infection was blocked by antichemokine antibodies. Another study found higher CCL3L1 (MIP-1α) gene copies among HIV-1 exposed uninfected infants compared to HIV-1 infected South African infants in the absence of maternal nevirapine, however CCL3 production was reduced in nevirapine exposure (Kuhn et al., 2007). Meadows-Taylor et al (2006) showed that CCL3 production was significantly higher in HIV-1 EU infants compared to HIV-1 infected and unexposed South African infants, suggesting a protective role of CCL3 against HIV-1 infection (Meadows-Taylor et al., 2006). Another previous study by Oliva et al (1998) evaluated the production of CC chemokines by the NK cells isolated from HIV-1

infected individuals. They discovered that stimulation of NK cells with IL-2 significantly increase CC chemokine production, particularly MIP-1α, MIP-1β and RANTES which have been shown to suppress HIV-1 replication (Oliva et al., 1998). Based on these findings, we speculate that *KIR2DL1* interacts with *HLA-Cw*04* resulting in inhibition of NK cell responses. Consequently, CC-chemokine production is reduced and CCR5 receptors are not engaged, thus HIV-1 epitopes bind and enter the $CD4^+$ T cells (Fig 4.2 A). Together these findings suggest that CCL3 may play a crucial role in protecting African HIV-1 exposed infants against HIV-1 infection. However, these are speculations, NK cell functional studies are required to further investigate the mechanism behind the *HLA-Cw*0401*-mediated susceptibility to acquisition of HIV-1 in exposed infants.

The second potential mechanism is that inhibition of NK cell responses suppresses production of cytolytic granules such as perforins, granzyme B and granulolysin. These events may lead into inhibition of NK cell–mediated natural cytotoxicity and lack of destruction of HIV-1 infected cells and results in increased HIV-1 replication and infection of naïve CD4 T cells (Fig 4.2 B). The third potential mechanism is that *KIR2DL1-HLA-Cw*04* interactions decrease type 1 immunoregulatory cytokine production, particularly IFN-γ, TNF-α, GM-CSF (Fig 4.2 C). Consequently, adaptive immune responses such as CD4 T cell differentiation and activation of effector cells would be inhibited, allowing HIV-1 replication to increase further. Normally, high levels of IFN-γ enhance antiviral activity and induce tat-induced LTR transactivation antagonism (Emilie et al., 1992; Fauci et al., 2005). However, due to inhibition of NK cell responses, production of IFN-γ is reduced (Fauci et al., 2005). Previously, Mantoya et al (2006) found higher IFN-γ production by the $CD3^+$ $CD56^+$ NK cells in HIV-1 exposed, uninfected individuals compared to healthy unexposed controls.

We concluded that increased susceptibility to HIV-1 acquisition in infants that had *HLA-Cw4* might be caused by reduced IFN-γ production, which antagonizes tat-induced LTR transactivation.

Another possible mechanism for higher risk of HIV-1 acquisition in infants that carry *HLA-Cw4* allele is that, *HLA-Cw4* binds to activating receptors such as *KIR2DS1* and *KIR2DS4* and results in activation of NK cells. However over-activation of NK cells has been shown to cause mucosal inflammation due to increased immune activation such as production of cytolytic granules and cytokines (Merino et al., 2011). Furthermore, we speculate that increased immune activation may lead into increased viral replication, increased inflammatory response resulting in higher risk of HIV-1 acquisition.

More recently, a genome-wide association study showed that SNP *sr924942* and *HLA-Cw*04:01* have a synergistic effect towards faster HIV-1 disease progression to AIDS (Fellay et al., 2009). This may suggest that the observed association between *HLA-Cw*04:01* with HIV-1 acquisition could also be influenced by an additional effect of SNP *rs924942*, however, further studies to investigate this effect are required.

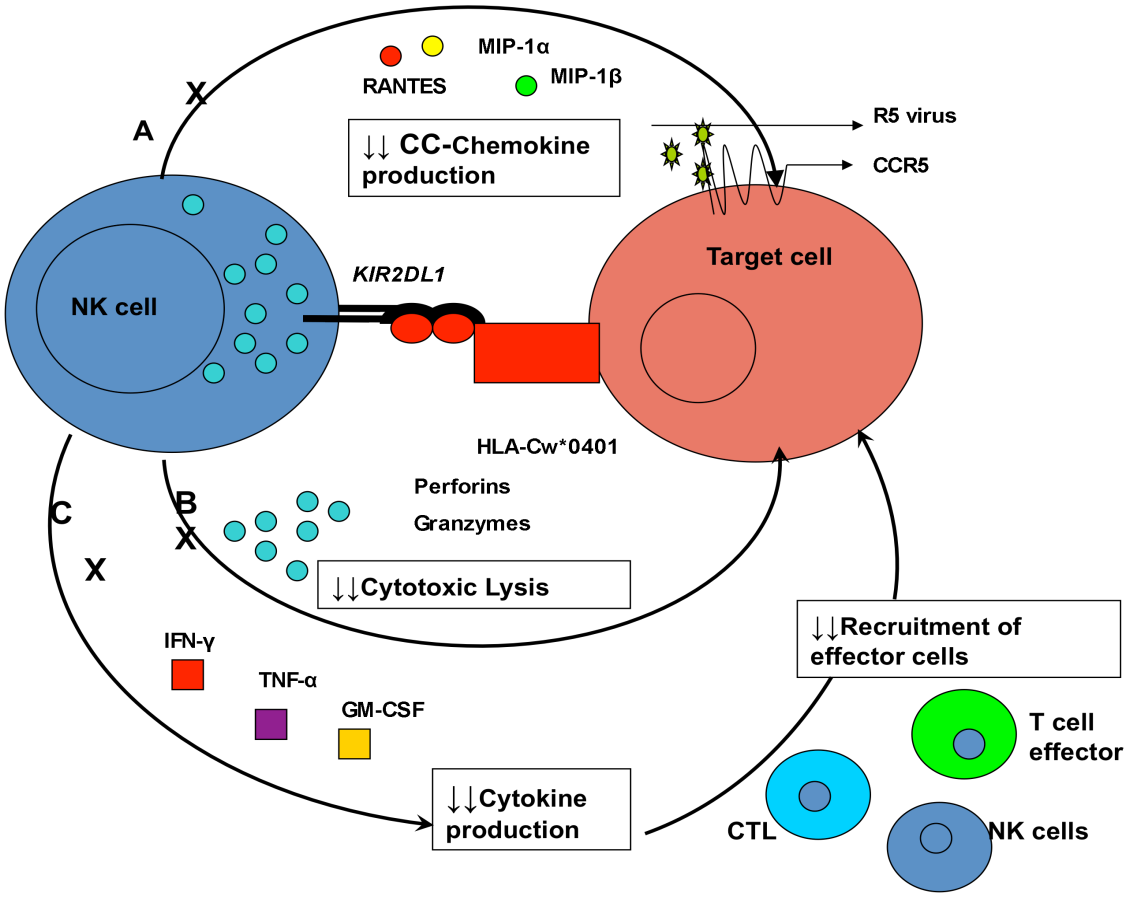


Figure 4.2: Study model; *HLA-Cw*04:01* and *KIR2DL1* interactions may lead into increased susceptibility to mother-to-child acquisition of HIV-1 infection through various mechanisms such as **A:** decreased chemokine production, **B:** reduction of cytolytic lysis or **C:** decreased cytokine production.

We also found various trends between HLA-class I and HIV-1 acquisition, the frequency of *HLA-A*02:05* and *HLA-A*03:01* tended to be consistently associated with protection from HIV-1 acquisition among exposed, uninfected infants, however these results did not reach statistical significance. We found the following trend of protection against HIV-1 infection, the frequency of *HLA-A*02:05* and *HLA-A*03:01;* EU> UN> EI infants which suggests consistent protection against HIV-1 acquisition. Our findings were consistent with previous research, which evaluated the role of HLA-class I in HIV-1 mother-to-child transmission.

This study found that *HLA-A*02:05* under the A2/6802 supertype among infants was associated with a 7-fold reduced risk of perinatal HIV-1 acquisition in Nairobi (MacDonald et al., 2001). Similar findings were also obtained when female sex workers were followed up, MacDonald et al (2000) revealed that *HLA-A*02:02, A*02:05, A*02:14* and *A*68:02* (A2/6802 supertype) were associated with protection from HIV-1 seroconversion among discordant female sex workers in Nairobi (MacDonald et al., 2000). MacDonald et al (2001) only investigated the role of HLA-class I, they excluded HLA's cognate ligand, KIRs and did not control for maternal viral load, which has been shown to influence mother-to-child HIV-1 transmission. We extended their findings by controlling for maternal viral load, we also included KIR gene analysis and negative controls.

We speculate that *HLA-A*03:01* interacts with *KIR3DL2* receptor and results in inhibition of NK cell responses. We also proposed that *HLA-A*02:05* gene present highly conserved viral recombinant epitopes to the cytotoxic T lymphocytes which prevent HIV-1 acquisition by enhancing diverse immune response against HIV-1. Taken together, our data suggest that *HLA-A*02:05* and *HLA-A*03:01* may be associated with resistance from HIV-1 acquisition. However, these would need to be confirmed in larger cohorts and would need to be demonstrated in functional studies that monitor CTL responses to highly conserved viral epitopes in the presence of these marginally protective HLA-alleles. Although not significant, these findings have important implications in the development of preventative and therapeutic vaccines for infants.

The frequency of *HLA-Cw*02:10* tended to be associated with reduced HIV-1 acquisition in HIV-1 exposed, uninfected infants. However this protection was not found in unexposed, uninfected infants.

This is the first study to find associations between the frequency of *HLA-Cw*02:10* and HIV-1 infant's acquisition. *HLA-Cw*02:10* is a group C2 allotype and it has an Asn at position 77 and a Lys at position 80 (Kulpa et al., 2011). We speculate that *HLA-C*02:10* binds to *KIR2DS1* and lead into activation of NK cell responses which results in increased production of CC-chemokines that block CCR5 receptors and lead to decreased susceptibility of $CD4^+$ cells to HIV-1 infection. An alternative explanation could be that, *HLA-C*02:01* present highly conserved epitopes to cytotoxic T lymphocytes for activation of immune responses which results in inhibition of HIV-1 acquisition only in exposed infants. We also revealed that the frequency of *HLA-B*08:01* was associated with a trend towards a higher risk of infant HIV-1 acquisition. *HLA-B*08:01* is a -Bw6 allele which has been associated with higher risk of HIV-1 transmission among heterosexual couples probably, due to it's ability to present mutant viral epitopes to the CTLs.

However, inconsistent with our findings, in another South African mother-infant cohort, HLA-*B*08:01* was likely lower in HIV-1 infected infants compared to exposed uninfected infants (Paximadis et al., 2011). The difference between Paximadis et al., 2011 and our findings could possibly be due to allelic variation between ethnic groups; their study was conducted in a Sotho tribe while our study was conducted in Zulu/Xhosa tribes. They also did not take viral load into consideration, while we stratified exposed uninfected infants by maternal viral load.

However, our study was consistent with studies in adults, Yindom et al (2010) assessed the association between KIR-HLA compound genotypes and heterosexual HIV-2 transmission, they found that *HLA-B*08:01* was associated with higher risk of heterosexual acquisition of HIV-2 (Yindom et al., 2010) and HIV-1 disease progression (Fellay et al., 2009). Taken together, our data suggest that *HLA-B*08:01* may be associated with increased risk of HIV-1

mother-to-child acquisition, however, further studies are required to fully address the effect of *HLA-B*08:01* allele in HIV-1 acquisition.

Previous studies on HIV-1 mother-to-child acquisition only investigated a limited number of KIR and HLA alleles. Winchester et al (2004) evaluated the association between HLA-B and HIV-1 mother-to-child acquisition, they revealed that infant's HLA-B alleles were not associated with transmission risk. They only investigated HLA-B and excluded the effect of other HLA-class I alleles or their cognate KIR receptors. Furthermore, Ballan et al (2007) evaluated the association between activating, inhibitory KIR receptors and mother-to-child HIV-1 acquisition or disease severity, they found higher frequencies of *KIR2DL1* and *KIR3DL1* among HIV-1 infected infants compared to exposed uninfected infants. However, they had small numbers with less statistical power and they only evaluated KIR receptors without cognate HLA ligands. Recently, Paximadis et al., (2011) evaluated the association between HLA-B, HLA-C and HIV-1 mother-to-child acquisition. They found higher frequencies of *KIR2DL3* in combination with HLA-C1/C2 in HIV-1 exposed uninfected infants compared to HIV-1 infected infants (Paximadis et al., 2011). However, they did not correct for maternal viral load or adjust for multiple comparisons. We extended their findings by including the entire KIR and HLA-class I profile on DBS derived DNA. We stratified our controls of exposed uninfected infants based on maternal viral load and we adjusted our findings for multiple comparisons. Our analysis also included a group of healthy unexposed controls to represent the general population of South African infants.

A lot of evidence in exposed, seronegative individuals suggest that KIR genes function independently, or in combination with cognate HLA-class I ligands to influence HIV-1 acquisition. A recent study that assessed the associations between KIR/HLA compound

genotypes and HIV-1 mother-to-child transmission in South African mother-infant pairs demonstrated that *KIR2DL3* in combination with HLA-C1/C2 among exposed infants was associated with protection from HIV-1 acquisition (Paximadis et al., 2011).

Similarly, another study evaluated the associations between KIR/HLA genotypes and HIV-1 seroconversion in African female sex workers and they found that *KIR2DL2/KIR2DL3* in the absence of cognate ligand, HLA-C1 were associated with protection from HIV-1 seroconversion (Jennes et al., 2006). Moreover, *KIR3DL1* homozygosity in the absence of HLA-C1 was also associated with resistance to HIV-1 infection among *Cote d Ivoire* female sex workers (Jennes et al., 2006). More recently, Merino et al (2011) evaluated associations between *KIR2DS1-HLA-Cw*04* allotypes and HIV-1 seroconversion in Zambian discordant couples, they found that *KIR2DS4*001* alone or in combination with *HLA-Cw*04* were associated with increased risk of HIV-1 seroconversion on serodiscordant Zambian couples (Merino et al., 2011). In contrast, infant's individual activating or inhibitory KIR genes and KIR haplotypes were not associated with HIV-1 acquisition among exposed infants. A possible reason for these different findings could be due to allelic variation between ethnic groups; most of these studies were conducted in West Africa and our study was conducted in Southern Africa. The distribution of KIR genes could vary in different regions. Paximadis et al (2011) did not correct for multiple comparisons therefore, these could be trends after adjustment for multiple comparisons. Another alternative explanation is that, we did not analyze *KIR2DL2/KIR2DL3* homozygosity or heterozygosity in our cohort, we might have missed the *KIR2DL2/KIR2DL3* associations with mother-to-child acquisition of HIV-1.

Previous research found that *KIR3DL1* homozygosity in the absence of cognate ligand, HLA-Bw4 was associated with protection from HIV-1 seroconversion (Jennes et al., 2006). A previous study by Boulet et al (2008) compared genetic distribution of *KIR3DL1 *h/*y* and *HLA-B*57* between HIV-1 infected and exposed uninfected individuals, they found that co-expression of *KIR3DL1 *h/*y* and *HLA-B*57* was associated with lower risk of HIV-1 infection among HIV-1 exposed uninfected individuals (Boulet et al., 2008). Recently, Boulet et al (2010) stimulated *KIR3DL1 *h/*y* plus *HLA-B*57* with HLA-devoid K562 cells and found that co-expression of *KIR3DL1 *h/*y HLAB*57* in combination with HLA-Bw4 increased NK cell function. On the other hand, homozygosity for *KIR3DS1* has been shown to enhance activation of NK responses and lead to resistance to HIV-1 acquisition in exposed, uninfected individuals (Boulet et al., 2008). In contrast, in our study, *KIR3DL1/S1* alone or in combination with HLA-Bw4 had no effect in HIV-1 mother-to-child acquisition, probably due to low frequencies of *KIR3DS1* in Sub-Saharan African populations (Norman and Parham, 2005). We show that heterozygous HLA-C1/C2 tended to be associated with resistance from HIV-1 acquisition, contrasting to a previous study that did not find a significant difference in heterozygosity of HLA-C1/C2 alone among the infant groups (Paximadis et al., 2011). However, they revealed that *KIR2DL3* in combination with HLA-C1/C2 was associated with protection from HIV-1 acquisition.

Recently, Kulkarni et al (2011) compared the frequency of insertions/ deletions at position 263 of the *HLA-C 3'UTR* between elite controllers and non-controllers in a cohort of European Americans. Their analysis found that miR 148a binding site of HLA-C 3'UTR was in strong linkage disequilibrium with alleles downstream of 35 SNP, which has been associated with slower HIV-1 disease progression to AIDS (Kulkarni et al., 2011; Thomas et

al., 2009). They also found that elite controllers had higher levels of deletions, whereas non-controllers had higher levels of insertions at position 263 of the *HLA-C 3'UTR* (Kulkarni et al., 2011). In contrast, in our analysis, insertions or deletions at position 263 of *HLA-C 3'UTR* were not associated with the risk of HIV-1 acquisition.

The possible explanation of the difference in findings could be ethnic difference, their study was conducted in European Americans while our study was conducted in the African population, allele distribution could be different between the two populations. We found that homozygous/heterozygous high (HH, HL) expression of specific HLA-C alleles, particularly *Cw*02:10, C*08:02, C*03:04, C*05:01, C*07:01, C*07:02* and *C*17* was associated with the risk of HIV-1 acquisition. Whereas, homozygous low (LL) expression of specific HLA-C alleles such as *Cw*01:02, C*02:02, C*04:01, C*06:02, C*08:02, C*12:03, C*14:02, C*16:01* and *C*18* was associated with protection from HIV-1 acquisition. This is the first study to find associations between levels of expression of the HLA-C alleles with the risk of HIV-1 mother-to-child acquisition.

All together our findings suggest that infants who display *HLA-Cw4* are at higher risk of acquiring HIV-1 compared to the infants that lack the gene, indicating that *HLA-Cw4* is a high risk factor of mother-to-child acquisition of HIV-1. Reciprocally, infants that carry *HLA-A*02:05, HLA-A*03:01* and *HLA-C*02:10* are marginally protected from HIV-1 acquisition. However, KIR genotypes had no impact on mother-to-child acquisition of HIV-1 in our study setting. These findings have important implications in the development of preventative and therapeutic vaccines, these HLA-alleles could be used to present highly

conserved recombinant viral epitopes to the cytotoxic T lymphocytes and enhance memory immune activation towards HIV-1 virus to prevent acquisition of HIV-1. HLA alleles could also interact with KIR receptors and lead into activation or inhibition of NK cell responses. However, these are all speculations, to test these ideas, we would design functional studies and monitor either NK cells or CTL responses to highly conserved viral epitopes in the presence of these marginally protective HLA-alleles.

4.2 DISEASE PROGRESSION IN HIV-1 INFECTED INFANTS

Previous studies revealed that HIV-1 clinical outcome variation could be influenced by factors such as virus, host and environmental responses (Fellay et al., 2009). Genetic variants located at HLA-B and HLA-C loci are the strongest determinants of HIV-1 control compared to other genes (Fellay et al., 2009). We investigated the role of KIR/HLA genotypes in disease progression among HIV-1 infected infants. We revealed that *HLA-A*23:01* tended to be associated with faster HIV-1 disease progression, while *HLA-B*81* tended to be associated with slower disease progression to AIDS. Consistent with our findings, a previous study by MacDonald et al (2000) compared the frequency of HLA-class I alleles between HIV-1 exposed, seronegative and HIV-1 infected female sex workers and found that *HLA-A*23:01* was associated with increased risk of HIV-1 seronconversion. Another study also evaluated the associations between HLA-class I and HIV-1 disease progression in HIV-1 infected children, they found that *HLA-A*23:01* was associated with rapid progression to AIDS (Chen et al., 1997). Similarly, a previous study by Thobakgale et al (2009) evaluated the impact of HLA-class I in HIV-1 disease progression in HIV-1 infected children and found higher frequency of *HLA-B*81* in non-progressors compared to rapid progressors (Thobakgale et al., 2009).

Furthermore, they revealed that if the virus mutates, that mutation would result in fitness cost in the virus (Thobakgale et al., 2009). Taken together, these findings suggest that *HLA-A*23:01* may be associated with faster disease progression, while, *HLA-B*81* might be associated with slower HIV-1 disease progression. CD4 T lymphocyte count has been previously found to be a better clinical predictor of HIV-1 disease progression than viral load in HIV-1 infected children (Ballan et al., 2007; Mock et al., 1998). Based on these findings, we speculate that increased disease progression in infants that have *HLA-A*23:01* is due to inability of these alleles to mount protective immune response against HIV-1. Furthermore, we speculate that *HLA-A*23:01* present HIV-1 epitopes that are associated with escape mutations. Whereas reciprocally, *HLA-B*81* present highly conserved viral epitopes to the CTLs which in turn enhance protective immune response against HIV-1. Taken together, these findings imply that *HLA-B*81* can be used for the development of HIV-1 therapeutic vaccine for infants, recombinant HIV-1 epitopes can be created and it would be presented by *HLA-B*81* to the infant CTLs and activate an immune response against HIV-1. All together these data support the role of HLA-class I alleles in influencing the infant immune response against HIV-1 infection and furthermore, these findings indicate the significance of genetic markers in the development of preventative HIV-1 vaccines for infants. However, functional studies are required to investigate the HLA-derived protective or high-risk mechanisms.

The frequency of *HLA-A*68:02* was associated with a trend towards higher CD4 lymphocyte count while *HLA-B*35:01* showed a trend towards lower CD4 lymphocyte count. Our findings were in line with a previous study that revealed that *HLA-A2/68:02* among infants was associated with lower risk of perinatal HIV-1 acquisition (Macdonald et al., 2000) and decreased susceptibility to HIV-1 infection among HIV-1 exposed female sex workers in Kenya (MacDonald et al., 2001). We speculate that *HLA-A*68:02* may present highly

conserved viral epitopes to the cytotoxic T cells and results in immune activation which then reduce susceptibility to HIV-1 acquisition.

We also noted a weak negative association between *HLA-B*35:01* and CD4 T lymphocyte count. Consistent with our findings, previous studies found that *HLA-B*35* was associated with increased HIV-1 disease progression in adults (Carrington et al., 1999; Gao et al., 2005; Carrington and O'Brien 2003; Huang et al, 2009). More specifically, *HLA-B*35-Px (B*35:02, B*35:03, B*35:04, B*53:01)* has been associated with faster disease progression to AIDS compared to *HLA-B*35PY (B*35:01, B*35:08)* among HIV-1 infected individuals although they only have one amino acid difference (Gao et al., 2001; Huang et al., 2009). These studies support our findings that *HLA-B*35:01* may be associated with faster HIV-1 disease progression among HIV-1 infected infants. We speculate that *HLA-B*35:01* may present HIV-1 epitopes that are associated with escape mutations which allow viral entry and replication to occur.

Our analysis of associations between HLA-class I alleles and viral load suggested that *HLA-A*29:11, B*13:02* and *C*16:01* tended to be associated with increased viral load. Whereas, the frequency of *HLA-B*39:10* tended to be associated with decreased viral load in HIV-1 infected infants. This is the first study to find trends of associations between *HLA-A*29:11, B*13:02, B*39:10, C* 16:01* and HIV-1 disease progression. However, our findings are in line with previous findings by Winchester et al (2004), that evaluated the effect of HLA-B alleles in HIV-1 mother-to-child infections and found that *HLA-B*13:02* among the mothers has been associated with higher risk of HIV-1 transmission (Winchester et al., 2004). These findings imply that *HLA-A*29:11, B*13:02* and *C*16:01* may be associated with faster

disease progression, while *HLA-B*39:10* may be associated with slower disease progression to AIDS.

In our analysis of KIR associations, *KIR2DL3* was associated with a trend towards lower CD4 T lymphocyte count, while *KIR2DS1* and *KIR2DL5* were both associated with a trend towards higher viral load. This is the first study to find association between *KIR2DS1, KIR2DL5* and HIV-1 disease progression. All together, these findings suggest that these KIR genes were all associated with faster disease progression to AIDS. A previous study by Ballan et al. (2007) compared KIR receptors between HIV-1 infected and exposed uninfected infants and showed a negative correlation between *KIR2DL3* and the CD4+ T cells among infants (Ballan et al., 2007). We speculate that *KIR2DL3* and *KIR2DL5* interact with their cognate HLA ligands and inhibit NK cell responses which could lead into increased HIV-1 replication and faster disease progression to AIDS. Whereas, on the other hand, *KIR2DS1* interacts with its strong ligand, *HLA-Cw*0401* and results in stimulation of NK cells which could lead into inflammation and viral shedding and increase HIV-1 replication and consequently increase viral load.

CHAPTER 5: CONCLUSIONS

Although previous investigators have determined specific KIR/HLA genotypes associated with HIV-1 mother-to-child acquisition, their analysis has been limited to selected KIR or HLA alleles. They also did not have sufficient numbers to reach statistical power. Here, we extended their findings by evaluating the role of KIR repertoire and HLA-class I alleles, particularly HLA-A, B and C in HIV-1 mother-to-child acquisition in an African cohort. We found that *HLA-Cw*04* tended to be associated with higher risk of HIV-1 mother-to-child acquisition and we found no associations between KIR genotypes and mother-to-child transmission of HIV-1. We also found a tendency towards negative correlations between *HLA-A*23:01* and CD4 T lymphocyte count. Whereas, *HLA-B*81* tended to correlate with higher CD4 T cell count. *HLA-A*29:11, -B*13:02* and *-C*16:01* tended to be associated with higher viral load, whereas *B*39:10* tended to be associated with lower viral load. Our findings suggest that *HLA-A*02:05* and *HLA-*03:01* might be useful in presenting HIV-1 epitopes to the CTLs for the development of an effective preventative vaccines for infants, while *HLA-B*81* and *B*39:10* may be used in the development of therapeutic vaccines against HIV-1 disease progression in HIV-1 infected infants. We rejected our hypotheses on the role of KIR/HLA genes in HIV-1 mother-to-child acquisition. Activating KIR and/ or KIR B haplotypes did not influence HIV-1 acquisition or disease progression among HIV-1 exposed infants. Furthermore, specific HLA-B alleles did not influence HIV-1 mother-to-child acquisition, however, they were associated with slower HIV-1 disease progression to AIDS. We accepted our hypothesis on the role of KIR/HLA genes is HIV disease progression on HIV-1 infected infants. HIV rapid progressors had lower frequencies of activating *KIR2DS1*, *HLA-B*39:10* and *HLA-B*81* compared to slow progressors. These findings have important implications for the vaccine development studies.

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CHAPTER 7: APPENDIX

Appendix 7.1

Dried blood spot sample collection

Dried blood spot samples were collected previously from the mother-infant pairs by the study clinicians and pediatricians (Bland et al., 2009). Whatman-Bioscience #903 10534612 filter paper card was labelled with patient's identity, date and visit identity. The unistick two devices were prepared and then used to prick the patient's finger. The droplets of blood were collected by touching the incision area with five designated circles on the filter paper. The finger was squeezed to maximise blood volumes from the patient's finger. Once finished, the sterile pad was gently pressed on the incision area to stop bleeding. Whatman-Bioscience card was held without touching circles on the filter paper, blood spots were allowed to air-dry for approximately 4 hours or overnight. The card was placed in an impermeable bag with dessicant pack and stored at -80^{o} C for 1 year, room temperature for 1 year and -70^{o} C for approximately 6 years.

Appendix 7.2

Supplementary Table 2.3: Qiagen QIAamp produced the most optimal results for PCR detection of IL-10 SNP-592 in both the first and the second extraction, these results were concordant with that obtained from whole blood. Grey squares indicate indeterminant results

-592 Extraction 1					-592 Extraction 2			
PID	**Whole Blood**	**QIAamp**	**MiniMag**	**prepGem**	**Whole Blood**	**QIAamp**	**MiniMag**	**prepGem**
102120	AC	AC			AC			AC
104140	AC	AC			AC	AC		AC
103460	CC	CC			CC	CC		
105900	CC	CC			CC	CC		
106960	CC	CC			CC	CC		
107480	AA	AA			AA	AA	AA	
109040	CC	CC			CC	CC		
112100	AA	AA			AA	AA		
155490	CC	CC		CC	CC	CC		

Note: -592 : Wild type genotype = CC

-592: Mutation = AA

-592: Heterozygous = AC

Appendix 7.3

Supplementary Table 2.4: Qiagen QIAamp produced the most optimal results for PCR detection of IL-10 SNP-1082 in both the first and the second extraction, these results were concordant with that obtained from whole blood. Grey square indicates indeterminant results and red square indicates discordant results.

-1082 Extraction 1					**-1082 Extraction 2**			
PID	**Whole Blood**	**QIAamp**	**MiniMag**	**prepGem**	**Whole Blood**	**QIAamp**	**MiniMag**	**prepGem**
102120	AA	AA			AA		AG	AA
104140	AA	AA			AA	AA	AA	AA
103460	AG	AG			AG	AG		
105900	AG	AG			AG	AG	AG	
106960	AG	UN			AG	AG		
107480	AA	AA			AA	AA	AG	AA
109040	AG	AG			AG	AG	AA	
112100	AA	AA			AA	AA		
155490	AG	UN	AG		AG	AG		

Note: -1082 : Wild type genotype = AA

-1082: Mutation = GG

-1082: Heterozygous = AG

Appendix 7.4

Supplementary Table 2.5: HLA-class I genotyping from wgaDNA had a higher frequency of false homozygous alleles and samples that failed to amplify, however, HLA-B alleles produced the highest percentage of successful HLA determination compared to HLA-A and HLA-C alleles.

NO	HGAL	PID	A1	A2	B1	B2	C1	C2
1	CAR42232	102120wb	0205	6601	3910	5801	0701	1203
2	CAR42232	102120WGA	0205	0205	F	F	1203	1203
3	CAR42233	104140wb	0205	0205	4201	5801	0701	1700
4	CAR46233	104140WGA	0205	0205	5801	5801	0701	1700
5	CAR42234	103460wb	6602	7400			0210	1700
6	CAR42234	103460WGA	6602	6602			0210	0210
7	CAR42236	106960wb	3004	6802	0702	1401	0702	0804
8	CAR42236	106960WGA	3004	6802	0702	1401	0804	0804
9	CAR42237	107480wb	3001	7400	0801	1503	0210	1700
10	CAR42237	107480WGA	3001	7400	0801		1700	1700
11	CAR42238	109040wb	0201	2301	1503	5801	0210	0302
12	CAR42238	109040WGA	0201	2301	1503	5801	0210	0302
13	CAR42239	112100wb	0201	3004	1503	3501	0401	0704
14	CAR42239	112100WGA	0201	0201	1503	3501	F	F
15	CAR42240	155490wb	0202	3002	3910	5301		
16	CAR42240	155490WGA	0202	3002	3910	5301		
17	CAR42241	156130wb	2301	6802	1510	5702	0401	1800
18	CAR42241	156130WGA	6802	6802	1510	5702	0401	0410
19	CAR42242	156260wb	3002	7400	1503	1801	0210	0704
20	CAR42242	156260WGA	3002	3002	F	F	0210	0704
21	CAR42243	159250wb	2911	6801	1302	5802	0602	0602
22	CAR42243	159250WGA	2911	2911	F	F	F	F
23	CAR42244	176640wb	0301	2301	5801	8100	0302	0804
24	CAR42244	176640WGA	0301	0301	5801	8100	0302	0804
25	CAR42245	178030wb	2902	8001	1801	3501	0202	0401
26	CAR42245	178030WGA	F	F	F	F	F	F
27	CAR42246	193650wb	0101	2911	1302	8100	0602	1800
28	CAR42246	193650WGA	F	F	F	F	F	F
29	CAR42247	200670wb	0205	3002	1510	4501	0804	1601
30	CAR42247	200670WGA	0205	3002	1510	1510	0804	0804
31	CAR42248	200770wb	0205	3001	1401	5801	0701	0804
32	CAR42248	200770WGA	F	F	1401	5801	F	F
33	CAR42249	202180wb	2301	2902	0801	5301	0304	0701
34	CAR42249	202180WGA	2301	2902	0801	5301	F	F
35	CAR42250	208140wb	2911	3004	1302	1402	0602	1700
36	CAR42250	208140WGA	2911	2911	F	F	F	F
37	CAR42251	230130wb	0205	0205	1401	5801	0701	0804
38	CAR42251	230130WGA	F	F	F	F	F	F

F Failed PCR in wgaDNA

concordant for 1 allele

Failed PCR in both wb and wgaDNA

Appendix 7.5

Supplementary Table 2.6: There was no significant difference between gDNA yields produced when a single or two punches of DBS samples were used as a template of gDNA isolation by Qiagen QIAamp DNA mini kit.

	QIAGEN kit	**PREPGEM**	**PHENOL CHLOROFORM**
DBS 2 punches	4.6 ng/µl Total= 690 ng		425.3ng/µl Total= 42500 ng **Whole blood**
DBS 1 punch	3.8ng/µl Total= 570 ng	121.7 ng/µl Total=5354.8 ng	249.7 ng/µl Total= 24970 ng **Buffy coat**

Appendix 7.6

Supplementary Table 2.7: KIR genotyping on 38 additional samples from HIV-1 infected infants, wgaDNA was suitable for genotyping of 9 genes except *KIR2DL2, KIR2DL3, KIR2DL5, KIR2DS1* and *KIR2DS5.*

	KIR2DL2	***KIR2DL3***	***KIR2DL5***	***KIR2DS1***	***KIR2DS5***
False negative	**2**	**10**	**3**	**1**	**6**
False positive	**1**	**1**	**0**	**0**	**2**
Sensitivity (%)	**92**	**63**	**89.3**	**83.3**	**63**
Specificity	**91**	**88**	**100**	**100**	**90**
Frequency	**0.703**	**0.50**	**0.714**	**0.166**	**0.314**

Appendix 7.7

Supplementary Table 7.7: HLA frequeny between HIV-1 exposed infected and exposed-uninfected infants.

Logistic analysis on HLA data

	mother+/child+		mother+/child-			no adjusting				Bonferroni
	N	%	N	%		p value	OR	95% CI		corrected p
A0101	3	2.54	6	5.22	A0101 vs others	0.30	0.47	0.12	1.94	1.00
	115	97.46	109	94.78						
A0201	12	10.17	17	14.78	A0201 vs others	0.29	0.65	0.30	1.44	1.00
	106	89.83	98	85.22						
A0202	1	0.85	4	3.48	A0202 vs others	0.20	0.24	0.03	2.15	1.00
	117	99.15	111	96.52						
A0205	5	4.24	14	12.17	A0205 vs others	**0.03**	0.32	0.11	0.92	1.00
	113	95.76	101	87.83						
A0301	8	6.78	18	15.65	A0301 vs others	**0.04**	0.39	0.16	0.94	1.00
	110	93.22	97	84.35						
A2301	23	19.49	20	17.39	A2301 vs others	0.68	1.15	0.59	2.23	1.00
	95	80.51	95	82.61						
A2402	5	4.24	3	2.61	A2402 vs others	0.50	1.65	0.39	7.08	1.00
	113	95.76	112	97.39						
A2601	3	2.54	2	1.74	A2601 vs others	0.67	1.47	0.24	8.99	1.00
	115	97.46	113	98.26						
A2902	20	16.95	17	14.78	A2902 vs others	0.65	1.18	0.58	2.38	1.00
	98	83.05	98	85.22						
A2911	5	4.24	5	4.35	A2911 vs others	0.97	0.97	0.27	3.46	1.00
	113	95.76	110	95.65						
A3001	23	19.49	29	25.22	A3001 vs others	0.30	0.72	0.39	1.33	1.00
	95	80.51	86	74.78						
A3002	15	12.71	10	8.70	A3002 vs others	0.32	1.53	0.66	3.56	1.00
	103	87.29	105	91.30						
A3004	6	5.08	0	0.00	A3004 vs others					
	112	94.92	115	100.00						
A3201	3	2.54	6	5.22	A3201 vs others	0.30	0.47	0.12	1.94	1.00
	115	97.46	109	94.78						
A3303	1	0.85	2	1.74	A3303 vs others	0.55	0.48	0.04	5.40	1.00
	117	99.15	113	98.26						
A3402	16	13.56	10	8.70	A3402 vs others	0.24	1.65	0.71	3.80	1.00
	102	86.44	105	91.30						
A4301	10	8.47	5	4.35	A4301 vs others	0.21	2.04	0.67	6.15	1.00
	108	91.53	110	95.65						
A6601	13	11.02	7	6.09	A6601 vs others	0.19	1.91	0.73	4.98	1.00
	105	88.98	108	93.91						
A6602	1	0.85	2	1.74	A6602 vs others	0.55	0.48	0.04	5.40	1.00
	117	99.15	113	98.26						
A6801	6	5.08	5	4.35	A6801 vs others	0.79	1.18	0.35	3.97	1.00
	112	94.92	110	95.65						
A6802	14	11.86	20	17.39	A6802 vs others	0.23	0.64	0.31	1.34	1.00
	104	88.14	95	82.61						
A7400	11	9.32	11	9.57	A7400 vs others	0.95	0.97	0.40	2.34	1.00
	107	90.68	104	90.43						
A8001	7	5.93	2	1.74	A8001 vs others	0.12	3.56	0.72	17.53	1.00
	111	94.07	113	98.26						
B0702	10	8.33	10	8.77	B0702 vs others	0.90	0.95	0.38	2.36	1.00
	110	91.67	104	91.23						

B0705	3	2.50	3	2.63	B0705 vs others	0.95	0.95	0.19	4.80	1.00
	117	97.50	111	97.37						
B0801	17	14.17	8	7.02	B0801 vs others	0.08	2.19	0.90	5.29	1.00
	103	85.83	106	92.98						
B1302	1	0.83	5	4.39	B1302 vs others	0.12	0.18	0.02	1.59	1.00
	119	99.17	109	95.61						
B1401	4	3.33	5	4.39	B1401 vs others	0.68	0.75	0.20	2.87	1.00
	116	96.67	109	95.61						
B1402	4	3.33	3	2.63	B1402 vs others	0.75	1.28	0.28	5.83	1.00
	116	96.67	111	97.37						
B1503	23	19.17	26	22.81	B1503 vs others	0.49	0.80	0.43	1.51	1.00
	97	80.83	88	77.19						
B1510	22	18.33	24	21.05	B1510 vs others	0.60	0.84	0.44	1.61	1.00
	98	81.67	90	78.95						
B1516	1	0.83	3	2.63	B1516 vs others	0.31	0.31	0.03	3.03	1.00
	119	99.17	111	97.37						
B1801	8	6.67	11	9.65	B1801 vs others	0.41	0.67	0.26	1.73	1.00
	112	93.33	103	90.35						
B3501	2	1.67	1	0.88	B3501 vs others	0.60	1.92	0.17	21.41	1.00
	118	98.33	113	99.12						
B3910	5	4.17	4	3.51	B3910 vs others	0.79	1.19	0.31	4.56	1.00
	115	95.83	110	96.49						
B4101	3	2.50	0	0.00	B4101 vs others					
	117	97.50	114	100.00						
B4201	27	22.50	25	21.93	B4201 vs others	0.92	1.03	0.56	1.92	1.00
	93	77.50	89	78.07						
B4202	4	3.33	5	4.39	B4202 vs others	0.68	0.75	0.20	2.87	1.00
	116	96.67	109	95.61						
B4403	25	20.83	20	17.54	B4403 vs others	0.52	1.24	0.64	2.38	1.00
	95	79.17	94	82.46						
B4501	9	7.50	9	7.89	B4501 vs others	0.91	0.95	0.36	2.47	1.00
	111	92.50	105	92.11						
B5301	8	6.67	7	6.14	B5301 vs others	0.87	1.09	0.38	3.11	1.00
	112	93.33	107	93.86						
B5703	3	2.50	5	4.39	B5703 vs others	0.43	0.56	0.13	2.39	1.00
	117	97.50	109	95.61						
B5801	8	6.67	11	9.65	B5801 vs others	0.41	0.67	0.26	1.73	1.00
	112	93.33	103	90.35						
B5802	27	22.50	20	17.54	B5802 vs others	0.35	1.36	0.72	2.60	1.00
	93	77.50	94	82.46						
B8100	9	7.50	7	6.14	B8100 vs others	0.68	1.24	0.45	3.45	1.00
	111	92.50	107	93.86						
C0202	6	4.92	5	4.39	C0202 vs others	0.85	1.13	0.33	3.80	1.00
	116	95.08	109	95.61						
C0210	20	16.39	32	28.07	C0210 vs others	**0.03**	0.50	0.27	0.94	1.00
	102	83.61	82	71.93						
C0302	3	2.46	1	0.88	C0302 vs others	0.37	2.85	0.29	27.79	1.00
	119	97.54	113	99.12						
C0304	17	13.93	21	18.42	C0304 vs others	0.35	0.72	0.36	1.44	1.00
	105	86.07	93	81.58						
C0401	43	35.25	18	15.79	C0401 vs others	**0.001**	2.90	1.55	5.43	0.05
	79	64.75	96	84.21						
C0602	33	27.05	29	25.44	C0602 vs others	0.78	1.09	0.61	1.94	1.00

	89	72.95	85	74.56						
C0701	25	20.49	28	24.56	C0701 vs others	0.45	0.79	0.43	1.46	1.00
	97	79.51	86	75.44						
C0702	11	9.02	9	7.89	C0702 vs others	0.76	1.16	0.46	2.90	1.00
	111	90.98	105	92.11						
C0704	2	1.64	5	4.39	C0704 vs others	0.23	0.36	0.07	1.91	1.00
	120	98.36	109	95.61						
C0802	8	6.56	5	4.39	C0802 vs others	0.47	1.53	0.49	4.82	1.00
	114	93.44	109	95.61						
C0804	3	2.46	2	1.75	C0804 vs others	0.71	1.41	0.23	8.61	1.00
	119	97.54	112	98.25						
C1203	5	4.10	4	3.51	C1203 vs others	0.81	1.17	0.31	4.49	1.00
	117	95.90	110	96.49						
C1601	13	10.66	13	11.40	C1601 vs others	0.85	0.93	0.41	2.09	1.00
	109	89.34	101	88.60						
C1700	33	27.05	29	25.44	C1700 vs others	0.78	1.09	0.61	1.94	1.00
	89	72.95	85	74.56						
C1800	7	5.74	11	9.65	C1800 vs others	0.26	0.57	0.21	1.53	1.00
	115	94.26	103	90.35						

*total 60 alleles were tested for HLA-A, B and C. Therefore, a Bonferroni adjusted p-value is the normal p-value multiplied by 60 (the number of outcomes being tested).

Appendix 7.8

Supplementary Table 7.8 HLA-C alleles between HIV-1 infected and unexposed uninfected infants.

Logistic analysis on HLA data — Feb. 28,2010

	mother+/child+		mother-/child-			no adjusting				Bonferroni corrected p
	N	%	N	%		p value	OR	95% CI		
A0101	3	2.54	6	4.92	A0101 vs others	0.34	0.50	0.12	2.07	1.00
	115	97.46	116	95.08						
A0201	12	10.17	16	13.11	A0201 vs others	0.48	0.75	0.34	1.66	1.00
	106	89.83	106	86.89						
A0202	1	0.85	1	0.82	A0202 vs others	0.98	1.03	0.06	16.73	1.00
	117	99.15	121	99.18						
A0205	5	4.24	10	8.20	A0205 vs others	0.21	0.50	0.16	1.50	1.00
	113	95.76	112	91.80						
A0301	8	6.78	14	11.48	A0301 vs others	0.21	0.56	0.23	1.39	1.00
	110	93.22	108	88.52						
A2301	23	19.49	21	17.21	A2301 vs others	0.65	1.16	0.61	2.24	1.00
	95	80.51	101	82.79						
A2402	5	4.24	4	3.28	A2402 vs others	0.70	1.30	0.34	4.98	1.00
	113	95.76	118	96.72						
A2601	3	2.54	5	4.10	A2601 vs others	0.51	0.61	0.14	2.61	1.00
	115	97.46	117	95.90						
A2902	20	16.95	18	14.75	A2902 vs others	0.64	1.18	0.59	2.36	1.00
	98	83.05	104	85.25						
A2911	5	4.24	2	1.64	A2911 vs others	0.25	2.65	0.50	13.95	1.00
	113	95.76	120	98.36						
A3001	23	19.49	28	22.95	A3001 vs others	0.51	0.81	0.44	1.51	1.00
	95	80.51	94	77.05						
A3002	15	12.71	19	15.57	A3002 vs others	0.53	0.79	0.38	1.64	1.00
	103	87.29	103	84.43						
A3004	6	5.08	4	3.28	A3004 vs others	0.49	1.58	0.43	5.75	1.00
	112	94.92	118	96.72						
A3201	3	2.54	4	3.28	A3201 vs others	0.74	0.77	0.17	3.51	1.00
	115	97.46	118	96.72						
A3303	1	0.85	4	3.28	A3303 vs others	0.22	0.25	0.03	2.29	1.00
	117	99.15	118	96.72						
A3402	16	13.56	10	8.20	A3402 vs others	0.19	1.76	0.76	4.05	1.00
	102	86.44	112	91.80						
A4301	10	8.47	8	6.56	A4301 vs others	0.57	1.32	0.50	3.47	1.00
	108	91.53	114	93.44						
A6601	13	11.02	7	5.74	A6601 vs others	0.15	2.03	0.78	5.29	1.00
	105	88.98	115	94.26						
A6602	1	0.85	2	1.64	A6602 vs others	0.59	0.51	0.05	5.73	1.00
	117	99.15	120	98.36						
A6801	6	5.08	8	6.56	A6801 vs others	0.63	0.76	0.26	2.27	1.00
	112	94.92	114	93.44						
A6802	14	11.86	19	15.57	A6802 vs others	0.41	0.73	0.35	1.53	1.00
	104	88.14	103	84.43						
A7400	11	9.32	18	14.75	A7400 vs others	0.20	0.59	0.27	1.32	1.00
	107	90.68	104	85.25						
A8001	7	5.93	6	4.92	A8001 vs others	0.73	1.22	0.40	3.74	1.00
	111	94.07	116	95.08						
B0702	10	8.33	11	9.48	B0702 vs others	0.76	0.87	0.35	2.13	1.00
	110	91.67	105	90.52						

B0705	3	2.50	4	3.45	B0705 vs others	0.67	0.72	0.16	3.28	1.00
	117	97.50	112	96.55						
B0801	17	14.17	13	11.21	B0801 vs others	0.50	1.31	0.60	2.83	1.00
	103	85.83	103	88.79						
B1302	1	0.83	3	2.59	B1302 vs others	0.32	0.32	0.03	3.09	1.00
	119	99.17	113	97.41						
B1401	4	3.33	7	6.03	B1401 vs others	0.33	0.54	0.15	1.89	1.00
	116	96.67	109	93.97						
B1402	4	3.33	2	1.72	B1402 vs others	0.44	1.97	0.35	10.94	1.00
	116	96.67	114	98.28						
B1503	23	19.17	14	12.07	B1503 vs others	0.14	1.73	0.84	3.55	1.00
	97	80.83	102	87.93						
B1510	22	18.33	22	18.97	B1510 vs others	0.90	0.96	0.50	1.85	1.00
	98	81.67	94	81.03						
B1516	1	0.83	1	0.86	B1516 vs others	0.98	0.97	0.06	15.63	1.00
	119	99.17	115	99.14						
B1801	8	6.67	10	8.62	B1801 vs others	0.57	0.76	0.29	1.99	1.00
	112	93.33	106	91.38						
B3501	2	1.67	4	3.45	B3501 vs others	0.39	0.47	0.09	2.64	1.00
	118	98.33	112	96.55						
B3910	5	4.17	3	2.59	B3910 vs others	0.51	1.64	0.38	7.01	1.00
	115	95.83	113	97.41						
B4101	3	2.50	2	1.72	B4101 vs others	0.68	1.46	0.24	8.91	1.00
	117	97.50	114	98.28						
B4201	27	22.50	34	29.31	B4201 vs others	0.23	0.70	0.39	1.26	1.00
	93	77.50	82	70.69						
B4202	4	3.33	4	3.45	B4202 vs others	0.96	0.97	0.24	3.95	1.00
	116	96.67	112	96.55						
B4403	25	20.83	17	14.66	B4403 vs others	0.22	1.53	0.78	3.02	1.00
	95	79.17	99	85.34						
B4501	9	7.50	10	8.62	B4501 vs others	0.75	0.86	0.34	2.20	1.00
	111	92.50	106	91.38						
B5301	8	6.67	10	8.62	B5301 vs others	0.57	0.76	0.29	1.99	1.00
	112	93.33	106	91.38						
B5703	3	2.50	8	6.90	B5703 vs others	0.12	0.35	0.09	1.34	1.00
	117	97.50	108	93.10						
B5801	8	6.67	11	9.48	B5801 vs others	0.43	0.68	0.26	1.76	1.00
	112	93.33	105	90.52						
B5802	27	22.50	23	19.83	B5802 vs others	0.62	1.17	0.63	2.19	1.00
	93	77.50	93	80.17						
B8100	9	7.50	8	6.90	B8100 vs others	0.86	1.09	0.41	2.94	1.00
	111	92.50	108	93.10						
C0202	6	4.92	5	4.03	C0202 vs others	0.74	1.23	0.37	4.14	1.00
	116	95.08	119	95.97						
C0210	20	16.39	19	15.32	C0210 vs others	0.82	1.08	0.55	2.15	1.00
	102	83.61	105	84.68						
C0302	3	2.46	2	1.61	C0302 vs others	0.64	1.54	0.25	9.37	1.00
	119	97.54	122	98.39						
C0304	17	13.93	16	12.90	C0304 vs others	0.81	1.09	0.52	2.28	1.00
	105	86.07	108	87.10						
C0401	43	35.25	27	21.77	C0401 vs others	0.02	1.96	1.11	3.44	1.00
	79	64.75	97	78.23						
C0602	33	27.05	35	28.23	C0602 vs others	0.84	0.94	0.54	1.65	1.00

	89	72.95	89	71.77						
C0701	25	20.49	28	22.58	C0701 vs others	0.69	0.88	0.48	1.62	1.00
	97	79.51	96	77.42						
C0702	11	9.02	22	17.74	C0702 vs others	0.05	0.46	0.21	0.99	1.00
	111	90.98	102	82.26						
C0704	2	1.64	5	4.03	C0704 vs others	0.28	0.40	0.08	2.09	1.00
	120	98.36	119	95.97						
C0802	8	6.56	6	4.84	C0802 vs others	0.56	1.38	0.46	4.10	1.00
	114	93.44	118	95.16						
C0804	3	2.46	3	2.42	C0804 vs others	0.98	1.02	0.20	5.14	1.00
	119	97.54	121	97.58						
C1203	5	4.10	2	1.61	C1203 vs others	0.26	2.60	0.50	13.69	1.00
	117	95.90	122	98.39						
C1601	13	10.66	14	11.29	C1601 vs others	0.87	0.94	0.42	2.09	1.00
	109	89.34	110	88.71						
C1700	33	27.05	41	33.06	C1700 vs others	0.30	0.75	0.43	1.30	1.00
	89	72.95	83	66.94						
C1800	7	5.74	9	7.26	C1800 vs others	0.63	0.78	0.28	2.16	1.00
	115	94.26	115	92.74						

*total 60 alleles were tested for HLA-A, B and C. Therefore, a Bonferroni adjusted p-value is the normal p-value multiplied by 60 (the number of outcomes being tested).

Appendix 7.9

Table 7.9.1 Correlations between KIR/HLA genes and HIV-1 Viral Load

ANOVA on log VL data — Mar. 03, 2011

	child's mean VL for group mother+/child+							Bonferroni
Child HLA/KIR	N	Mean	SE	p	ifferenc	95%CI		corrected p
A0101	3	5.34	0.42	0.68	0.18	-0.66	1.02	1.00
	111	5.16	0.07					
A0201	12	5.45	0.21	0.15	0.32	-0.12	0.75	1.00
	102	5.13	0.07					
A0202	1	5.29	0.72	0.86	0.13	-1.32	1.57	1.00
	113	5.16	0.07					
A0205	5	4.88	0.32	0.37	-0.30	-0.95	0.36	1.00
	109	5.18	0.07					
A0301	8	5.19	0.26	0.92	0.03	-0.50	0.55	1.00
	106	5.16	0.07					
A2301	22	5.21	0.15	0.76	0.05	-0.29	0.39	1.00
	92	5.15	0.08					
A2402	4	5.50	0.36	0.35	0.34	-0.39	1.07	1.00
	110	5.15	0.07					
A2601	3	5.75	0.41	0.16	0.60	-0.23	1.43	1.00
	111	5.15	0.07					
A2902	20	5.16	0.16	0.98	-0.01	-0.36	0.35	1.00
	94	5.17	0.07					
A2911	4	5.99	0.35	**0.02**	0.86	0.14	1.57	1.00
	110	5.13	0.07					
A3001	23	5.05	0.15	0.39	-0.15	-0.48	0.19	1.00
	91	5.19	0.08					
A3002	15	5.06	0.19	0.56	-0.12	-0.52	0.28	1.00
	99	5.18	0.07					
A3004	5	5.64	0.32	0.13	0.49	-0.16	1.15	1.00
	109	5.14	0.07					
A3201	3	5.07	0.42	0.81	-0.10	-0.94	0.74	1.00
	111	5.17	0.07					
A3303	1	4.44	0.72	0.32	-0.73	-2.17	0.71	1.00
	113	5.17	0.07					
A3402	14	5.09	0.19	0.67	-0.09	-0.50	0.32	1.00
	100	5.18	0.07					
A4301	10	5.09	0.23	0.73	-0.08	-0.56	0.39	1.00
	104	5.17	0.07					
A6601	13	5.15	0.20	0.93	-0.02	-0.44	0.40	1.00
	101	5.17	0.07					
A6602	1	6.43	0.72	0.08	1.27	-0.15	2.70	1.00
	113	5.15	0.07					
A6801	5	5.14	0.32	0.95	-0.02	-0.68	0.64	1.00
	109	5.17	0.07					
A6802	14	4.89	0.19	0.13	-0.31	-0.72	0.09	1.00
	100	5.20	0.07					
A7400	10	4.80	0.23	0.09	-0.40	-0.87	0.07	1.00
	104	5.20	0.07					
A8001	7	5.07	0.27	0.74	-0.10	-0.66	0.46	1.00
	107	5.17	0.07					
B0702	10	5.14	0.23	0.84	-0.05	-0.52	0.42	1.00
	106	5.19	0.07					
B0705	3	5.37	0.42	0.65	0.19	-0.64	1.03	1.00

	113	5.18	0.07					
B0801	15	5.36	0.19	0.32	0.20	-0.19	0.59	1.00
	101	5.16	0.07					
B1302	1	6.70	0.71	**0.03**	1.52	0.12	2.93	1.00
	115	5.17	0.07					
B1401	4	4.85	0.36	0.34	-0.35	-1.07	0.37	1.00
	112	5.20	0.07					
B1402	4	5.18	0.36	0.98	-0.01	-0.74	0.72	1.00
	112	5.19	0.07					
B1503	22	5.31	0.15	0.38	0.15	-0.19	0.49	1.00
	94	5.16	0.07					
B1510	21	5.39	0.16	0.15	0.25	-0.09	0.59	1.00
	95	5.14	0.07					
B1516	1	5.08	0.72	0.88	-0.11	-1.54	1.33	1.00
	115	5.19	0.07					
B1801	8	4.85	0.25	0.17	-0.36	-0.88	0.16	1.00
	108	5.21	0.07					
B3501	2	5.24	0.51	0.92	0.05	-0.96	1.07	1.00
	114	5.18	0.07					
B3910	5	4.55	0.32	**0.04**	-0.66	-1.30	-0.02	1.00
	111	5.21	0.07					
B4101	3	5.40	0.42	0.61	0.22	-0.62	1.05	1.00
	113	5.18	0.07					
B4201	27	5.10	0.14	0.51	-0.11	-0.42	0.21	1.00
	89	5.21	0.08					
B4202	4	5.43	0.36	0.48	0.26	-0.47	0.98	1.00
	112	5.18	0.07					
B4403	24	5.07	0.15	0.37	-0.15	-0.47	0.18	1.00
	92	5.22	0.07					
B4501	9	5.45	0.24	0.24	0.29	-0.20	0.78	1.00
	107	5.16	0.07					
B5301	7	5.04	0.27	0.57	-0.16	-0.72	0.40	1.00
	109	5.20	0.07					
B5703	3	4.84	0.41	0.39	-0.36	-1.19	0.47	1.00
	113	5.19	0.07					
B5801	8	5.17	0.25	0.94	-0.02	-0.54	0.50	1.00
	108	5.19	0.07					
B5802	25	5.07	0.14	0.38	-0.14	-0.46	0.18	1.00
	91	5.22	0.08					
B8100	9	4.90	0.24	0.21	-0.31	-0.80	0.18	1.00
	107	5.21	0.07					
C0202	6	4.73	0.29	0.13	-0.46	-1.05	0.14	1.00
	112	5.19	0.07					
C0210	19	5.18	0.17	0.94	0.01	-0.35	0.37	1.00
	99	5.17	0.07					
C0302	3	5.22	0.42	0.90	0.06	-0.78	0.90	1.00
	115	5.17	0.07					
C0304	16	5.20	0.18	0.87	0.03	-0.35	0.42	1.00
	102	5.16	0.07					
C0401	42	5.30	0.11	0.14	0.20	-0.07	0.48	1.00
	76	5.10	0.08					
C0602	30	5.14	0.13	0.83	-0.03	-0.34	0.27	1.00
	88	5.18	0.08					

C0701	24	5.24	0.15	0.58	0.09	-0.24	0.42	1.00
	94	5.15	0.07					
C0702	11	5.10	0.22	0.76	-0.07	-0.52	0.38	1.00
	107	5.17	0.07					
C0704	2	5.21	0.51	0.94	0.04	-0.99	1.06	1.00
	116	5.17	0.07					
C0802	8	4.98	0.26	0.44	-0.21	-0.73	0.32	1.00
	110	5.18	0.07					
C0804	3	5.17	0.42	1.00	0.00	-0.84	0.84	1.00
	115	5.17	0.07					
C1203	5	4.74	0.32	0.18	-0.45	-1.10	0.21	1.00
	113	5.19	0.07					
C1601	12	5.64	0.20	**0.02**	0.53	0.10	0.95	1.00
	106	5.11	0.07					
C1700	33	5.08	0.13	0.40	-0.13	-0.42	0.17	1.00
	85	5.20	0.08					
C1800	7	4.81	0.27	0.18	-0.38	-0.93	0.18	1.00
	111	5.19	0.07					
bw4	63	5.09	0.09	0.12	-0.21	-0.47	0.06	
others	53	5.30	0.10					
bw480i	43	5.04	0.11	0.08	-0.26	-0.54	0.03	
others	53	5.30	0.10					
bw480t	25	5.13	0.15	0.36	-0.16	-0.52	0.19	
others	53	5.30	0.10					
C1/C1	22	5.08	0.15	0.19	-0.24	-0.61	0.13	
C1/C2	46	5.33	0.10					
C1/C1	22	5.08	0.16	0.89	0.03	-0.36	0.41	
C2/C2	50	5.06	0.11					
C1/C2	46	5.33	0.10	0.06	0.27	-0.01	0.55	
C2/C2	50	5.06	0.10					
DD	16	5.19	0.20	1.00	0.00	-0.45	0.45	
II	48	5.19	0.11					
DD	16	5.19	0.18	0.79	0.05	-0.36	0.47	
DI	54	5.14	0.10					
DD/DI	70	5.15	0.09	0.75	-0.04	-0.31	0.23	
II	48	5.19	0.10					
DI	54	5.14	0.09	0.69	-0.06	-0.33	0.22	
II	48	5.19	0.10					
II/DI	16	5.19	0.18	0.88	0.03	-0.36	0.41	
DD	102	5.16	0.07					
HH	26	5.17	0.14	0.74	0.07	-0.34	0.47	
LL	26	5.10	0.14					
HH	26	5.17	0.15	0.77	-0.05	-0.40	0.30	
HL	58	5.22	0.10					
HH/HL	84	5.20	0.08	0.53	0.10	-0.22	0.42	
HL	26	5.10	0.14					
HL	58	5.22	0.09	0.46	0.12	-0.20	0.44	
LL	26	5.10	0.13					
2DL1	115	5.18	0.06	0.81	0.17	-1.21	1.54	
	1	5.01	0.69					
2DL2	80	5.20	0.08	0.53	0.09	-0.19	0.36	
	36	5.11	0.11					
2DL3	84	5.21	0.07	0.34	0.14	-0.15	0.42	

	32	5.08	0.12				
2DL4	116	5.18	0.06				
	0						
2DL5	80	5.26	0.08	**0.04**	0.29	0.02	0.56
	36	4.98	0.11				
2DS1	18	5.51	0.16	**0.02**	0.40	0.06	0.74
	98	5.11	0.07				
2DS2	72	5.18	0.08	0.87	0.02	-0.24	0.28
	44	5.16	0.10				
2DS3	37	5.30	0.11	0.20	0.18	-0.10	0.45
	78	5.12	0.08				
2DS5	55	5.25	0.09	0.28	0.14	-0.11	0.39
	61	5.11	0.09				
3DL1	116	5.18	0.06		0.14	-0.11	0.39
	0						
3ds1	12	5.47	0.20	0.11	0.33	-0.08	0.75
	104	5.14	0.07				
3DL2	115	5.17	0.06	0.45	-0.53	-1.90	0.84
	1	5.70	0.69				
2DP1	115	5.18	0.06	0.81	0.17	-1.21	1.54
	1	5.01	0.69				
2ds4 197/197	17	4.99	0.17	0.62	-0.10	-0.48	0.29
2ds4 197/219	44	5.08	0.10	0.10	-0.31	-0.69	0.06
2ds4 219/219	54	5.30	0.09	0.12	-0.22	-0.49	0.06
2ds4 197/219, 219/219	98	5.20	0.07	0.23	0.22	-0.14	0.57
others	17	4.99	0.17				
kir kaplotype "A"	29	4.99	0.12	0.11	-0.23	-0.51	0.05
kir kaplotype "B"	85	5.21	0.07				

Appendix 7.10

Supplementary Table 7.10.1: High-resolution HLA typing Primers

PCR primers & conditions			
Locus/choice	**Primer 1**	**Primer 2**	**Taq**
A/1	5A.1	3A.1	Amplitaq
A/1	5A.1	3A.1	**KAPARobust**
A/2	5A1N1	3A1N3	Amplitaq
A/2	5A1N1	3A1N3	**KAPARobust**
B/1	5B.1	3B.1	Amplitaq
B/1	5B.1	3B.1	**KAPARobust**
*B/2	5B3	3B1	KAPARobust
C/1	5C.1-2	3C.1-2	KAPARobust
C/2	CEX1F-2	DMEX3	KAPARobust

*For this PCR product's sequence, use INT2R 8

Supplementary Table 7.10.2: High-resolution HLA PCR and DNA sequencing cocktails

HLA-A with AmpliTaq			
	x1	X105	80
d-H2O	11.8	1239.0	944.0
10x Buffer	2.0	210.0	160.0
10mM d-NTP	0.4	42.0	32.0
25mM MgCl2	1.6	168.0	128.0
10uM Primer 1	0.4	42.0	32.0
10uM Primer 2	0.4	42.0	32.0
AmpliTaq	0.2	21.0	16.0
BSA	0.2	21.0	16.0
DMSO	1.0	105.0	80.0
DNA	2.0		
total (ul)	20.0		

HLA-B with AmpliTaq			
	x1	X105	80
d-H2O	10.8	1134.0	864.0
10x Buffer	2.0	210.0	160.0
10mM d-NTP	0.4	42.0	32.0
25mM MgCl2	1.6	168.0	128.0
10uM Primer 1	0.4	42.0	32.0
10uM Primer 2	0.4	42.0	32.0
AmpliTaq	0.2	21.0	16.0
BSA	0.2	21.0	16.0
DMSO	1.0	105.0	80.0
DNA	3.0		
total (ul)	20.0		

HLA-A, B & C with KAPARobust			
	x1	X105	52
d-H2O	12.3	1291.5	639.6
5x Buffer A	4.0	420.0	208.0
10mM d-NTP	0.4	42.0	20.8
25mM MgCl2	0.4	42.0	20.8
10uM Primer 1	0.4	42.0	20.8
10uMPrimer 2	0.4	42.0	20.8
KAPARobust	0.1	10.5	5.2
DNA	2.0		
total (ul)	20.0		

Sequence			
	x1	x105	80
d-H2O	5.675	595.875	454.000
5X BigDye Buffe	1.875	196.875	150.000
BigDye	0.250	26.250	20.000
10uM Primer	0.200	21.000	16.000
DNA(cleaned)	2.000		
total (ul)	10.000		

Supplementary Table 7.10.3: HLA typing PCR special primers cocktails.

HLA-A/C Special Primer		
	x1	15
d-H2O	11.4	171.0
10x Buffer	2.0	30.0
10mM d-NTP	0.4	6.0
25mM MgCl2	1.6	24.0
2uM SP	2.0	
10uM Primer 2	0.4	6.0
AmpliTaq	0.2	3.0
DNA	2.0	
total (ul)	20.0	

HLA-B Special Primer		
	x1	15
d-H2O	10.4	156.0
10x Buffer	2.0	30.0
10mM d-NTP	0.4	6.0
25mM MgCl2	1.6	24.0
2uM SP	2.0	
10uM Primer 2	0.4	6.0
AmpliTaq	0.2	3.0
DNA	3.0	
total (ul)	20.0	

HLA-A, B & C with KAPARobust		
	x1	52
d-H2O	11.3	587.6
5x Buffer A	4.0	208
10mM d-NTP	0.4	20.8
25mM MgCl2	0.4	20.8
10uM Primer 1	0.4	20.8
10uMPrimer 2	0.4	20.8
DMSO	1.0	52
KAPARobust	0.1	5.2
DNA	2.0	
total (ul)	20.0	

PCR conditions You can use 30sec extension with KAPA taq

Stepdown A/B

95 3:00	95 :15	62 :15 x5	72 1:00	95 :15	58 :15 x26	72 1:00	95 :15	55 1:00 x4	72 2:00	72 7:00

Regular HLA-C

95 2:00	95 :15	70 :15 x40	72 1:00	72 7:00	4 ∞

HLA allele frequence

	all data					Mother+/child+					
Locus	Allele	requenc	SE	95% CI		Allele	Frequency	SE	95% CI		Allele
HLA-A	0101	0.02	0.01	0.01	0.03	0101	0.01	0.01	0.00	0.03	0101
	0201	0.07	0.01	0.05	0.08	0201	0.06	0.02	0.03	0.09	0201
	0202	0.01	0.00	0.00	0.02	0202	0.00	0.00	0.00	0.01	0202
	0205	0.04	0.01	0.03	0.06	0205	0.02	0.01	0.00	0.04	0205
	0214	0.00	0.00	0.00	0.01	0214	0.01	0.01	0.00	0.02	0301
	0220	0.00	0.00	0.00	0.00	0220	0.00	0.00	0.00	0.01	2301
	0301	0.06	0.01	0.04	0.07	0301	0.03	0.01	0.01	0.06	2402
	2301	0.10	0.01	0.08	0.12	2301	0.11	0.02	0.07	0.16	2601
	2402	0.02	0.00	0.01	0.03	2402	0.02	0.01	0.00	0.04	2901
	2601	0.01	0.00	0.01	0.02	2601	0.01	0.01	0.00	0.03	2902
	2612	0.00	0.00	0.00	0.00	2612	0.00	0.00	0.00	0.01	2911
	2901	0.00	0.00	0.00	0.01	2901	0.00	0.00	0.00	0.01	3001
	2902	0.09	0.01	0.07	0.11	2902	0.09	0.02	0.06	0.13	3002
	2911	0.02	0.00	0.01	0.03	2911	0.02	0.01	0.00	0.04	3009
	3001	0.12	0.01	0.10	0.15	3001	0.11	0.02	0.07	0.16	3201
	3002	0.07	0.01	0.05	0.09	3002	0.07	0.02	0.04	0.11	3301
	3004	0.01	0.00	0.01	0.02	3004	0.03	0.01	0.01	0.05	3303
	3009	0.00	0.00	0.00	0.01	3009	0.00	0.00	0.00	0.01	3402
	3101	0.00	0.00	0.00	0.00	3201	0.01	0.01	0.00	0.03	3601
	3201	0.02	0.01	0.01	0.03	3301	0.00	0.00	0.00	0.01	4301
	3301	0.00	0.00	0.00	0.01	3303	0.00	0.00	0.00	0.01	6601
	3303	0.01	0.00	0.00	0.02	3401	0.00	0.00	0.00	0.01	6602
	3401	0.00	0.00	0.00	0.00	3402	0.07	0.02	0.04	0.11	6603
	3402	0.05	0.01	0.04	0.07	3601	0.00	0.00	0.00	0.01	6801
	3601	0.01	0.00	0.00	0.01	4301	0.04	0.01	0.02	0.07	6802
	4301	0.03	0.01	0.02	0.05	6601	0.06	0.02	0.03	0.10	7400
	6601	0.04	0.01	0.03	0.06	6602	0.00	0.00	0.00	0.01	8001
	6602	0.01	0.00	0.00	0.01	6801	0.03	0.01	0.01	0.05	
	6603	0.00	0.00	0.00	0.01	6802	0.06	0.01	0.03	0.09	
	6801	0.03	0.01	0.02	0.04	7400	0.05	0.02	0.03	0.08	
	6802	0.08	0.01	0.06	0.10	8001	0.03	0.01	0.01	0.05	
	7400	0.06	0.01	0.04	0.08						
	8001	0.02	0.01	0.01	0.03						
HLA-B	0702	0.05	0.01	0.03	0.06	0702	0.05	0.01	0.02	0.08	0702
	0704	0.00	0.00	0.00	0.00	0705	0.01	0.01	0.00	0.03	0705
	0705	0.01	0.00	0.01	0.02	0801	0.07	0.02	0.04	0.10	0801
	0801	0.06	0.01	0.04	0.08	1302	0.00	0.00	0.00	0.01	1302
	1302	0.01	0.00	0.01	0.02	1401	0.02	0.01	0.00	0.03	1401
	1303	0.00	0.00	0.00	0.00	1402	0.02	0.01	0.00	0.04	1402
	1401	0.02	0.01	0.01	0.03	1501	0.01	0.01	0.00	0.02	1503
	1402	0.01	0.00	0.01	0.02	1503	0.10	0.02	0.06	0.14	1510
	1501	0.01	0.00	0.00	0.01	1510	0.10	0.02	0.07	0.14	1516
	1503	0.10	0.01	0.08	0.12	1516	0.00	0.00	0.00	0.01	1517
	1510	0.10	0.01	0.08	0.13	1801	0.03	0.01	0.01	0.06	1801
	1516	0.01	0.00	0.00	0.01	3501	0.01	0.01	0.00	0.02	2705
	1517	0.00	0.00	0.00	0.00	3910	0.02	0.01	0.00	0.04	3501
	1801	0.04	0.01	0.03	0.06	4101	0.01	0.01	0.00	0.03	3910
	2705	0.00	0.00	0.00	0.00	4201	0.13	0.02	0.08	0.17	4201
	3501	0.01	0.00	0.00	0.02	4202	0.02	0.01	0.00	0.03	4202

HLA allele frequence

	all data					Mother+/child+					
Locus	Allele	requenc	SE	95% CI		Allele	Frequency	SE	95% CI		Allele
HLA-A	0101	0.02	0.01	0.01	0.03	0101	0.01	0.01	0.00	0.03	0101
	0201	0.07	0.01	0.05	0.08	0201	0.06	0.02	0.03	0.09	0201
	0202	0.01	0.00	0.00	0.02	0202	0.00	0.00	0.00	0.01	0202
	0205	0.04	0.01	0.03	0.06	0205	0.02	0.01	0.00	0.04	0205
	0214	0.00	0.00	0.00	0.01	0214	0.01	0.01	0.00	0.02	0301
	0220	0.00	0.00	0.00	0.00	0220	0.00	0.00	0.00	0.01	2301
	0301	0.06	0.01	0.04	0.07	0301	0.03	0.01	0.01	0.06	2402
	2301	0.10	0.01	0.08	0.12	2301	0.11	0.02	0.07	0.16	2601
	2402	0.02	0.00	0.01	0.03	2402	0.02	0.01	0.00	0.04	2901
	2601	0.01	0.00	0.01	0.02	2601	0.01	0.01	0.00	0.03	2902
	2612	0.00	0.00	0.00	0.00	2612	0.00	0.00	0.00	0.01	2911
	2901	0.00	0.00	0.00	0.01	2901	0.00	0.00	0.00	0.01	3001
	2902	0.09	0.01	0.07	0.11	2902	0.09	0.02	0.06	0.13	3002
	2911	0.02	0.00	0.01	0.03	2911	0.02	0.01	0.00	0.04	3009
	3001	0.12	0.01	0.10	0.15	3001	0.11	0.02	0.07	0.16	3201
	3002	0.07	0.01	0.05	0.09	3002	0.07	0.02	0.04	0.11	3301
	3004	0.01	0.00	0.01	0.02	3004	0.03	0.01	0.01	0.05	3303
	3009	0.00	0.00	0.00	0.01	3009	0.00	0.00	0.00	0.01	3402
	3101	0.00	0.00	0.00	0.00	3201	0.01	0.01	0.00	0.03	3601
	3201	0.02	0.01	0.01	0.03	3301	0.00	0.00	0.00	0.01	4301
	3301	0.00	0.00	0.00	0.01	3303	0.00	0.00	0.00	0.01	6601
	3303	0.01	0.00	0.00	0.02	3401	0.00	0.00	0.00	0.01	6602
	3401	0.00	0.00	0.00	0.00	3402	0.07	0.02	0.04	0.11	6603
	3402	0.05	0.01	0.04	0.07	3601	0.00	0.00	0.00	0.01	6801
	3601	0.01	0.00	0.00	0.01	4301	0.04	0.01	0.02	0.07	6802
	4301	0.03	0.01	0.02	0.05	6601	0.06	0.02	0.03	0.10	7400
	6601	0.04	0.01	0.03	0.06	6602	0.00	0.00	0.00	0.01	8001
	6602	0.01	0.00	0.00	0.01	6801	0.03	0.01	0.01	0.05	
	6603	0.00	0.00	0.00	0.01	6802	0.06	0.01	0.03	0.09	
	6801	0.03	0.01	0.02	0.04	7400	0.05	0.02	0.03	0.08	
	6802	0.08	0.01	0.06	0.10	8001	0.03	0.01	0.01	0.05	
	7400	0.06	0.01	0.04	0.08						
	8001	0.02	0.01	0.01	0.03						
HLA-B	0702	0.05	0.01	0.03	0.06	0702	0.05	0.01	0.02	0.08	0702
	0704	0.00	0.00	0.00	0.00	0705	0.01	0.01	0.00	0.03	0705
	0705	0.01	0.00	0.01	0.02	0801	0.07	0.02	0.04	0.10	0801
	0801	0.06	0.01	0.04	0.08	1302	0.00	0.00	0.00	0.01	1302
	1302	0.01	0.00	0.01	0.02	1401	0.02	0.01	0.00	0.03	1401
	1303	0.00	0.00	0.00	0.00	1402	0.02	0.01	0.00	0.04	1402
	1401	0.02	0.01	0.01	0.03	1501	0.01	0.01	0.00	0.02	1503
	1402	0.01	0.00	0.01	0.02	1503	0.10	0.02	0.06	0.14	1510
	1501	0.01	0.00	0.00	0.01	1510	0.10	0.02	0.07	0.14	1516
	1503	0.10	0.01	0.08	0.12	1516	0.00	0.00	0.00	0.01	1517
	1510	0.10	0.01	0.08	0.13	1801	0.03	0.01	0.01	0.06	1801
	1516	0.01	0.00	0.00	0.01	3501	0.01	0.01	0.00	0.02	2705
	1517	0.00	0.00	0.00	0.00	3910	0.02	0.01	0.00	0.04	3501
	1801	0.04	0.01	0.03	0.06	4101	0.01	0.01	0.00	0.03	3910
	2705	0.00	0.00	0.00	0.00	4201	0.13	0.02	0.08	0.17	4201
	3501	0.01	0.00	0.00	0.02	4202	0.02	0.01	0.00	0.03	4202

Mother+/child-				Mother-/child-				
Frequency	SE	95% CI		Allele	Frequency	SE	95% CI	
0.02	0.01	0.00	0.04	0101	0.03	0.01	0.01	0.05
0.07	0.02	0.04	0.11	0201	0.07	0.02	0.04	0.10
0.02	0.01	0.00	0.04	0202	0.00	0.00	0.00	0.01
0.06	0.02	0.03	0.09	0205	0.04	0.01	0.02	0.07
0.08	0.02	0.05	0.11	0301	0.06	0.01	0.03	0.09
0.09	0.02	0.06	0.12	2301	0.09	0.02	0.06	0.13
0.01	0.01	0.00	0.03	2402	0.02	0.01	0.00	0.03
0.01	0.01	0.00	0.02	2601	0.02	0.01	0.00	0.04
0.00	0.00	0.00	0.01	2902	0.08	0.02	0.05	0.12
0.09	0.02	0.05	0.13	2911	0.01	0.01	0.00	0.02
0.02	0.01	0.00	0.04	3001	0.12	0.02	0.08	0.16
0.13	0.02	0.09	0.18	3002	0.08	0.02	0.05	0.11
0.05	0.02	0.02	0.08	3004	0.02	0.01	0.00	0.04
0.00	0.00	0.00	0.01	3101	0.00	0.00	0.00	0.01
0.03	0.01	0.01	0.05	3201	0.02	0.01	0.00	0.03
0.00	0.00	0.00	0.01	3301	0.00	0.00	0.00	0.01
0.01	0.01	0.00	0.02	3303	0.02	0.01	0.00	0.03
0.04	0.01	0.02	0.07	3402	0.04	0.01	0.02	0.07
0.01	0.01	0.00	0.03	4301	0.03	0.01	0.01	0.06
0.02	0.01	0.00	0.04	6601	0.03	0.01	0.01	0.05
0.03	0.01	0.01	0.05	6602	0.01	0.01	0.00	0.02
0.01	0.01	0.00	0.02	6603	0.00	0.00	0.00	0.01
0.00	0.00	0.00	0.01	6801	0.03	0.01	0.01	0.06
0.02	0.01	0.00	0.04	6802	0.08	0.02	0.05	0.12
0.10	0.02	0.06	0.14	7400	0.08	0.02	0.05	0.11
0.05	0.01	0.02	0.08	8001	0.03	0.01	0.01	0.05
0.01	0.01	0.00	0.02					
0.05	0.02	0.02	0.08	0702	0.05	0.01	0.02	0.07
0.01	0.01	0.00	0.03	0704	0.00	0.00	0.00	0.01
0.04	0.02	0.02	0.08	0705	0.02	0.01	0.00	0.04
0.02	0.01	0.00	0.04	0801	0.06	0.01	0.03	0.09
0.02	0.01	0.00	0.04	1302	0.01	0.01	0.00	0.03
0.01	0.01	0.00	0.03	1303	0.00	0.00	0.00	0.01
0.12	0.02	0.08	0.17	1401	0.03	0.01	0.01	0.06
0.11	0.02	0.08	0.16	1402	0.01	0.01	0.00	0.02
0.01	0.01	0.00	0.03	1501	0.01	0.01	0.00	0.02
0.00	0.00	0.00	0.01	1503	0.06	0.02	0.03	0.10
0.05	0.01	0.03	0.08	1510	0.09	0.02	0.06	0.13
0.00	0.00	0.00	0.01	1516	0.00	0.00	0.00	0.01
0.00	0.00	0.00	0.01	1801	0.04	0.01	0.02	0.07
0.02	0.01	0.00	0.04	3501	0.02	0.01	0.00	0.04
0.11	0.02	0.08	0.16	3910	0.01	0.01	0.00	0.03
0.02	0.01	0.00	0.04	4101	0.01	0.01	0.00	0.02

0.10	0.02	0.06	0.14	4201	0.15	0.02	0.11	0.19
0.04	0.01	0.02	0.07	4202	0.02	0.01	0.00	0.04
0.00	0.00	0.00	0.01	4403	0.07	0.02	0.04	0.11
0.00	0.00	0.00	0.01	4501	0.04	0.01	0.02	0.07
0.01	0.01	0.00	0.02	4901	0.00	0.00	0.00	0.01
0.03	0.01	0.01	0.05	5101	0.01	0.01	0.00	0.02
0.02	0.01	0.00	0.04	5301	0.04	0.01	0.02	0.07
0.05	0.01	0.02	0.08	5703	0.03	0.01	0.01	0.06
0.09	0.02	0.05	0.12	5801	0.05	0.01	0.02	0.07
0.03	0.01	0.01	0.05	5802	0.11	0.02	0.07	0.16
				8100	0.03	0.01	0.01	0.06

0.02	0.01	0.00	0.04	0102	0.00	0.00	0.00	0.01
0.15	0.02	0.11	0.20	0202	0.02	0.01	0.00	0.04
0.00	0.00	0.00	0.01	0210	0.08	0.02	0.05	0.12
0.10	0.02	0.06	0.14	0302	0.01	0.01	0.00	0.02
0.09	0.02	0.05	0.13	0303	0.01	0.01	0.00	0.02
0.01	0.01	0.00	0.03	0304	0.07	0.02	0.04	0.09
0.13	0.02	0.09	0.18	0401	0.12	0.02	0.08	0.17
0.14	0.02	0.09	0.18	0602	0.15	0.02	0.11	0.20
0.04	0.01	0.02	0.07	0701	0.11	0.02	0.08	0.16
0.02	0.01	0.00	0.04	0702	0.09	0.02	0.06	0.13
0.00	0.00	0.00	0.01	0704	0.02	0.01	0.00	0.04
0.02	0.01	0.00	0.04	0802	0.02	0.01	0.01	0.04
0.01	0.01	0.00	0.02	0804	0.01	0.01	0.00	0.03
0.02	0.01	0.00	0.04	1203	0.01	0.01	0.00	0.02
0.06	0.01	0.03	0.09	1505	0.00	0.00	0.00	0.01
0.13	0.02	0.09	0.18	1601	0.06	0.01	0.03	0.09
0.05	0.01	0.02	0.08	1700	0.17	0.02	0.13	0.21
				1800	0.04	0.01	0.02	0.06

	3910	0.02	0.00	0.01	0.03	4403	0.11	0.02	0.07	0.14	4403
	4101	0.01	0.00	0.00	0.01	4501	0.04	0.01	0.02	0.06	4501
	4201	0.13	0.01	0.10	0.15	5001	0.00	0.00	0.00	0.02	4901
	4202	0.02	0.01	0.01	0.03	5301	0.03	0.01	0.01	0.05	5001
	4403	0.09	0.01	0.07	0.11	5702	0.01	0.01	0.00	0.03	5101
	4501	0.04	0.01	0.03	0.05	5703	0.01	0.01	0.00	0.03	5301
	4901	0.00	0.00	0.00	0.01	5801	0.03	0.01	0.01	0.06	5703
	5001	0.00	0.00	0.00	0.01	5802	0.12	0.02	0.08	0.16	5801
	5101	0.01	0.00	0.00	0.01	8100	0.04	0.01	0.02	0.06	5802
	5301	0.03	0.01	0.02	0.05	8202	0.01	0.01	0.00	0.02	8100
	5702	0.00	0.00	0.00	0.01						
	5703	0.02	0.01	0.01	0.04						
	5801	0.04	0.01	0.03	0.06						
	5802	0.11	0.01	0.09	0.13						
	8100	0.03	0.01	0.02	0.05						
	8202	0.00	0.00	0.00	0.01						
HLA-C	0102	0.00	0.00	0.00	0.01	0202	0.02	0.01	0.01	0.04	0202
	0202	0.02	0.01	0.01	0.03	0210	0.09	0.02	0.05	0.12	0210
	0210	0.11	0.01	0.08	0.13	0302	0.02	0.01	0.00	0.04	0302
	0302	0.01	0.00	0.00	0.02	0303	0.01	0.01	0.00	0.02	0304
	0303	0.01	0.00	0.00	0.01	0304	0.07	0.02	0.04	0.11	0401
	0304	0.08	0.01	0.06	0.10	0401	0.19	0.02	0.14	0.24	0501
	0401	0.13	0.01	0.11	0.16	0602	0.14	0.02	0.10	0.18	0602
	0501	0.00	0.00	0.00	0.01	0701	0.11	0.02	0.07	0.15	0701
	0602	0.14	0.01	0.12	0.17	0702	0.05	0.01	0.02	0.07	0702
	0701	0.12	0.01	0.10	0.14	0704	0.01	0.01	0.00	0.02	0704
	0702	0.06	0.01	0.04	0.08	0802	0.03	0.01	0.01	0.06	0801
	0704	0.02	0.00	0.01	0.03	0804	0.01	0.01	0.00	0.03	0802
	0801	0.00	0.00	0.00	0.01	1203	0.02	0.01	0.00	0.04	0804
	0802	0.03	0.01	0.02	0.04	1505	0.00	0.00	0.00	0.01	1203
	0804	0.01	0.00	0.00	0.02	1601	0.05	0.01	0.02	0.08	1601
	1203	0.02	0.00	0.01	0.02	1700	0.15	0.02	0.10	0.20	1700
	1505	0.00	0.00	0.00	0.01	1800	0.03	0.01	0.01	0.05	1800
	1601	0.06	0.01	0.04	0.07						
	1700	0.15	0.01	0.13	0.18						
	1800	0.04	0.01	0.02	0.05						