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KIR-HLA GENES AND MATERNAL INFANT HIV-1 TRANSMISSION

Heather Alanna Hong

A thesis submitted to the faculty of Health Sciences, School of Pathology, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree Doctor of Philosophy (Virology)

Johannesburg, April 2015

DECLARATION

I hereby declare that:

1) The research reported in this thesis, except where otherwise indicated, is my own original work submitted for the degree of Doctor of Philosophy (Virology) at the University of Witwatersrand, Johannesburg, South Africa.

2) This thesis has not been submitted before for any degree or examination to any other University.

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29 April 2015

Heather Alanna Hong — Date

DEDICATION

I would like to dedicate this PhD to my parents

Norman and Lily Hong

And to my husband

Clinton Fok

Thank you for your continuous love and support throughout the course of my studies

ABSTRACT

Natural Killer (NK) cells are an essential part of the immune system capable of controlling several viral infections, including HIV-1. Moreover, several studies have linked specific NK cell receptors, termed killer immunoglobulin-like receptors (KIR), and their human leukocyte antigen (HLA) ligands to favourable clinical outcomes in HIV-1 infected individuals. Recent studies have also highlighted the role of KIR and HLA in modulating the risk of adult HIV-1 transmission and disease progression; however, little is known about the role of KIR and HLA in mother-to-child transmission (MTCT) of HIV-1. Therefore, this thesis specifically explores *KIR/HLA-ligand* combinations in HIV-1 infected mothers and their infants, as well as the *KIR/HLA-ligand* relationship between mother and infant, in the context of MTCT of HIV-1. The study of HIV-1 exposed uninfected (EU) infants in comparison with HIV-1 *in utero* (IU) or intrapartum (IP) infected infants provides important information regarding the correlates of protection and/or susceptibility to HIV-1 acquisition. Moreover, having data on HIV-1 transmitting (TR) and non-transmitting (NT) mothers may also highlight maternal factors that increase or decrease HIV-1 transmission.

Using samples from two distinct previously recruited Black South African mother-infant cohorts (MTCT1, N=217 and MTCT2, N=79) which differed in terms of antiretroviral therapy (ART) regimens (monotherapy and dual therapy, respectively) and overall rate of HIV-1 transmission (9.8% and 5.2%, respectively), *KIRs* and *HLA-ligands* (*HLA-A*, *-B*, *-C* and *-G*) were genotyped. Given the costly and laborious nature of both *KIR* genotyping by gel electrophoresis and *HLA* genotyping using a sequence based typing method, we developed, validated and published a real-time PCR detection assay that allowed for the quick, easy and cost effective detection of all 16 *KIRs* and their respective *HLA-ligands* (*HLA-A*, *-B*, *-C*). Thus, these real-time *KIR* and *HLA-ligand* PCR assays were used to genotype the second cohort, MTCT2, while MTCT1 samples were previously genotyped using the commercial Olerup *KIR* genotyping kit and published *HLA-B* and *-C* genotyping methods (Paximadis et al., 2011). Therefore, using these methods, the following were investigated (1) the effect of paired *KIR/HLA* gene combinations amongst mother and infant, (2) the role of *KIR2DS4* allelic variance, and (3) the role of the *HLA-G* (the HLA-ligand for KIR2DL4) in vertical transmission of HIV-1.

We identified that certain *KIR/HLA-ligand* combinations between mother and infant are able to influence the risk of vertical transmission of HIV-1. In MTCT1, concordance amongst

mother and infant for *KIR2DL3/KIR2DL3* in combination with *C2/C2* significantly increased the risk for IP transmission (*P*=0.043, OR=4.48), while "matched" allorecognition between infant *KIR2DL2/KIR2DL3* genotype with cognate *HLA-C1/C1* ligands from the mother significantly associated with increased risk for IU acquisition of HIV-1 (*P*=0.047, OR=4.02). Moreover, both these associations maintained significance post adjustment for maternal viral load (VL) and ART administration. In MTCT2, where NT and TR mothers were matched according to VL, CD4+ T cell count and ART regimen, only one *KIR* genotype showed a significant association with risk of MTCT. Both IU-TR and IP-TR mothers had significantly higher representation of the Bx32 genotype compared to NT mothers (*P*=0.005 and *P*=0.038, respectively). Interestingly, although the AA1 haplotype was significantly under-represented in the maternal group compared to the infant group (13.9% vs. 33.7%, *P*=0.005), and the AA1 haplotype frequency was significantly lower in MTCT2 mothers compared to MTCT1 mothers (13.9% vs. 27.2%, *P*=0.029), the AA1 haplotype did not significantly associate with risk of vertical transmission.

Regarding *HLA-ligand* (*HLA-A, -B* and *–C*) comparisons, in MTCT2 both maternal and infant possession of *HLA-A Bw4:80I* significantly increased the risk for IU transmission and IU acquisition of HIV-1 (*P*=0.005, OR=3.67 and *P*=0.001, OR=7.99, respectively). Moreover, the risk of maternal IU transmission remained significant when *KIR3DL1* was present (i.e. *KIR3DL1+Bw4:80I*, *P*=0.039, OR=6.25). Thus, these findings highlighted that the presence or absence of particular *KIR* genes and their *HLA-ligand*s in mother and infant, and the *KIR/HLA-ligand* pairings between mother and infant could influence the risk of HIV-1 transmission.

The influence of *KIR* allelic variance in transmission of HIV-1 is largely unknown, and no studies have addressed this in the context of MTCT of HIV-1. Thus, we evaluated the singular activating *KIR* gene that is present in the group A haplotype, *KIR2DS4*, as allelic variants of *KIR2DS4* can encode either functional membrane bound (KIR2DS4-f) or non-functional truncated (KIR2DS4-v) receptors. In MTCT1, we identified that maternal possession of *KIR2DS4-f* but absent in their infants (i.e. M+I- discordance) increased the risk for IP transmission (*P*=0.005, OR=3.84). While infant possession of *KIR2DS4-v* significantly increased the risk for IU acquisition of HIV-1 (*P*=0.022, OR=2.88) and this was enhanced when infants were homozygous for the group A haplotype (*P*=0.004, OR=18.40). In MTCT2, although the frequencies of *KIR2DS4* variants did not significantly differ in the maternal, infant and paired mother-infant TR and NT groups, in infants there was a weak trend towards

higher representation of *KIR2DS4-f* in IU infected infants compared to EU infants (*P*=0.080, OR=2.61). Scatter plot comparisons of maternal *KIR2DS4-v* and the AA1 haplotype showed that the two MTCT cohorts differed considerably and this difference was largely due to the maternal AA1 haplotype. It could be postulated that the lack of a significant *KIR2DS4* association in MTCT2 was because other activating *KIR* genes or Bx haplotypes may be negating the effects of *KIR2DS4* variants evident in MTCT1. Overall, these data highlighted the importance of evaluating *KIR* alleles in HIV-1 transmission.

In addition, given that HLA-G is an immunotolerogenic molecule primarily expressed at the maternal-foetal interface and it is the cognate ligand for the framework *KIR* gene, *KIR2DL4* (virtually present in all individuals), we questioned whether *HLA-G* alleles and/or polymorphisms with the 3' untranslated region (UTR) of HLA-G might influence vertical transmission of HIV-1 in our two mother-infant cohorts. In MTCT1, we identified two independent *HLA-G* factors that associated with increased risk for maternal IU transmission: *G*01:01:02* allele (*P*=0.036, OR=2.26) and the 3' UTR1 haplotype (*P*=0.011, OR=2.96). Linkage disequilibrium (LD) analysis identified that *G*01:01:02* was in strong LD with a 14-bp insertion (*Ins*) within the 3'UTR, and *G*01:01:01* was in strong LD with the UTR1 haplotype that included several polymorphisms that have been associated with higher expression of HLA-G, including a 14-bp deletion (*Del*). While maternal *HLA-G* associated with risk of HIV-1 transmission, infant *HLA-G* alleles and 3'UTR haplotypes were not found to associate with risk of HIV-1 acquisition. Therefore, for the MTCT2 cohort we selected to focus exclusively on maternal *HLA-G* polymorphisms. Interestingly, maternal possession of *HLA-G* genotype *G*01:01:01/G*01:04:04* (in strong LD with the UTR1 and UTR3 haplotypes) associated with increased risk for IP transmission (*P*=0.001 OR=29.2). In addition, the frequency of the UTR1 haplotype was significantly higher in IP-TR mothers compared to NT mothers (*P*=0.024, OR=3.27). These findings suggest that maternal *HLA-G* alleles and/or polymorphisms within the 3'UTR that might alter expression of HLA-G potentially influence risk of mother-to-child transmission of HIV-1.

To conclude, these immunogenetic findings add further to our understanding of factors that contribute to the vertical transmission of HIV-1 and highlight the importance of genetic studies in future attempts to unravel the influence of host genes in an ever-changing environment of antiviral drug usage for HIV-1 prevention.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
3TC	Lamivudine
AIDS	Acquired Immunodeficiency Syndrome
ABC	Abacavir
ADCC	Antibody dependent cellular cytotoxicity
ALB	Albumin
ART	Antiretroviral therapy
AZT	Azidothymidine also known as Zidovudine (ZDV)
BCR	B cell receptor
bp	Base pair
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CNV	Copy number variation
CRFs	Circulating recombinant forms
CSW	Commercial sex workers
CTL	Cytotoxic T lymphocyte
d4T	Stavudine
DBS	Dried blood spot
dd	Daily dose
ddATP	2',3'-dideoxyadenosine triphosphate
ddC	Zalcitabine
ddI	Didanosine
DLV	Delavirdine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
EIs	Entry inhibitors
Env	HIV envelope protein
ESP	Exposed seronegative persons
ETR	Etravirine
EU	Exposed uninfected
FTC	Emtricitabine
GALC	Galactosylceramidase
HAART	Highly Active Antiretroviral Therapy
HEPS	Highly exposed persistently seronegative
HESN	Highly exposed seronegative
HIV-1	Human Immunodeficiency virus 1
HIV-2	Human Immunodeficiency virus 2
HLA	Human Leukocyte Antigen
HWE	Hardy-Weinberg Equilibrium
i.e.	That is
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILT	Immunoglobulin-like transcript
INIs	Integrase inhibitors
IP	Intrapartum
IP-10	Interferon gamma-induced protein 10

ITAMs	Immunoreceptor tyrosine-based activating motifs
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
IU	*In utero*
KIR	Killer Immunoglobulin-like Receptor
KIR2DS4-f	Full length functional *KIR2DS4* alleles
KIR2DS4-v	Truncated non-functional *KIR2DS4* alleles
KLR	Killer cell lectin-like receptors
LCR	Leukocyte Receptor Complex
LD	Linkage disequilibrium
LILR	Leukocyte immunoglobulin-like receptors
LPS	Lipopolysaccharide
LTNP	Long term nonprogressor
MHC	Major Histocompatibility Complex
MHC	Major histocompatibility complex
MTCT	Mother-to-child transmission of HIV
MTCT1	Mother-infant cohort 1
MTCT2	Mother-infant cohort 2
NCR	Natural cytotoxicity receptors
NK cell	Natural Killer cell
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
NT	HIV-1 non-transmitting
NtRTIs	Nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PEP	Post-exposure prophylaxis
PreP	Pre-exposure prophylaxis
PIs	Protease inhibitors
PMTCT	Prevention of mother-to-child transmission of HIV
PP	Post partum
PP-VCT	Post-partum voluntary counselling and testing
Reg	HIV regulatory protein
RNA	Ribonucleic acid
RT	Reverse transcriptase
SBT	Sequence-based typing
sd	Single dose
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
STD	Sexually transmitted disease
STIs	Sexually transmitted infections
TCR	T cell receptor
TDF	Tenofovir disoproxil fumarate
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
TR	HIV-1 transmitting
TRAIL	TNF-related apoptosis-inducing ligand
uNK	Uterine NK cell
VL	Viral load
vs.	Versus
WHO	World Health Organisation
ZDV	Zidovudine also known as Azidothymidine (AZT)

PUBLICATIONS

Some of the findings presented in this thesis have been published or are in preparation for publication.

Published:

1. **Hong HA**, Paximadis M, Gray GE, Kuhn L and Tiemessen CT. (2015) Maternal human leukocyte antigen-G (HLA-G) genetic variants associate with *in utero* mother-to-child transmission of HIV-1 in Black South Africans. *Infection, Genetics and Evolution*, 30: 147-158.

2. **Hong HA**, Paximadis M, Gray GE, Kuhn L and Tiemessen CT. (2013) *KIR2DS4* allelic variants: Differential effects on *in utero* and intrapartum HIV-1 mother-to-child transmission. *Clinical Immunology*, 149: 498-508.

3. **Hong HA**, Loubser AS, de Assis Rosa D, Naranbhai V, Carr W, Paximadis M, Lewis DA, Tiemessen CT and Gray CM. (2011) *KIR* genotyping and *HLA KIR-ligand* identification by real-time PCR. *Tissue Antigens*, 78:3 185-194.

In preparation:

1 **Hong HA**, Paximadis M, Gray GE, Kuhn L and Tiemessen CT. Matched allorecognition of infant *KIR* and maternal *HLA-ligands* associate with risk of HIV-1 acquisition.

Contribution to other publications:

1. Gray CM, **Hong HA**, Young K, Lewis DA, Fallows D, Manca C and Kaplan G. Plasma interferon-gamma-inducible protein-10 (IP-10) can be used to predict viral load in HIV-1 infected individuals. (2013) *Journal of Acquired Immune Deficiency Syndromes*, 63(3): e115-e116.

PRESENTATIONS

1. **Poster presentation**: HIV R4P Conference, Cape Town, South Africa, 28 - 31 Oct, 2014. Characterization of the 3' untranslated region of *HLA-G* in HIV-1 infected Black South African mothers and their infants.

2. **Poster presentation**: AIDS Vaccine Conference, Barcelona, Spain. 7 - 10 Oct, 2013. *KIR2DS4* confers susceptibility in mother-to-child HIV-1 transmission.

3. **Oral Presentation**: 5th Cross Faculty Graduate Symposium, 1 - 2 Aug, 2013. *KIR2DS4* confers susceptibility in mother-to-child transmission of HIV-1

4. **Poster presentation**: 8th Conference of the Federation of African Immunological Societies (FAIS), Durban, South Africa, 2 - 5 Dec, 2012. *KIR2DL2*, *KIR2DL3* and *HLA-C* genes in mother-to-child HIV-1 transmission.

5. **Oral presentation**: University of Witwatersrand, Faculty of Health Sciences, Biennial Research Day & Postgraduate Expo, 19 Sep, 2012. The role of KIR/HLA concordance in mother-to-child HIV-1 transmission.

6. **Poster presentation**: 2nd Conference of the South African Immunological Society (SAIS), Johannesburg, South Africa, 5 - 8 Dec, 2010. Real-time PCR assay for the detection of killer cell immunoglobulin-like receptor (KIR) ligands (HLA-Bw4, -C1 and -C2).

7. **Poster presentation**: AIDS Vaccine Conference, Atlanta Georgia, USA, 28 Sep – 1 Oct, 2010. Sustainable plasma IP-10 levels mark chronic viral replication and correlate with viral load.

8. **Poster presentation**: 1st Conference of the South African Immunological Society (SAIS), Cape Town, South Africa, 9 - 11 Dec, 2009. Measurement of killer immunoglobin-like receptors (KIR) in South Africa using real-time PCR.

CHAPTER 1
Literature Review

1.1 Introduction

It has been more than three decades since the discovery of the human immunodeficiency virus (HIV) as the causative agent of acquired immunodeficiency syndrome (AIDS); however HIV/AIDS continues to be a global health concern. It is estimated that more than 25 million people have died of AIDS and more than 33 million are currently living with HIV/AIDS (Dieffenbach and Fauci, 2011). Sub-Saharan Africa remains the region most heavily affected by HIV and accounts for more than two thirds of all people living with HIV (UNAIDS, 2011).

South Africa was ranked as the country having the most people living with HIV/AIDS in 2011, with an estimated figure of 5.6 million (UNAIDS, 2012). In the same year, over 2 million deaths were attributed to AIDS related causes, and, while this number reflects many lives lost, this was still less than the figure reported in 2001, demonstrating the vast improvement in HIV-attributed mortality through the provision of antiretroviral therapy (ART). Nevertheless, HIV prevalence remains high at 17.3% with greatest burden amongst young adults, particularly, women. Approximately one in three women aged 25-29 are living with HIV, more than four times greater than that of men in the same age group. In addition, HIV in women is the leading cause of HIV infection in children, primarily acquired through vertical transmission of the virus from mother to child (Newell, 1998).

Prior to preventative mother-to-child transmission (PMTCT) intervention, such as ART and selective caesarean section before labour and/or membrane rupture, MTCT rates ranged from 12-45% (Bryson, 1996). This is surprisingly low in comparison to other congenital viral infections, such as rubella (Louvain de Souza et al., 2012). These HIV-1 seronegative and polymerase chain reaction (PCR) negative infants born to HIV-infected mothers represent a unique cohort of exposed but uninfected (EU) infants. Mechanisms that provide EU infants with “natural” protection/resistance to HIV-1 infection might provide relevant clues towards better vaccine and drug design. Recent evidence suggests that both virus and host factors play a role in MTCT. This study focuses on select host (both mother and infant) factors affecting the risk of MTCT, in particular the role of killer immunoglobulin like receptors (KIR) and their human leukocyte antigens (HLA) ligands.

1.2 The Biology of HIV Infection

1.2.1 HIV Origin

Based on genomic sequence similarity to simian immunodeficiency virus (SIV), two distinct types of HIV can be identified: HIV-1 and HIV-2. HIV-1 is the designation given to forms linked to SIV originating from the common chimpanzee, *Pan troglodytes* (SIVcpz,) and the gorilla, *Gorrilla gorilla* (SIVgor); and HIV-2 denotes human viruses related to virus from the sooty mangabey, *Cercocebus atys* (SIVsmm) (Sharp and Hahn, 2011, Hemelaar, 2012). Cross-species transmission likely occurred as result of hunting and butchering of these primates for bushmeat and/or their capture and trade as pets. While both types of HIV share many similarities including their basic gene arrangement, the modes of transmission, intracellular replication pathways and the same opportunistic infections, HIV-2 is less easily transmitted and appears to progress more slowly than HIV-1 (Nyamweya et al., 2013). Globally, HIV-1 is the predominant virus, while HIV-2 is mainly found in West African nations, such as Guinea Bissau, The Gambia, Senegal, Cape Verde, Cote d'Ivoire, Mali, Sierra Leone, and Nigeria (Campbell-Yesufu and Gandhi, 2011).

HIV-1 can be classified into four viral groups: M (main), N (non-M/non-O), O (outlier) and the newly discovered, P (**Figure 1.1**). These groups represent four separate introductions of SIV into humans. Group M accounts for the majority of HIV/AIDS cases, and can be divided into nine distinct subtypes (or clades) designated A, B, C, D, F, G, H, J and K. The clades E and I are no longer in use, as they likely resulted from the recombination of two viral subtypes, known as circulating recombinant forms (CRFs) (Robertson et al., 1995). Clade C is the most prevalent transmitted virus and its wide transmissibility is likely the result of a higher viral set point and higher viral load (VL) in the genital fluids compared to other clades (John-Stewart et al., 2005, Levy, 2009). Furthermore, clade C has been reported to be more frequently transmitted from mother-to-child compared to clade D, which in turn is more frequently transmitted than clade A in the absence of ART (Renjifo et al., 2004).

Molecular epidemiological studies show that there is a specific geographic distribution pattern for HIV-1 subtypes (**Figure 1.1**); and this distribution pattern seems to be the consequence of either accidental trafficking (viral migration), with a resulting "founder effect," or a prevalent route of transmission, which results in a strong advantage for and local predominance of the prevalent subtype transmitted in that population (Buonaguro et al., 2007).

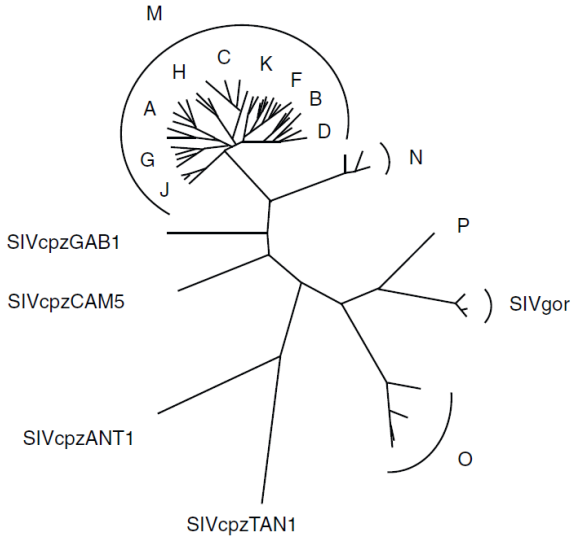
Phylogenetic tree of HIV & SIV	HIV-1	Geographic distribution
	A	Central Africa, sub-Saharan Africa
	B	South America, Brazil, United States, Thailand, Europe, Caribbean, India, Japan
	C	Brazil, India, South Africa
	D	Central Africa, sub-Saharan Africa
	F	Brazil, Romania, Democratic Republic of Congo (Zaire)
	G	Democratic Republic of Congo (Zaire), Gabon, Thailand, Russia, Central Africa
	H	Democratic Republic of Congo (Zaire), Gabon, Russia, Central Africa
	J	North, Central and West Africa, Caribbean
	K	Democratic Republic of Congo (Zaire), Cameroon

Figure 1.1 Phylogenetic tree of the SIV and HIV viruses as well as the geographic distribution of HIV-1 group M subtypes A D, F–H, J and K. Reproduced and modified from (Hemelaar, 2012).

1.2.2 HIV-1 Life Cycle

HIV-1 is an enveloped retrovirus from the *Lentivirus* genus. Characteristic of HIV-1 and other retroviruses are their ability use reverse transcriptase (RT), a DNA polymerase enzyme, to transcribe single-stranded RNA into single stranded DNA. Typical lentivirus infections show a chronic course of disease, with a long period of clinical latency, persistent viral replication and involvement of the central nervous system (Fanales-Belasio et al., 2010). The HIV-1 virion is about 110 nm in diameter and has a cone-shaped viral core that contains two single stranded RNAs of approximately 9.2 kb in length as well as the enzymes reverse transcriptase, protease, ribonuclease and integrase, all encased in an outer lipid envelope. The genome consists of nine genes, including two regulatory (*rev*, *tat*), four accessory (*vif*, *vpu*, *nef* and *vpr*) and three structural (*env*, *gag* and *pol*) genes.

HIV-1 infection is usually initiated with a single virion infecting a target cell upon entry across the mucosal lining of either genital, rectal or oral tract (Coffin and Swanstrom, 2013). In **Figure 1.2**, HIV-1 uses its envelope glycoproteins, gp120 and gp41 to bind to target cells, CD4+ T cells and/or monocytes, macrophages, and dendritic cells. Both proteins undergo a conformational change allowing gp120 to interact with either an α- or CXC-chemokine or β- or CC-chemokine coreceptor, CXCR4 or CCR5, respectively, depending on viral tropism.

Macrophage-tropic isolates tend to use CCR5 for cellular entry, while T cell tropic isolates tend to use CXCR4 (Gorry and Ancuta, 2011). Following chemokine coreceptor engagement, other conformational changes are then initiated to expose the binding domain of the transmembrane protein, gp41. This change forms a stable structure that allows fusion of HIV and host cell membrane, with a fusion pore through which the viral core can enters the host cell cytoplasm (Simon et al., 2006).

Once in the host cell cytoplasm, the viral core disassembles and the viral RNA is reverse transcribed into single-stranded DNA by RT. The single-stranded DNA is again reverse transcribed and synthesized as double-stranded DNA (Moss, 2013). Of note, in comparison to other viral and host DNA polymerases, HIV-1 RT lacks "proof-reading" ability and is extremely error prone (Preston et al., 1988, Roberts et al., 1988) and is thus responsible for the high mutation and recombination rates of HIV-1. This ability allows HIV-1 to evolve rapidly in response to selection pressures either within individual hosts or within populations (Zhang et al., 2010b).

At the midpoint of infection, double-stranded viral DNA enters the nucleus and integrates with the host's chromosomal DNA by means of the viral enzyme, integrase. The integrated viral genome, or proviral DNA, is then transcribed by the cellular RNA polymerase II into messenger RNA (mRNA) when NF-kβ binds to proviral long terminal repeats (LTRs) that flank the viral genome. Transcribed mRNA is then spliced and results in the translation of viral proteins (Tat, Rev, and Nef). The Rev protein assists in transporting singly spliced or unspliced RNA transcripts into the cytoplasm, where mRNA is translated into structural HIV-1 proteins (i.e. the nucleocapsid). The nucleocapsid then associates with genomic viral RNA, which is followed by processing of several Gag polyproteins that assemble at the plasma membrane to initiate budding.

The released virion undergoes subsequent processing of viral proteins by viral protease to transform the immature virion into a mature infectious virion, ready to infect other cells. However, in some cases viral budding from monocytes and macrophages results in intracellular vacuoles which remain in the host cell resulting in latent reservoirs of HIV-1 (Fanales-Belasio et al., 2010). This creates one of the greatest challenges in the combat against HIV-1 infection, as ART is ineffective at targeting these reservoirs.

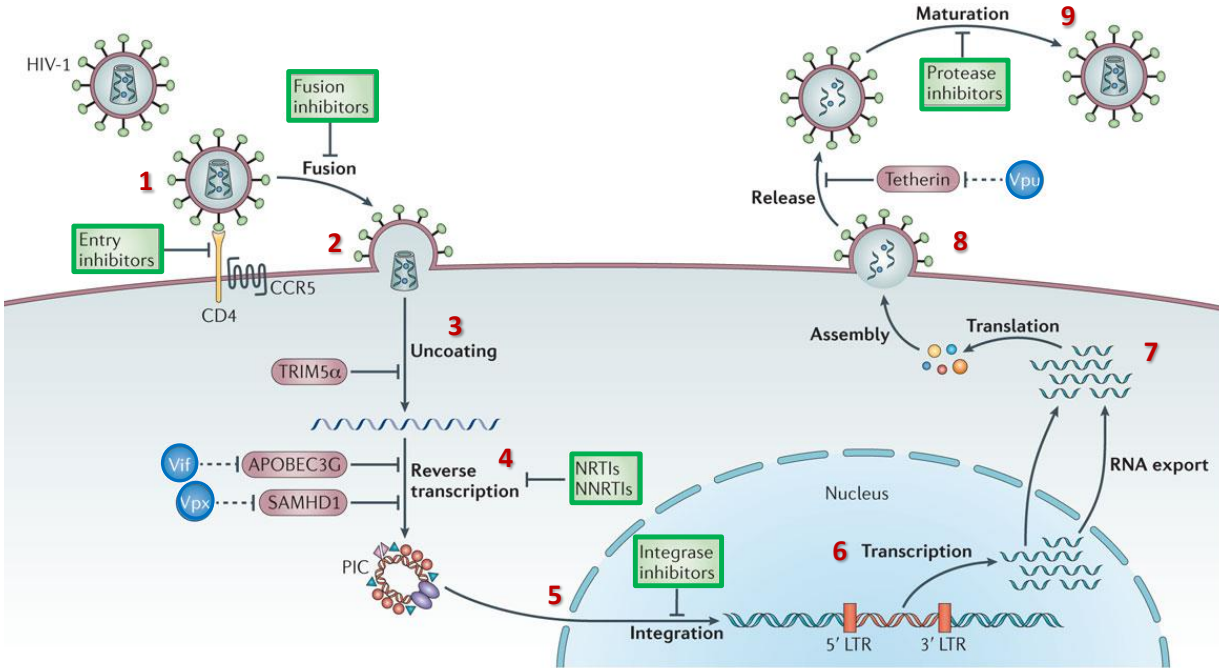


Figure 1.2 The main steps in the HIV-1 replication cycle: (1) binding to the CD4 receptor and co-receptors; (2) fusion with the host cell membrane; (3) uncoating of the viral capsid with release of the HIV RNA and proteins into the cytoplasm; (4) reverse transcription of HIV RNA to DNA; (5) formation of the pre-integration complex (PIC) and translocation into the nucleus; (6) provirus integration and protein synthesis; (7) viral proteins that translocate to the cell surface to assemble into new immature virus forms; (8) budding and (9) maturation. Also shown are the major families of antiretroviral drugs (green) and the step of the life cycle that they block; host HIV restriction factors (pink); and their corresponding viral antagonists (blue). CCR5, CC-chemokine receptor 5; LTR, long terminal repeat; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors. Reproduced and modified from (Barre-Sinoussi et al., 2013).

1.2.3 HIV-1 Pathogenesis

Transmission of HIV-1 is a function of both where the virus appears in the body and how it is shed (Klatt, 2013). HIV-1 can be present in a variety of body fluids and secretions, such as blood, semen, vaginal fluid, pre-seminal fluid or breast milk and as such transmission can occur through sexual intercourse (anal, vaginal or oral), blood transfusion, intravenous drug use as well as mother-to-child transmission (MTCT, further discussed in section 1.3). Worldwide, sexual transmission across the genital mucosa accounts for the majority of cases of HIV-1 infection. Moreover, risk of HIV-1 sexual transmission as well as acquisition is increased when a person has a sexually transmitted disease (STD), as large amounts of HIV can be found in ulcers and the genital fluid of those infected with genital herpes, syphilis, and gonorrhoea (Moss, 2013). In addition, other factors include high viral load (VL) of the index partner, anal vs. vaginal intercourse and lack of male circumcision. Nevertheless, in each of the transmission routes exchange of fluids with either cell-free or cell-associated virus is

necessary for transmission. It has been suggested that cell-associated viruses may be the more efficient mediators of transmission because they can continue releasing virus while penetrating deeply into the recipient's tissues, whilst cell-free virus would need to cross the mucosal barriers of the genital tract as well as gain access to a suitable target cell (Jennes and Kestens, 2014).

Clinically, there are three major phases of adult HIV-1 infection: acute, chronic, and AIDS (**Figure 1.3**). The eclipse phase is the initial stage of the acute infection before systemic viral dissemination. Within the first week, HIV is freely replicating and spreading from the initial site of infection, and viraemia is not yet detectable (Coffin and Swanstrom, 2013). This phase is followed by the acute phase (also known as primary infection) which is characterized by flu-like symptoms with fever, fatigue, muscle aches, nausea and/or diarrhoea (which may persist for 1 to 2 weeks). Acute infection is marked by high viraemia of up to 10^6 copies of viral RNA per millilitre of blood, peaking one to two weeks after infection and severe CD4+ T cell loss. At this time point, the immune response starts to appear in the form of HIV-1 antibodies (known as seroconversion) and cytotoxic T-lymphocytes (CTLs, in the form of CD8+ T cells) that target HIV-1 antigens expressed on infected cells (Coffin and Swanstrom, 2013). During this acute phase of peak viraemia, the high titres of HIV-1 can be detectable in the blood using a p24 antigen test; however, HIV antibody tests (such as enzyme immunoassay) are often negative in the first three weeks (Klatt, 2013). Individuals in this stage of infection are the most infectious because of the high VL in blood and genital secretions, correspondingly, over half of all HIV infections may be transmitted during this period (Shaw and Hunter, 2012).

At the end of the acute phase there is a marked drop in VL with the establishment of a viral set point, likely as the result of partial immune control as well as exhaustion of activated CD4+ T cells. Viral set point has been inversely associated with the rate of disease progression (Mellors et al., 1995). The asymptomatic chronic phase, or the latency period, ranges from 1-10 years and is distinguished by a constant, slow level of viraemia with a gradual decrease in CD4+ T cells (Bashirova et al., 2011). The final stage, AIDS, is characterised by progressive loss of the CD4+ T cells below 200 cells/μl of blood, severe impairment of immune function and high levels of viraemia; consequently, allowing opportunistic infections (such as Candidiasis, cytomegalovirus and herpes simplex virus), neurological complications (AIDS dementia complex), and neoplasms (Kaposi's sarcoma)

that would seldom occur in persons with intact immune function (Hutchinson, 2001). On average, there is a period of 8 to 10 years from initial infection to clinical AIDS in adults, although about 10% of persons will rapidly progress to AIDS in 2 to 3 years following HIV infection (Klatt, 2013). Typically, the survival time of an untreated individual diagnosed with AIDS is approximately 1 year but can vary according to the AIDS defining condition.

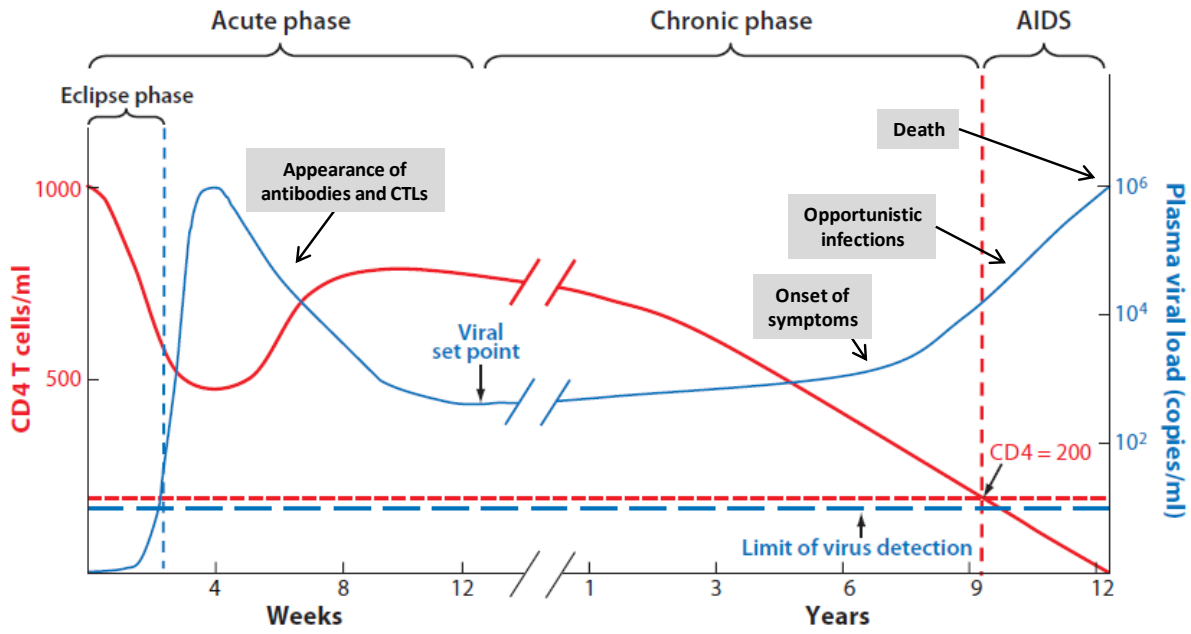


Figure 1.3 The clinical course of adult HIV-1 infection showing the dynamics of peripheral blood CD4+ T cell counts and plasma viral load during a typical course of HIV-1 infection. The three major phases of infection are shown: acute, chronic, and AIDS. Reproduced and modified from (Bashirova et al., 2011).

1.3 Mother-to-child transmission (MTCT) of HIV-1

MTCT of HIV-1, also termed vertical or perinatal transmission, is the spread of HIV-1 from an HIV-1-infected woman to her child during pregnancy (*in utero*), labour and/or delivery (intrapartum), or breastfeeding (post partum) (Ahmad, 1996, Newell, 1998, UNAIDS, 1998). In the absence of intervention, the overall risk of HIV-1 MTCT is approximately 15-30% during pregnancy and/or labour, with an additional risk of 10-20% with prolonged breastfeeding (Teasdale et al., 2011), making MTCT of HIV-1 the major contributor of HIV/AIDS in children. It is estimated that of the 35 million people living with HIV-1, 3.3 million are children (UNAIDS, 2013). Moreover, in 2012, the overall HIV-1 prevalence estimate among antenatal women in South Africa was 29.5% (one of the highest in the world) with 260,280 pregnant women requiring treatment for prevention of mother-to-child transmission (PMTCT) of HIV-1 (National Department of Health, 2012). Without effective

treatment, morbidity and mortality among infants born to HIV-1-positive women is high (Kuhn et al., 2005), and more than half of all infants born with HIV-1 will die before their second birthday (WHO, 2010).

Clinically paediatric HIV-1 infection differs markedly from adult HIV-1 infection. In the weeks following acute infection in adults, plasma viraemia decreases by 100 to 1000-fold and is relatively stable at this 'viral load set-point' for many years; however, during the first few months post-infection, infant plasma viraemia generally increases 10-fold to levels that are much higher than those in adults (Tobin and Aldrovandi, 2013). Disease progression is generally faster with rapid decline of CD4+ T cells and onset of recurrent infections, failure to thrive and delayed neurodevelopment (Muenchhoff et al., 2014). Moreover, the timing and route of infant infection also influences the disease progression; IU infants were reported to have a median survival time of 208 days compared to 380 days for infants infected at birth or > 500 days for infants with postnatal infection (Marinda et al., 2007). Similarly, premature infants delivered prior to 34 weeks gestation were twice as likely to become infected IP or PP through breast milk than full term infants born after 37 weeks of gestation (Fawzi et al., 2001). While the exact reasons for these differences remain unknown it has been suggested that infant immunologic immaturity, high viraemia and/or the rapid expansion of CD4+ T cells that accompany somatic growth drive increased disease progression (Tobin and Aldrovandi, 2013). In agreement with this, peak viraemia was found to be lower in postnatally infected children compared to IP-infected children, suggesting that improved viral control may be due to the more mature immune system (Obimbo et al., 2009, Muenchhoff et al., 2014).

Nevertheless, effective PMTCT and ART have transformed paediatric HIV-1 into a chronic disease. In South Africa, PMTCT guidelines were amended to the World Health Organization (WHO) 2010 guidelines and since then MTCT rates have dropped dramatically to 2.7% in 2011 (Goga et al., 2012). Early virologic suppression due to ART has been associated with normalization of B and T-cell numbers and function as well as enhanced immunologic recovery. Moreover, early ART may also influence the size and half-life of latent-viral reservoirs established early in infection, as is evident in the case of the 'Mississippi Child' who was IU-infected and appeared to have been "functionally cured" after the early initiation of ART (Persaud et al., 2013). In this scenario, the infant initiated ART within 30 h of life and stayed on ART until 18 months of age when ART was

discontinued by the mother. However, in subsequent visits to the clinic half a year later and in the successive follow-up period, VL remained undetectable in the absence of ART and showed no signs of ongoing viral replication or HIV-1 specific T-cell or antibody responses were detected, thus presenting as "functionally cured." Unfortunately though, after > 2 years follow up with undetectable HIV-1 RNA levels in the absence of ART, there was a rebound viraemia in this child. Nevertheless, this case still represents hope that one day novel therapeutic strategies can be developed to target the viral reservoir to potentially achieve drug-free remission. It should be noted that despite the current PMTCT strategies and ART for HIV-1-infected women and their children, in the absence of ART large proportions of infants remained HIV-1-uninfected and appeared to have "natural protection". These exposed uninfected (EU) infants seem to evade infection despite an immature immune system and, in the case of breastfeeding prolonged repetitive, exposure (Tobin and Aldrovandi, 2013). Thus, the study of the mechanisms of natural resistance to HIV-1 in these EU infants might aid in the development of an effective HIV-1 vaccine.

1.3.1 Modes of MTCT

While the exact mechanisms and timing of MTCT remains poorly understood, three stages of MTCT have been defined based on the detection of viral DNA in the infant's blood, namely: *in utero* (IU), intrapartum (IP) and post-partum (PP). HIV-1 detection by DNA PCR in infant's blood collected at birth suggests that transmission occurred prior to delivery and indicates IU transmission, while IP transmission is considered if DNA PCR is negative at birth but positive 6 or more days later in the absence of breastfeeding. However, in breastfed infants the timing of IP or PP transmission can be difficult, although it is generally assumed that PP infected infants are DNA PCR negative at birth but positive approximately 3 to 5 months later within the breastfeeding period (Toth et al., 2001). Knowledge about the timing of transmission and factors involved is crucial to planning of intervention strategies to decrease vertical transmission (John and Kreiss, 1996).

1.3.1.1 *In utero* (IU) transmission

Despite 9 months of exposure at the maternal-foetal interface and evidence of bi-directional cellular trafficking between mother and foetus, only 5–10% of children become IU-infected, demonstrating that IU or transplacental HIV-1 transmission is the least efficient form of MTCT (Tobin and Aldrovandi, 2013). Nevertheless, a number of maternal, infant and viral factors have been found to increase the risk of IU transmission, of which the strongest

evidence for an association includes high maternal VL and the presence of ascending infections with chorioamnionitis (inflammation of the foetal membranes) (Lehman and Farquhar, 2007, Ellington et al., 2011, Tobin and Aldrovandi, 2013). High maternal VL during primary HIV-1 infection has been consistently associated with increased risk of MTCT, especially in the absence of ART (Magder et al., 2005, Taha et al., 2011). In a South African study, women who seroconverted during pregnancy were shown to be 2.3 times more likely to transmit HIV-1 to their infants as opposed to women with established HIV-1 infection (Moodley et al., 2011). Similarly, women acquiring HIV-1 post partum were reported to have a two-fold increased risk of transmitting HIV-1 via breast milk (Dunn and Newell, 1992), suggesting that primary infection increases the risk of transmission. Additionally, co-infections with sexually transmitted infections (STIs), such as syphilis and gonorrhoea, have also been associated with increased risk for IU transmission. These ascending bacterial infections cause inflammation of the placenta, chorion and amnion and possibly allow the migration of HIV-1-infected maternal cells into the amniotic cavity, predisposing infants to HIV-1 acquisition (Chi et al., 2006, Lehman and Farquhar, 2007).

Similarly Kumar et al. (2012) found an association between elevated levels of interleukin (IL)-4, IL-5, IL-6, IL-7, IL-9, eotaxin, IL-1Ra and interferon gamma-induced protein 10 (IP-10) in placental plasma and IU transmission, but not IP transmission. Moreover, IP-10 levels were independently associated with IU MTCT, and this association remained after controlling for maternal CD4+ T cell count and other confounding variables such as maternal age and gestational age (Kumar et al., 2012). Despite these two important factors that contribute to the risk IU transmission, interestingly, infant gender has also been implicated. Several reports found female infants to have a twofold increased risk of HIV-1 infection at birth when compared to male infants (Galli et al., 2005, Taha et al., 2005, Biggar et al., 2006b). It was suggested that Y chromosome-derived antigens, present in male and absent in female foetuses, activate maternal lymphocytes and either cause release of cytokines that have direct antiviral effects or limit maternal HIV-1-infected lymphocyte survival in male infants (Biggar et al., 2006b). Other evidence of IU transmission comes from (1) the early detection (as early as 8 weeks gestation) of HIV-1 in foetal specimens, placental tissue and amniotic fluid, (2) viral isolation from 20-60% of infected infants at the time of birth, (3) the presence of p24 antigen in foetal serum and (4) IU onset of symptomatic HIV-1 disease (Sprecher et al., 1986, Mundy et al., 1987, Rudin et al., 1993, Newell, 1998, UNAIDS, 1998). However, others have suggested that a high proportion of IU infections

occur over the last 1 to 2 months of pregnancy (Chouquet et al., 1999) or in the days just before delivery (Rouzioux et al., 1995, Kourtis et al., 2001, Lehman and Farquhar, 2007). In both cases, infants have detectable virus within the first 48 hours of birth (Bryson et al., 1992).

1.3.1.2 Intrapartum (IP) transmission

In the absence ART, the majority of MTCT occurs at the time of delivery (Tobin and Aldrovandi, 2013). The primary factors that influence IU transmission, such as high maternal VL and chorioamnionitis, have also been associated in IP transmission; however, other factors that have been found to increase IP transmission. These include viral subtype and level of viral shedding in genital tract, the presence of genital ulcers, maternal-foetal microtransfusions (i.e. placental tears) as well as complications during delivery, such as prolonged membrane rupture and cervicovaginal lacerations (Lehman and Farquhar, 2007). IP transmission risk was higher in pregnant women infected with HIV-1 subtype C than were those infected with subtype A or D, because of increased shedding of HIV-1-infected vaginal cells (John-Stewart et al., 2005) as well as mothers having high cervicovaginal VL and the presence of genital ulcers (John et al., 2001). In addition, when there was damage to the placental barrier, maternal–foetal microtransfusions increased the risk for IP MTCT (Kwiek et al., 2006). In each of these, IP transmission likely occurs when there is (1) direct contact of the foetus/infant with infectious maternal blood and genital secretions during passage through the birth canal, (2) through ascending infection from the vagina or cervix to the foetal membranes and amniotic fluid, and/or (3) through absorption in the foetal–neonatal digestive tract (Nielsen et al., 1996).

Early evidence of IP transmission came from the study of twins born to HIV-1 infected mothers which reported that the first born twin had a two-fold higher risk of contracting HIV-1 than the second born twin (Goedert et al., 1991). The authors suggested that the increased risk of HIV-1 transmission in the first born twin was likely due to increased exposure to infected blood and/or mucus in the birth channel, or if surgical delivery followed rupture of the mother's membranes, the first born twin (who would be closer to the uterine opening) would also have greater exposure to material fluids entering the uterus. Therefore, Goedert et al. (1991) proposed that risk of MTCT may be reduced by performing surgical deliveries before membranes rupture. Since then elective caesarean section before the onset of labour and/or membrane rupture has reduced the incidence of IP transmission (The European Collaborative Study, 1994, Kourtis et al., 2001, Teasdale et al., 2011).

1.3.1.3 Post-partum (PP) transmission

While the mechanism of PP transmission is still not completely understood, breast milk HIV-1 transmission, like sexual transmission, depends on (1) breaching an epithelial barrier that limits the amount of HIV-1 in the transmitting fluid, (2) remaining in an infectious form within the secretion, and (3) traversing another mucosal surface to infect a new host (Tobin and Aldrovandi, 2013). More importantly, these are dependent on HIV-1 viral levels in breast milk and the duration of breastfeeding. HIV-1 in breast milk can originate either from blood cell–free virus released into breast milk or can be produced by local replication in macrophages and in ductal and alveolar mammary epithelial cells, as such HIV-1 can be detected both in the cellular compartment of breast milk and in cell-free milk, and is at its highest concentration in the colostrums (Bulterys et al., 2010). In general, breast milk HIV-1 RNA concentrations are 2–3 $\log_{10}$ lower than levels in plasma, ranging from undetectable to greater than 10^5 $\log_{10}$ copies/ml, and are highly correlated with plasma HIV-1 VL (Rousseau et al., 2003a). Additionally, the presence of mastitis (clinical or subclinical) or breast abscess can also increase breast milk virus levels and PP MTCT risk (John et al., 2001).

Without ART, PP transmission accounts for 5-20% of MTCT (WHO et al., 2013), but with extended breastfeeding the risk of PP MTCT increased due to its cumulative nature (Fowler et al., 2012). In high-income countries where clean water is available WHO 2013 guidelines recommend that breastfeeding be avoided completely and infants be given commercial infant formula; in addition, these infants are also treated with once daily nevirapine (NVP) or twice daily azidothymidine (AZT) for 4 to 6 weeks (WHO et al., 2013). However, in low- and middle-income countries (where there is little access to clean water, sanitation and health services), early weaning prior to 6 months was associated with increased infant morbidity and mortality due to diarrheal disease, pneumonia, and other infectious diseases (Kafulafula et al., 2010). Furthermore, abrupt weaning was associated with increased risk of PP transmission if infants resumed breastfeeding after a period of cessation due to elevated levels breast milk VL (Thea et al., 2006). Thus, the WHO in 2013 revised the PMTCT guidelines and encouraged mothers who chose to breastfeed to do so exclusively for 6 months and if they could then safely use breast milk substitutes, wean over several weeks and then stop breastfeeding if suitable breast milk substitutes can be safely used and sustained. Mothers who cannot provide safe breast milk substitutes at 6 months should continue breastfeeding for 12 months or longer, along with providing nutritious locally available complementary foods. During this period, infants are also given once daily NVP for 6 weeks or the duration of

breastfeeding (WHO et al., 2013). In these resource limited settings breast milk provides nutrients and antibodies that protect the infant from childhood diseases; where replacement feeding with unsafe water puts the infant at greater risk for gastrointestinal diseases. Moreover, mixed feeding with the early introduction of non–breast milk foods was associated with a increased risk of transmission, likely due to the delayed closure of the enterocyte junctions in the intestinal mucosal barrier, or, alternatively, from intestinal immune activation resulting from early introduction of foreign antigens or pathogens (Bulterys et al., 2010). Current WHO guidelines now recommended that ART be provided for all pregnant and breastfeeding mothers; thus, breastfeeding is highly recommended in low- and middle-income countries. However, a caveat to this is that there must be 100% adherence to taking the drugs correctly, otherwise there is a risk that the baby can become infected with HIV-1 or become infected with drug-resistant virus (Fogel et al., 2011).

1.3.1.4 The exposed uninfected (EU) infant

Through successful implementation of PMTCT policies, children born to HIV-1 infected mothers are now much less likely to acquire HIV-1 infection and MTCT rates have been reduced from 30% to 2.7% (Afran et al., 2014). However, even in the absence of ART large proportions of infants escape infection and are called exposed-uninfected (EU) infants. These EU infants seem to have "natural protection" and have raised many questions: Why does HIV-1 transmission not occur effectively in every mother-infant pair? What is different in transmitting versus non-transmitting mothers? What is different in highly exposed HIV-1 uninfected children versus those who get infected? (Louvain de Souza et al., 2012).

EU infants are not the only ones who "escape" HIV-1 infection; a number of individuals with vastly different exposure profiles have also been reported, including health care workers with occupational exposure (Pinto et al., 1995), seronegative heterosexual or homosexual partners of subjects with HIV-1 infection (Clerici et al., 1992, Goh et al., 1999), commercial sex workers (CSW) (Fowke et al., 1996, Rowland-Jones et al., 1998, Songok et al., 2012), and intravenous drug users (Scott-Algara et al., 2003). Other terms that have been used to describe these individuals that resist infection despite multiple high risk exposure include: exposed seronegative persons (ESP), highly exposed persistently seronegative (HEPS) or highly exposed seronegative (HESN). Thus, studies of persons who remain uninfected despite extensive exposure to HIV-1 continue to provide valuable information on mechanisms of natural protection, which can then be applied to both novel drug and vaccine

design (Kulkarni et al., 2003). However, despite their escape from HIV-1 infection, there is an increasing body of evidence that EU infants/children suffer immunologic harm both with and without exposure to ART (Tobin and Aldrovandi, 2013). In resource-poor countries, EU children have higher mortality rates than do infants born to HIV-1 uninfected mothers, even when feeding patterns are similar (Newell et al., 2004). Nevertheless, these risks outweigh the mortality risk that is associated with being HIV-1 infected.

1.3.2 Factors involved in MTCT

Many factors have been identified to influence the risk of MTCT of HIV-1 (**Table 1.1**). Chief amongst these is high maternal VL and advanced disease (Kourtis and Bulterys, 2010).

Viral factors

High maternal VL (mVL), measured at delivery, has been described as the strongest risk factor for both IU and IP transmission (Mock et al., 1999). Similarly, in a multivariate Tanzanian study, mVL, CD4+ T cell count and clinical stage of infection were the most significant predictors of transmission, of which a mVL of 50 000 HIV-1 RNA copies/mL or more at delivery was associated with a four-fold increase in the risk MTCT (Fawzi et al., 2001). Correspondingly, higher mVL in the genital tract VL has also been independently associated with a higher risk of MTCT of HIV-1 (Tuomala et al., 2003). Likewise, adult transmission is also dependent on the biologic properties of the virus, its concentration in the exposed body fluid, and the nature of the host susceptibility both at the cellular and immunological levels (Levy, 2009). Furthermore, it has been reported that a strong genetic bottleneck occurs during MTCT of HIV-1. This is evident through population diversity and phylogenetic pattern analysis of the HIV-1 subtype C envelope glycoprotein, where a single viral variant appeared to be responsible for infection in the infants, and as a result the newly transmitted viruses were less diverse and harboured significantly less glycosylated envelope (Zhang et al., 2010a). This suggested that viruses with the restricted glycosylation in envelope glycoprotein appeared to be preferentially transmitted during HIV-1 subtype C perinatal transmission. Nevertheless, transmission of multiple variants have been described.

Table 1.1 Factors associated with risk of MTCT of HIV-1

Factors	Description
Viral	Viral load in plasma, cervicovaginal tract and breast milk
	Viral genotype and phenotype
	Viral resistance to antiretroviral therapy
	HIV-1 co-receptor (tropism)
Maternal	Clinical stage of mothers infection (AIDS)
	Maternal CD4+ T cell count
	Nutritional status (vitamin A deficiency)
	Antiviral treatment
	Maternal immune factors (the level of neutralizing antibodies and cytokines)
	Sexually transmitted diseases (Herpes simplex virus type 2 and syphilis)
	Co-infections (malaria, TB)
	Behavioural factors (cigarette smoking, drug use)
	Cracked or bleeding nipples
	Clinical or subclinical mastitis
	The type of breast milk (colostrums vs. later milk)
	Duration of breast-feeding
Obstetric	Chorioamnionitis and placental pathology
	Prolonged membrane rupture
	Timing of delivery
	Mode of delivery
	Invasive monitoring (amniocentesis) and other obstetric procedures (forceps)
	Duration of labour
Foetal/infant	Genetic factors (mother-infant HLA concordance)
	Background infections
	Birth order
	Birth weight
	Breastfeeding
	Infant gastrointestinal maturity
	Foetal immune response

Reproduced and modified from references (Bryson et al., 1992, The European Collaborative Study, 1994, Ahmad, 1996, Bryson, 1996, Nielsen et al., 1996, Newell, 1998, Garcia et al., 1999, Kuhn et al., 1999, Semba et al., 1999, The European Collaborative Study, 1999, International Perinatal HIV Group, 2001, Rousseau et al., 2003a, John-Stewart et al., 2004, Newell et al., 2004, Chen et al., 2005, John-Stewart et al., 2005, Chi et al., 2006, Kourtis and Bulterys, 2010, Ahmad, 2011, Louvain de Souza et al., 2012).

IU transmitters were more likely to transmit single or multiple major maternal viral variants; whereas, IP transmitters were more likely to transmit minor HIV-1 variants, indicating that different selective pressures may be involved in determining the pattern of maternal HIV-1 variant transmission (Dickover et al., 2001). Similarly in another study, viral sequences from the blood and cervicovaginal fluid from HIV-1 transmitting mothers were compared to those in their infants and showed the presence of more than one HIV-1 variant in the neonate's plasma that derived from the maternal blood and vaginal compartment (Kourtis et al., 2011). This suggested that more than one episode of transmission with more than one viral strain from different maternal compartments occurred, which included both cell-free and cell-associated maternal virus. Other reports have also suggested that the HIV-1 subtype influences MTCT. In a Tanzanian study, subtype C was found to be preferentially transmitted IU when compared to subtypes A and D (Renjifo et al., 2004); while in Kenyan women, MTCT was more common among mothers infected with subtype D compared with subtype A (Yang et al., 2003). However, these findings have not been observed in other population groups (Martinez et al., 2006).

Host/genetic factors

Host factors can be broadly divided into innate and adaptive immune parameters and several components have been identified (**Table 1.2**). Innate factors include the chemokines and chemokine receptors. The β-chemokines CCL3 (macrophage inflammatory protein 1α, MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES, regulated on activation, normal T cell expressed and secreted) are natural ligands for CCR5 and therefore chemokine receptor-ligand interactions represent a barrier to HIV-1 binding to its coreceptor. Both qualitative and quantitative traits in either chemokine receptors or ligands have been described to influence susceptibility to HIV-1 MTCT (Louvain de Souza et al., 2012).

A well-known genetic factor that has received considerable attention over the last decade is the CCR5 locus and its *CCR5Δ*32 (rs333) allele. The 32-bp deletion within the coding region of the CCR5 gene generates a premature stop codon that forms a truncated protein that is not expressed on the cell surface. In this manner *CCR5Δ32* homozygosity has been found to confer near complete resistance to sexual transmission of HIV-1 infection by R5-type HIV-1 isolates (Liu et al., 1996, Samson et al., 1996), as well as protection against MTCT (Philpott et al., 1999). In addition, individuals with at least one copy of *CCR5Δ*32 exhibit improved

resistance relative to wild-type individuals, and if heterozygotes do become infected, they have reduced HIV-1 VL with slowed progression to AIDS by an additional 2–3 years (Zimmerman et al., 1997). However *CCR5Δ32* is rare and has an average allele frequency of 10%, translating into a homozygote frequency of about 1% in Europeans of Caucasian descent, while being virtually absent in African and Asian populations (Martinson et al., 1997).

Additionally, other CCR5 single nucleotide polymorphisms (SNPs) have also been associated with protection against HIV-1 transmission in adults and with delayed progression to AIDS. In a Malawian MTCT study, two CCR5 SNPs, -2459G and -2135T, were found to be protective among infants of mothers with low mVL (Pedersen et al., 2007). The authors proposed that protection might be due to reduced expression of the CCR5 receptor but was dependent on a delicate ratio of virus to receptor, above which changes in the receptor concentration would have no effect on viral infectivity. Conversely, high expression of CC chemokines (the natural ligands for CCR5) in EU infants has suggested that chemokines may possibly have a role in mediating inhibition of MTCT. In fact, copy number variation (CNV) in *CCL3L1* and *CCL4L2* chemokine genes has been linked to HIV-1 susceptibility and possessing a lower copy number of *CCL3L1* (relative to population mean) is associated with increased risk of HIV-1 infection and with HIV-1 disease progression in adults (Gonzalez et al., 2005). *CCL3L1* gene copy number also associated with CCL3 production and with vertical transmission (Meddows-Taylor et al., 2006). Moreover, high *CCL3L1* gene copies in the infant, but not maternal, were associated with reduced HIV transmission (Kuhn et al., 2007), and conversely MTCT was greatest if mother and infant both had low *CCL3L* or *CCL4L* copy numbers (Shostakovich-Koretskaya et al., 2009). More recently, two *CCL3* haplotypes (Hap-A1 and Hap-A3) were found to influence MTCT (Paximadis et al., 2013). The authors reported that Hap-A1 in infants (which also associated with higher *CCL3L* copy number) associated with protection from IU HIV-1 infection, whereas, Hap-A3 in mothers was associated with increased risk of IP transmission. These studies highlight the importance of understanding the gene content and gene copy number in disease susceptibility and/or resistance.

Table 1.2 Genetic variations associated with risk of MTCT of HIV-1

Gene	Polymorphism	Carrier	MTCT risk
CCR5	*CCR5Δ32/CCR5Δ32*	Infant	Decreased
	CCR5/CCR5Δ32	Mother	Decreased
	59029A or 59353T in promoter region	Infant	Increased
CCR2	*CCR2*- 64I	Mother	Decreased
CXCL12 (SDF-1)	3' UTR 801A	Mother	Contradictory
CCL3	Seven-SNP haplotype (Hap-A1)	Infant	Increased
CCL3L1	Reduced gene copy number	Infant	Increased
CCL4L2	Reduced gene copy number	M-I	Increased
HLA-A	*HLA-A*23:01*	Mother	Increased
	*HLA-A*2 (6802)*	Infant	Decreased
	*HLA-A*29*	Infant	Increased
HLA-B	*HLA-B*18*	Infant	Decreased
	*HLA-B*13:02*	Mother	Increased
	*HLA-B*35*	Infant	No effect
	*HLA-B*35:01*	Mother	Increased
	*HLA-B*35:03*	Mother	Increased
	*HLA-B*44:02*	Mother	Increased
	*HLA-B*49:01*	Mother	Decreased
	*HLA-B*50:01*	Mother	Increased
	*HLA-B*53:01*	Mother	Decreased
HLA-C	*HLA-Cw07*	Infant	Increased
	HLA-Cw08	Infant	Increased
TLR9	Haplotype AA and GG of the c.4- 44G/A and c.1635A/G alleles	Infant	Increased
APOBEC3G	rs8177832 A/G	Infant	No effect
	rs17496018 C/T	Infant	No effect
FCGR2A	rs1801274 A/A	Infant	Increased

Reproduced and modified from (Rowland-Jones et al., 1993, Rowland-Jones et al., 1995, Liu et al., 1996, Martinson et al., 1997, MacDonald et al., 1998, Philpott et al., 1999, Kulkarni et al., 2003, Kuhn et al., 2004, Gonzalez et al., 2005, Paximadis et al., 2009, Shostakovich-Koretskaya et al., 2009, Kourtis and Bulterys, 2010, Ahmad, 2011, Ellington et al., 2011, Louvain de Souza et al., 2012).

Immune factors

With regard to adaptive immune factors both humoral and cellular immune responses have been found to play an important part in influencing MTCT. Several studies have correlated the presence of neutralizing antibodies (nAbs) in maternal serum with protection from MTCT of HIV-1 (Scarlatti et al., 1993, Tranchat et al., 1999). Maternal anti-p24 and anti-gp120 antibodies were inversely associated with vertical transmission rates (Pitt et al., 2000). Whilst in another study, IU-transmitting mothers were significantly less likely to have autologous NABS to their own HIV-1 strains at delivery compared to non-transmitting mothers (Dickover et al., 2006). Furthermore, both heteroduplex and phylogenetic analyses showed that there was selective MTCT and/or outgrowth of maternal autologous neutralization escape HIV-1 variants, which indicated that maternal autologous NABS could exert powerful protective and selective effects in perinatal HIV-1 transmission.

With regards to cellular immune responses, a number of studies have identified HIV-1 specific CD4+ and CD8+ T cell responses in HIV-1 exposed but uninfected individuals, with the suggestion that these specific responses are a correlate of immune protection from HIV-1 infection. These HIV-1 specific responses have been observed and characterized in the Pumwani Kenyan cohort of sex workers both at systemic (Rowland-Jones et al., 1998, Alimonti et al., 2006) and mucosal levels (Kaul et al., 2000), in injecting drug users (Makedonas et al., 2002), in men who have sex with men (MSM) (Erickson et al., 2008) as well as EU infants (Rowland-Jones et al., 1993, McFarland et al., 1994, Kuhn et al., 2002). The detection of HIV-1 specific CTLs in ESN individuals thus seems to indicate that HIV-1 has managed to initially infect the host, but that its further propagation has been contained by immune mechanisms and completely eliminated.

In addition, specific *HLA* genes have also been implicated in risk of MTCT. One study found that mothers with *HLA-B* variants (*B*13:02*, *B*35:01*, *B*35:03*, *B*44:02*, *B*50:01*) transmitted HIV-1 to their infant even in the context of low VL, whereas mothers with other variants (*B*49:01, B*53:01*) did not transmit the virus despite high VL (Winchester et al., 2004). Furthermore, since the infant shares at least half of his or her *HLA* genes with the mother, both cell-free and cell-associated HIV-1 virions of maternal origin display maternal HLA, then foetal/newborn anti-HLA antibodies or alloreactive T cell responses could potentially protect against infection from the mother, if there is some degree of HLA discordance between mother and child. Indeed, mother-infant HLA concordance has been

associated with increased risk of MTCT (MacDonald et al., 1998, Polycarpou et al., 2002, Mackelprang et al., 2008). These studies suggested that infants whose HLA matched their mothers may be less able to recognize HIV-1 that has evolved to evade maternal immune responses via HLA-mediated selection, or that matched HLA might decrease the likelihood of infant alloimmune responses against maternally derived lymphocytes in that HLA molecules on the surface of HIV-1 infected or uninfected maternal cells will be recognized as "self" by infant cytotoxic T lymphocytes or NK cells, and will thus be less likely to be destroyed (Mackelprang et al., 2008). In addition, children who were homozygous or who shared both alleles with their mothers at more than one HLA class I locus were more likely to progress to AIDS or death than other children (Kuhn et al., 2004), which suggested the level of mother-infant concordance may compromise the child's capacity to control HIV-1 replication when the virus is acquired from the mother.

Our laboratory has described NK cell responses to HIV-1 Envelope and Regulatory peptides, in a whole blood assay, that were associated with reduced maternal-to-infant HIV-1 transmission (Tiemessen et al., 2009). These responses were significantly more highly represented in non-transmitting mothers and in exposed uninfected infants, and were not detected in uninfected control mothers or their infants. The detection of these HIV-1 specific NK cell responses in EU infants adds to the intriguing possibility that NK cells may possess memory of prior exposure to maternal HIV-1, or mediate these responses through interaction with maternal HIV-1 specific antibodies, a process termed antibody dependent cellular cytotoxicity (ADCC), either or both of which may have an important role in infant resistance to HIV-1 acquisition.

Obstetric factors

Several obstetric factors such as preterm delivery, prolonged membrane rupture and the use of invasive procedures (amniocentesis) have been associated with MTCT. However, elective caesarean section prior to onset of labour and rupture of the amniotic sac has dramatically reduced the risk of MTCT (The European Collaborative Study, 1994, The European Collaborative Study, 1999). This is likely due to the avoidance of microtransfusions of maternal blood to the foetus during labour contractions and of direct contact of the foetus's skin and mucosal membranes with infected secretions or blood in the maternal genital canal. Furthermore, in addition to elective caesarean section, if ART was provided antepartum, intrapartum, and post-partum, risk of MTCT was reduced even further (Kind et al., 1998).

1.3.3 Prevention of MTCT

With increasing knowledge about the underlying mechanisms of MTCT has come an increased emphasis on the search for interventions to prevent or reduce the risk of transmission (UNAIDS, 1998). Consequently, the WHO has promoted a comprehensive approach that includes four PMTCT components: (1) primary prevention of HIV-1 infection, (2) prevention of unintended pregnancies among HIV-1 infected women, (3) prevention of HIV-1 transmission from HIV-1 infected mothers to their infants and (4) care, treatment and support for HIV-1 infected mothers, their children and families (WHO, 2010). Most important has been administration of ART to mothers and their infants. It is estimated that in the absence of ART, 25% of infants infected with HIV-1 progress rapidly to AIDS or death within the first year of life (Teasdale et al., 2011). However, effective ART has reduced the rate MTCT to 2.7%, and where infants are infected, ART has transformed paediatric HIV-1 into a chronic disease (Tobin and Aldrovandi, 2013). ART can reduce MTCT in one or more of the following ways: (1) by reducing viral replication and thus lowering plasma VL in pregnant women, (2) through pre-exposure prophylaxis (PrEP) of babies by crossing the placenta, (3) through post-exposure prophylaxis (PEP) of babies after delivery and (4) through reducing transmission via breast-feeding (Siegfried et al., 2011).

1.3.3.1 Antiretroviral drugs

In the early 1990s, few ART options for HIV-1 infection existed (**Figure 1.4**), and ART largely consisted of monotherapy with zidovudine (ZDV), a nucleoside analog initially called azidothymidine (3'-azido-3'-deoxythymidine) or AZT. Initial HIV-1 treatment with AZT led to a sense of excitement in the medical field arising from the possibility that perhaps HIV-1 could be controlled, but it soon became clear that monotherapy was inadequate for long term viral suppression as HIV-1 with its high rate of replication, the low fidelity of reverse transcription and capacity for recombination lead to an elevated genetic diversity and the development of drug resistant strains (Boulet, 2009). As such treatment evolved over the years to dual therapy and combination therapy, also known as highly active antiretroviral therapy (HAART). To date, six distinct classes have been classified according to their effect on HIV-1 replication: (1) nucleoside reverse transcriptase inhibitors (NRTIs), (2) nucleotide reverse transcriptase inhibitors (NtRTIs), (3) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (4) protease inhibitors (PIs), (5) entry inhibitors (EIs) and (6) integrase inhibitors (INIs), of which the INIs represent the most recent antiviral drug class (De Clercq, 2010).

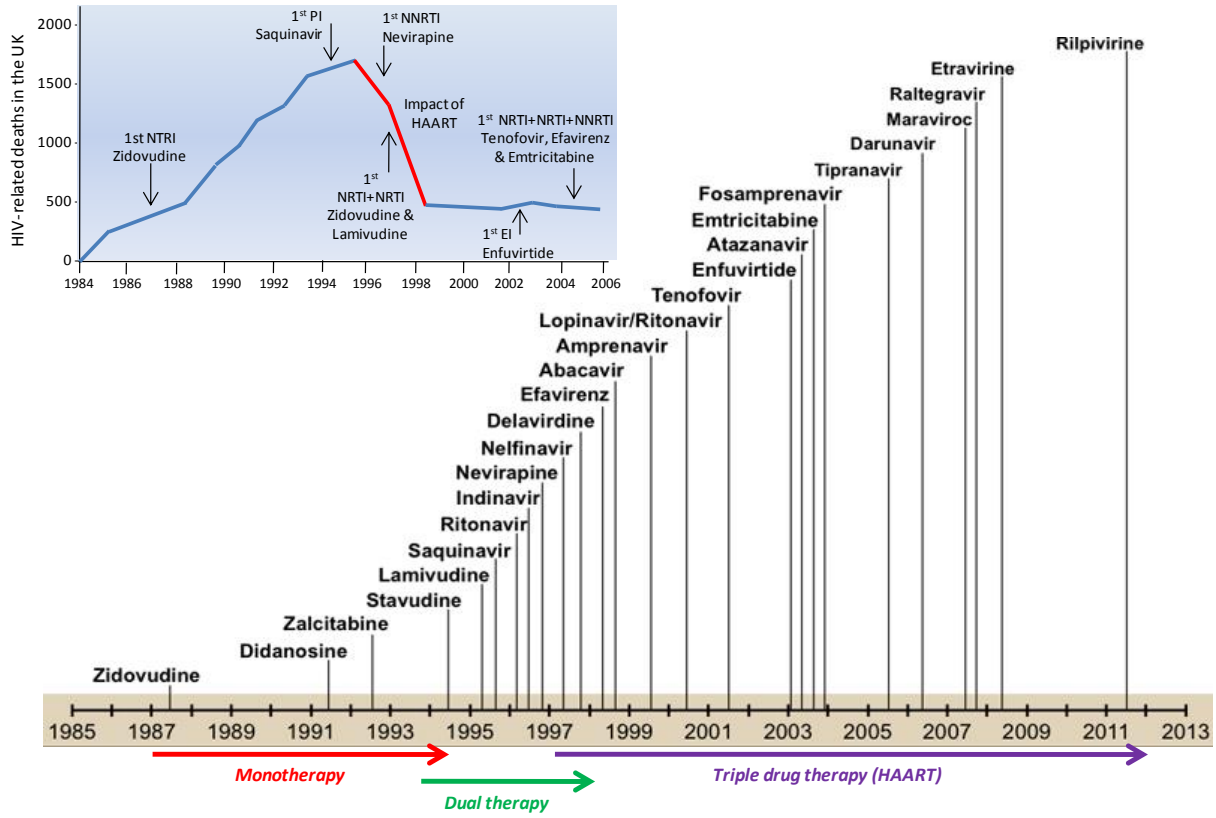


Figure 1.4 A timeline of FDA approved antiretroviral drugs used in the treatment of HIV-1 infection and the impact of these drugs on HIV-related deaths in the UK from 1894-2005. EI, entry inhibitors; HAART, highly active antiretroviral therapy; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor. Reproduced and modified from (Oversteegen et al., 2007) and (http://depts.washington.edu/hivaids/index.html).

Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)

At present, there are seven NRTIs licensed for clinical use in the treatment of HIV-1 infections: zidovudine (ZDV or AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC), and emtricitabine (FTC), as well as one NtRTI (tenofovir disoproxil fumarate, TDF) (De Clercq, 2013). Briefly, both NRTIs and NtRTIs (structural analogs of endogenous 2'-deoxy-nucleosides and –nucleotides but lack the 3'-hydroxyl group required for DNA elongation) act in a similar fashion. In their parent forms they are inactive and require successive phosphorylation steps by host cell kinases and phosphotransferases to form deoxynucleoside triphosphate (dNTP) analogs capable of viral inhibition (Cihlar and Ray, 2010). Following intracellular phosphorylation, NRTIs and NtRTIs compete with the endogenous substrates (dNTPs) for incorporation in the RT reaction where they serve as chain-terminators to prevent further chain linkages and inhibiting proviral synthesis. Thus, N(t)RTIs can be considered as pro-drugs that are active against HIV-1 RT as well as other

retroviral RTs, e.g. HIV-2 and SIV (de Bethune, 2010). The success of NRTIs in antiviral therapy is due, in part, to their intracellular persistence in their active triphosphoraleted form which allows for more constant viral inhibition. Some NRTI metabolites, for example ddI, despite having only a 1.5 h plasma half-life, is administered once a day because its active metabolite, 2',3'-dideoxyadenosine triphosphate (ddATP), has an intracellular half-life of 24 h (Cihlar and Ray, 2010).

Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

In contrast to NRTI and NtRTI, NNRTIs are active and do not require intracellular phosphorylation. NNRTIs prevent viral synthesis by directly binding to the RT enzyme in a hydrophobic pocket which prevents the normal dynamic movement of the enzyme complex (De Clercq, 2013). To date there are four NNRTIs: NVP, delavirdine (DLV), efavirenz (EFV) and etravirine (ETR). Of these four, NVP remains one of the most widely used PMTCT interventions as it is rapidly absorbed when given orally and readily crosses the placenta, it has a long half-life in pregnant women (61–66 h) and infants (45–54 h), its potent antiretroviral activity and because of its low cost (Siegfried et al., 2011). However, use of NVP as monotherapy leads to rapid development of resistant virus, which limits its usefulness when treating HIV-1 infection in the long term. Nevertheless, NNRTIs are highly specific inhibitors of HIV-1.

Protease inhibitors (PIs)

PIs selectively bind to and inhibit HIV-1 protease, the enzyme that cleaves viral Gag and GagPol precursor proteins, consequently inhibiting the viral maturation process (De Clercq, 2013). Currently there are ten PIs approved for the treatment of HIV-1 infections: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir, and darunavir, all of which, with the exception of tipranavir, are competitive inhibitors that mimic the natural substrate of the viral protease (Wensing et al., 2010).

Entry inhibitors (EIs)

Blocking HIV-1 entry into host cells is another pharmacologic strategy and drugs that block HIV-1 entry are collectively known as entry inhibitors. These include a complex group of drugs with multiple mechanisms of action depending on the stage of the entry process at which they act (Tilton and Doms, 2010). In general, EIs can target the different stages of viral entry: attachment and CD4 binding, coreceptor binding, and fusion. To date only two

antagonists have been approved: Maraviroc (a CCR5 antagonist) binds to the CCR5 coreceptor causing a conformational change in the coreceptor that prevents the HIV-1 gp120 from binding, and Enfuvirtide (a fusion inhibitor) which mimics the HIV-1 gp41 peptide, thereby preventing the conformational change needed to form a stable complex required for viral membrane fusion to target cells.

Integrase inhibitors

Lastly, HIV-1 integrase was the most recent HIV-1 enzyme to be successfully targeted for drug development. Raltegravir was the first approved integrase strand transfer inhibitor (InSTI) and specifically targets the final step of the three steps by which viral DNA is inserted in to the cellular genome, thus preventing the insertion of the viral genome into the DNA of the host cell (Hicks and Gulick, 2009). Raltegravir is currently administered in combination with other antiretrovirals for the treatment of HIV-1 infection in treatment-experienced adult patients with multi-drug-resistant HIV-1 strains.

Highly active antiviral therapy (HAART)

In general HAART consists of three or more HIV-1 drugs of which the two NRTIs form the backbone of current combinations in addition to a NNRTI, protease inhibitor or, more recently, integrase inhibitor (Cihlar and Ray, 2010). The first fixed-dose drug combinations used for the treatment of HIV-1 infections were Combivir (AZT+3TC) in 1997; Trizivir (AZT+3TC+ABC) in 2000; Epzicom (3TC+ABC) and Truvada (FTC+TDF) in 2004; Atripla (FTC+TDF+EFV) in 2006; and Complera/Eviplera (FTC+TDF and rilpivirine) in 2011 (De Clercq, 2013). To date, HAART has dramatically suppressed viral replication and the plasma HIV-1 VL to below the limits of detection of the most sensitive clinical assays (< 50 RNA copies/mL) resulting in a significant reconstitution of the immune system as measured by an increase in circulating CD4+ T cells (Arts and Hazuda, 2012).

As with ART therapy, the criteria for the initiation of ART have changed over the years. These criteria include plasma viraemia concentration, absolute or relative CD4+ T cell counts and clinical manifestations of HIV/AIDS (Simon et al., 2006). Historically, ART was initiated when patients presented with clinical signs of AIDS-defining illness or had CD4+ T cell count of ≤ 200 cells/μL (normal values being 500-1200 cells/μL). These individuals were already in advanced HIV-1 disease, and ART at this stage failed to improve CD4+ T cell counts to > 500 cells/μL. It was later reported that the magnitude of CD4+ T cell

recovery directly correlated with CD4+ T cell count at ART initiation (Moore and Keruly, 2007). Consequently, in 2010 the WHO guidelines changed ART initiation from 200 cells/μL to 350 cells/μL, and more recently recommended that HIV therapy start at CD4+ T cell counts less than 500 cells/μL.

Prior to 2010, the early South African PMTCT program included the administration of single-dose (sd) NVP to a mother during labour as well as sdNVP administration to the infant within 48 to 72 hours (National Department of Health, 2008). This was then updated to dual therapy, with a short-course of AZT added to the sdNVP protocol: mothers received daily AZT from 28 weeks gestation as well as sdNVP during labour/delivery, and infants received sdNVP at birth and either 7 or 28 days of AZT depending on whether the mother had received prophylaxis for ≤ 4 weeks (National Department of Health, 2008). In addition, lifelong ART was limited to mothers with CD4+ T cell counts ≤ 200 cells/μL. Subsequently in 2010, South African PMTCT guidelines were updated and aligned with WHO recommendations (WHO, 2009). Pregnant women were offered a single rapid HIV-1 antibody assay at their first antenatal care (ANC) visit. If negative, a second test was offered after 32 weeks gestation. Positive results were confirmed with a second rapid test and CD4+ T cell count dictated further management. Women with CD4+ T cell counts ≤ 350 cells/μL were initiated on lifelong ART and women with CD4+ T cell counts > 350 cells/μL received twice-daily AZT from 14 weeks gestation, with sdNVP, 3-hourly AZT and sd-FTC/TDF during labour. HIV-1 exposed infants received daily-dose (dd) NVP for 6 weeks or throughout the duration breastfeeding if mothers were not on ART (Technau et al., 2014). In addition, HIV-1 exposed infants had an HIV-1 DNA PCR test at their 6-week immunization visit. These guidelines were then further amended in March 2013 and combination ART was offered to all HIV-1 positive pregnant women irrespective of the CD4+ T cell count.

Although several studies reported that monotherapy with short-course AZT or sdNVP reduced the incidence of MTCT by 40% or more (Guay et al., 1999, Wiktor et al., 1999, Stringer et al., 2003), with dual therapy the reduction was more successful (Lallemant et al., 2004). Short-course AZT was found to reduce maternal VL in plasma and genital secretions (Chuachoowong et al., 2000, Mbori-Ngacha et al., 2003), and was also able to cross the placenta as determined by detection of therapeutic levels of AZT in cord blood (Bhadrakom et al., 2000). AZT likely reduced MTCT as a result of reduced maternal VL at delivery, as

well as acting as an infant prophylaxis against viral exposure during birth. However, maternal AZT would not likely reduce PP transmission due to its short half-life of 1–2 hours (Lehman and Farquhar, 2007). In contrast, sdNVP has a long half-life (60 hours in adults and 45 hours in neonates) and sdNVP given to the mother at the onset of labour is rapidly absorbed and crosses the placenta within 1 hour of oral ingestion resulting in levels well above the 100 ng/ml therapeutic target in both maternal breast milk and infant blood during the first few days of life (Musoke et al., 1999). Therefore, it is hypothesised that similar to short-course AZT, sdNVP may act as pre and/or post-exposure prophylaxis to the neonate (**Figure 1.5**).

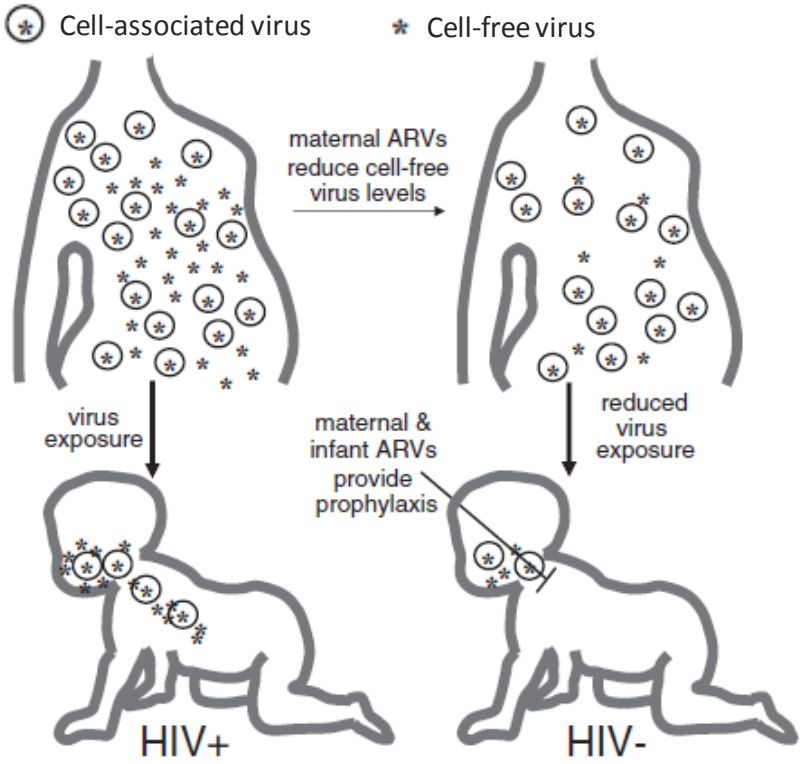


Figure 1.5 Short course antiretrovirals (ARVs) in PMTCT. Left panel: without maternal ARVs, virus ingested by the infant can result in infection. Right panel: ARVs given to the mother substantially reduce maternal viral load thereby reducing viral exposure to the infant. Reproduced from (Lehman and Farquhar, 2007).

In neonates whose mothers did not receive ART during pregnancy, prophylaxis with a two-drug ART regimen (AZT/NVP) or three-drug ART regimen (AZT/3TC/nelfinavir) was found to be far better than AZT alone for the prevention of IP transmission; moreover, the rates of IU transmission were lower with the two-drug (2.2%) and three-drug (2.4%) groups as compared to AZT (4.8%) alone (Nielsen-Saines et al., 2012). Additionally, a 1 week "tail" of 3TC or AZT following the sdNVP would prevent most NVP resistance (Van Dyke et al., 2012).

1.3.3.2 Drug resistance

Drug resistance evolution is a primary phenomenon that helps a pathogen survive and replicate even under harsh drug pressure (Das and Arnold, 2013). In the case of HIV-1, the high mutation rate that occurs during reverse transcription of HIV-1 to proviral DNA enhances the development of antiretroviral drug resistance. Clinical use of AZT or sdNVP revealed that treatment of HIV-1 infection with a single drug was not effective in keeping the VL down for a prolonged period; furthermore, drug resistance increased with the duration of therapy, as multiple amino acid changes accumulate over time to yield resistant viruses (Klatt, 2013). Resistance to NRTIs is mediated by two mechanisms: (1) ATP-dependent pyrophosphorolysis, which is the removal of NRTIs from the 3' end of the nascent chain and reversal of chain termination, and (2) increased discrimination between the native deoxyribonucleotide substrate and the inhibitor (Arts and Hazuda, 2012). Single dose NVP prophylaxis selects for NVP-resistant (NVP-R) variants in a high proportion of women (19-75%) and their infected infants (33–87%) and these variants remain detectable for a year or more (Palmer et al., 2006, Arrive et al., 2007, Martinson et al., 2007, Permar et al., 2013). Moreover, NVP-R variants emerge more frequently and persist longer after exposure to extended NVP prophylaxis; while sdNVP administration increases the risk of virologic failure to subsequent ARV treatment (Permar et al., 2013). Such transmitted drug resistance (TDR) has the potential to limit the response to subsequent treatment and can be transmitted to newly infected individuals. Factors contributing to acquired drug resistance in Africa include the lack of plasma VL monitoring, treatment interruptions due to drug shortages, drug interactions and the use of substandard antiretroviral regimens (Hamers et al., 2013).

Meta-analysis revealed the high prevalence of NVP resistant mutants in mothers and infants who had received sdNVP as compared to mothers and infants who had received sdNVP as well as additional ART, such as AZT+3TC (Arrive et al., 2007). Similarly, in the Kesho Bora randomized study, HIV-1 drug resistance was present in 17.1% of mothers who received sdNVP along with AZT versus 1.4% who received triple ART (Kesho Bora Study and de Vincenzi, 2011). Thus, PMTCT interventions were updated and advocated the use of combined ART to reduce NVP resistance. The WHO proposed a new strategy (Option B+) to treat all HIV-1 infected pregnant women with triple ART for life (WHO, 2012), but no studies have yet investigated the effects on resistance development. Furthermore the feasibility of this plan in low- to middle-income (resource poor) countries is questionable.

Overall, there is a great need to monitor drug resistance. However, very few molecular diagnostic laboratories in the region have the capacity to conduct HIV-1 genotypic resistance testing (Parkin et al., 2012). Nevertheless, other ways to address TDR is to improve national HIV-1 treatment programmes by optimizing their functioning and sustainability, including robust supply chains, access to routine VL monitoring and alternative regimens, strategies to maximize patient retention and enhanced surveillance of drug resistance (Hamers et al., 2013). Importantly, it must be stressed that failure to prevent and/or manage ART resistance can lead to greater evolution of viral resistance that can include cross-resistance that requires more complex antiretroviral regimens that may be less tolerable, making patient adherence more difficult, with a downward spiral to shorter duration of HIV-1 suppression and re-emergence of virologic failure (Klatt, 2013).

1.4 The Immune System

The human immune system has evolved a number of mechanisms to protect the host from a selection of pathogens and/or toxins. A key feature of the immune system is the recognition of "self" from "non-self", and as such host-pathogen or host-toxin discrimination is essential to permit the host to eliminate the threat without damaging its own tissues (Chaplin, 2010). However in the case of HIV-1 infection, the virus is able to overcome the immune system and in the absence of ART, infection is marked by the steady decline of CD4+ T cells and constant immune activation leading to an immunodeficient state. Therefore, by understanding how/why the immune system fails to remove this threat may help to develop more effective treatments. How does the immune system differentiate self from non-self? Overall, the immune system can be divided into two functional categories, namely innate and adaptive immunity.

1.4.1 Innate immunity

Innate immunity, also known as the first line of defence, is hardwired with germline-encoded receptors, such as toll like receptors (TLRs), that recognize either highly conserved structures expressed by large groups of microbes, or common biologic consequences of infection (Turvey and Broide, 2010). It can be further divided into two parts, namely physical and bloodborne barriers. Physical barriers prevent infection from establishing and includes intact skin, vigorous mucociliary clearance mechanisms of the respiratory, gastrointestinal and genitourinary tracts, as well as bacteriolytic lysozyme in tears, saliva, and other secretions (Turvey and Broide, 2010). Bloodborne barriers include haematopoietic cells such as

macrophages, dendritic cells, mast cells, neutrophils, eosinophils, and natural killer (NK) cells (NK cells are further discussed in section 1.4.3). These cells can be simplistically grouped according to their functional characteristics, i.e. neutrophils, monocytes, and macrophages are phagocytic cells that are able to engulf foreign particles; basophils, mast cells, and eosinophils release inflammatory mediators such as chemokines and cytokines that recruit other immune cells to the site of infection; and NK cells are able to mediate direct lysis of target cells with the release granzymes and perforin which induce apoptosis. To augment these cellular defences, innate immunity also has a humoral component that facilitates in the clearance of the infection, which includes complement proteins, lipopolysaccharide (LPS) binding protein, C-reactive protein and other antimicrobial peptides (Turvey and Broide, 2010). Furthermore, the innate immunity plays a central role in activating the adaptive immune response.

1.4.2 Adaptive immunity

Since the adaptive response is encoded by gene elements that somatically rearrange to assemble antigen-binding molecules with unique specificity for foreign structures, there is lag time between antigen exposure and maximal response ranging from hours to days (Chaplin, 2010). A key feature of the adaptive response is its capacity to produce long-lived memory cells that enables the host to mount a more rapid and efficient immune response upon subsequent exposure to the antigen (Warrington et al., 2011). Adaptive immunity can be divided into humoral and cell-mediated responses.

The humoral response involves antibody-producing cells, the B lymphocytes, which arise in the bone marrow (Bonilla and Oettgen, 2010). B cells constitute approximately 15% of peripheral blood lymphocytes and once mature, plasma cells express antigen specific membrane associated B cell antigen-binding receptors (BCR) that are secreted upon antigen binding and activation of the B cell. BCR are dimers of immunoglobulin heavy and light chains and upon secretion, BCR are referred to as antibodies/immunoglobulins (Ig). Based on structure and function, five classes of immunoglobulins exist, namely: IgG1-4, IgA1-2, IgM, IgE, and IgD. IgG is the most abundant class of immunoglobulins, constituting 80% to 85% of the immunoglobulins in the blood and accounting for most of the protective activity against infections. IgM is the largest immunoglobulin and usually exists as a pentamer that is stabilized by a J chain. It is also the first antibody produced during the initial response to antigens and is synthesized early in neonatal life. IgD is found in low concentrations in the

blood, and primarily acts as an antigen receptor on the surface of early B cells. IgE is typically seen in individuals who are having an allergic reaction or in parasitic infections. Antibodies can either act directly or indirectly to protect the host from infection. Directly, antibodies can affect infectious agents or their toxic products by neutralization, agglutination, or precipitation, and indirectly, antibodies activate components of innate resistance, such as complement cascade and phagocytes (Bonilla and Oettgen, 2010).

The cell-mediated immune response is regulated by the major histocompatibility complex (MHC), so named because it is responsible for graft rejection or tissue compatibility. The *MHC* genes encode the human leukocyte antigens (HLAs) on the cell surface. There are two major classes of MHC molecules that differ not only structure but also function within the immune system, namely: (1) MHC class I molecules (MHC I) expressed on all nucleated cells consisting of one membrane-spanning α chain (heavy chain) produced by *MHC* genes, and one β chain (light chain or β2-microglobulin) produced by the β2-microglobulin gene, and (2) MHC class II molecules (MHC II) which consist of two membrane-spanning chains, α and β, of similar size and both produced by *MHC* genes. MHC class II are only expressed on B lymphocytes, activated T lymphocytes, and antigen-presenting cells (APC) such as monocytes, macrophages, Langerhans cells, dendritic cells, endothelium, and epithelial cells.

Naive T lymphocytes (thymocytes) develop in the thymus and undergo positive and negative selection. Cells bearing a T cell receptor (TCR) that recognises self MHC are positively selected in the cortex and pass into the corticomedullary junction. T cells that react with self-antigens are deleted by apoptosis in a process known as negative selection (Chaplin, 2010). The cells that exit are self-tolerant but able to recognise foreign antigen when presented with self MHC. T lymphocytes can be broadly divided into cytotoxic CD8+ T lymphocytes (CTL) cells and CD4+ T helper (Th) cells that control both cell-mediated and humoral immune responses. Naive CD4+ and CD8+ T cells then traffic to lymph nodes where they encounter multiple APCs in the process of education. Mature T cells are activated on interaction of their TCRs with antigenic peptides complexed with MHC molecules (Bonilla and Oettgen, 2010).

TCRs on CD8+ T cells can interact with peptides on all nucleated cells expressing the classical MHC class I (HLA-A, HLA-B, and HLA-C). These MHC class I restricted peptides are generally produced from proteins translated within the cell (endogenous antigens)

encoded either in the host genome, by infecting viruses or other pathogens replicating intracellularly (Parkin and Cohen, 2001). In contrast, the TCRs of CD4+ T cells engage extracellular proteins (exogenous antigens) bearing MHC class II (HLA-DR, HLA-DQ, and HLA-DP). MHC class II molecules are present on APCs and are inducible by innate immune stimuli, including ligands for TLRs. APCs are present in large numbers in the skin and mucosal sites, where they actively sample exogenous proteins by means of phagocytosis or endocytosis. Endogenous antigens complexed with MHC class I molecules activate CD8+ cytotoxic T cells inducing targeted cell death. While these CD8+ T cell responses are highly specific to the cell that they recognise, CD4+ T cell activation leads to production of cytokines which in turn activates a wide range of cells around them.

The CD4+ Th cells can be subdivided functionally by the pattern of cytokines they produce. On stimulation, precursor Th0 lymphocytes become either Th1 or Th2 cells. The difference between these cells is only in the cytokines secreted; they are morphologically indistinguishable (Parkin and Cohen, 2001). However, the response they generate is very different. Th1 cells mainly produce interleukin (IL)-2 and interferon (IFN)-γ. IL-2 induces T cell proliferation (of both CD4+ and CD8+ T cells) and stimulates CD8+ T cell cytotoxicity, while IFN-γ activates macrophages to phagocytise pathogens such as mycobacteria, fungi, and protozoa and induces NK cell cytotoxicity. Th1 cytokines therefore induce mainly a cell-mediated inflammatory response. Conversely, Th2 cells produce IL-4, IL-5, IL-6 and IL-10 which favour antibody production.

Mounting evidence suggests that the early events following HIV-1 infection determine the course of disease progression in such a way that more robust control of viral replication in acute HIV-1 infection results in lower viral set-points and is associated with slower HIV-1 disease progression (Pantaleo et al., 1997, Carrington and Alter, 2012). Furthermore, it has been reported that during acute HIV-1 infection the innate response plays an important part in reducing viral replication even before the induction of adaptive immune responses (Alter et al., 2007b). In particular, the role of NK cells in the control and clearance of HIV-1 infection and other viral infections has become an important area of research.

1.4.3 Natural killer (NK) cells

NK cells are large bone marrow derived lymphocytes that constitute about 10-15% of the peripheral blood lymphocyte population (Farag and Caligiuri, 2006). NK cells are distinct from T and B cells in that they do not express receptors that require somatic gene rearrangements to generate receptor diversity and specificity. Instead, NK cell functions are controlled by a wide array of germline-encoded inhibitory and activating receptors that are expressed in a stochastic, variegated pattern, resulting in many subsets of functionally distinct NK cells (Orr and Lanier, 2010), several of which are specific for HLA class I ligands. Consequently, NK cells represent the major effector cell population of the innate immune system.

Upon recognition of target cells, either viral-infected or tumour cells, NK cells have the ability to respond rapidly in one of three ways: (1) by directed release of the contents of cytolytic granules (perforin and granzymes) onto target cells, (2) by expressing apoptotic ligands (*i.e.* Fas ligand and TNF-related apoptosis-inducing ligand, TRAIL) that engage death receptors on infected cells, and (3) by secreting cytokines, such as IFN-γ, that impede or prevent virus replication (Sowrirajan and Barker, 2011). In addition, NK cells, through their CD16 receptor (FcγRIIIa, a low-affinity receptor for the Fc portion of Ig-G), also engage with antibody-coated cells to induce the antibody-dependent cellular cytotoxicity (ADCC) response. NK cells also express TLRs, including TLR2, TLR3, TLR7/8 and TLR9, which can sense pathogen-associated molecular patterns (Sivori et al., 2004).

The importance of NK cells in controlling viral infection has been shown in individuals who lack or have dysfunctional NK cells that develop fatal viral infections, such as herpes or varicella infections (Biron et al., 1989, Etzioni et al., 2005). More importantly, NK cells have been shown to play an important role in the control and prevention of HIV-1 infection (Fauci et al., 2005, Carrington et al., 2008, Tiemessen et al., 2009, Biron, 2010, Berger and Alter, 2011, Tiemessen et al., 2011, Jost and Altfeld, 2013). Moreover, recent studies have described strategies evolved by HIV-1 to specifically evade NK cell-mediated immune responses, demonstrating that NK cells significantly impact HIV-1 evolution (Alter et al., 2011, Jost and Altfeld, 2012). Added to this, other studies have identified the expansion of pathogen specific NK cells and the generation of long-lasting "memory" cells that persist after cognate antigen encounter (Sun et al., 2009). Other groups have also shown that NK cells contain intrinsic adaptive immune features when primed using inflammatory cytokines

(Cooper et al., 2009). Thus, NK cells are no longer just confined to bridging the gap between the innate and the adaptive immune system, but actively play a crucial part in the adaptive immune response with their ability to mount an enhanced secondary recall response.

Using flow cytometry (**Figure 1.6**), peripheral NK cells have been classically defined as being $CD3^{-}CD56^{+}$, which can be further subdivided into three subsets with distinct functional properties, namely: $CD56^{bright}$, $CD56^{dim}$ and $CD56^{-}$ NK cells. In healthy individuals, over 90% of the peripheral blood NK cells consist of the $CD56^{dim}$ NK cells, which express CD16 and KIRs (Cooper et al., 2001, Caligiuri, 2008). These NK cells have strong cytotoxic activity with a modest ability to secrete cytokines. While in the lymph nodes, the dominant NK cell subset consists of $CD56^{bright}$ cells that lack the expression of CD16 and KIRs; these NK cells have high proliferation potential and are able to secrete large amounts of pro-inflammatory cytokines and thus might have important immunoregulatory functions (Jost and Altfeld, 2013). Additionally, an expanded subset of NK cells that are $CD56^{-}CD16^{+}$ has been reported in HIV-1 infection and other chronic viral infections (Mavilio et al., 2003, Mavilio et al., 2005). These NK cells do not respond to stimulation with HLA class I-devoid target cells or mitogens, despite the expression of KIRs, suggesting that these NK cells represent an exhausted/anergic subset of NK cells (Jost and Altfeld, 2013).

Of note, there also exists a unique set of NK cells found in the uterus, called uterine NK (uNK) cells, which represents up to 70% of the leukocyte population in first trimester deciduas (Moffett-King, 2002). Importantly, unlike their $CD56^{bright}$ peripheral counterparts, uNK cells have been phenotypically defined as being $CD56^{super\ bright}$ $CD16^{-}$ cells that also express KIRs, NKG2C and NKG2E, as well as CD69 and CD94 (Moffett-King, 2002). Although the exact function of uNK cells remains largely unknown, available evidence points to a role in regulating the complex process of placentation. In particular, they are thought to be involved in co-ordinating access of the placental trophoblast cells to the uterine arteries resulting in high conductance vessels, ensuring an adequate supply of oxygen and nutrients to the foetal-placental unit (Colucci et al., 2011). In addition, since the uNK cells can express activating and inhibitory KIRs and are in close proximity to interact with the invading foetal placental cells (or trophoblast cells) that express both maternal and paternal HLA-C allotypes (Chazara et al., 2011), they are likely candidates for fine-tuning the maternal immune responses that might regulate placentation as well as infectious disease.

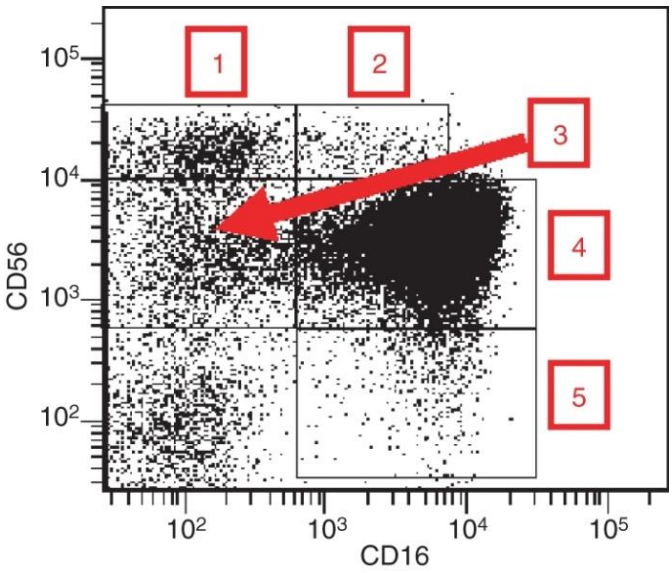


Figure 1.6 Natural killer (NK) cell subsets in human peripheral blood based on the relative expression of CD16 and CD56. Normal peripheral blood mononuclear cells (PBMC) were stained with anti-CD3, anti-CD14, anti-CD16, anti-CD19 and anti-CD56 antibodies conjugated to five different fluorochromes, a gate was set on $CD3^-$ $CD19^-$ cells (NK cells) and CD16 expression vs. CD56 expression is shown: (1) $CD56^{bright}$ $CD16^-$; (2) $CD56^{bright}$ $CD16^{dim}$; (3) $CD56^{dim}$ $CD16^-$; (4) $CD56^{dim}$ $CD16^+$; and (5) $CD56^-$ $CD16^+$. Reproduced from (Poli et al., 2009).

NK cells are regulated by a set of inhibitory and activating cell surface receptors that finely tune their potent effector functions. The NK cell receptors include: (1) killer immunoglobulin-like receptors (KIR), (2) killer cell lectin-like receptors (KLR) such as CD94/NKG2, (3) leukocyte immunoglobulin-like receptors (LILR), and (4) natural cytotoxicity receptors (NCR), such as NKp46, NKp44, and NKp30, and 2B4 (Rajalingam, 2012). These receptors have been shown to recognize both cellular and viral ligands, including major histocompatibility complex (MHC), MHC-like and non-MHC molecules. It is the dynamic interplay between these signals that ultimately determines the outcome of NK cell–target cell encounters (Li and Mariuzza, 2014). Simplistically, NK cell activity is controlled as a consequence of binding to their cognate ligands, NK cell cytolysis is induced when the dominant signal received is activating or NK cell cytolysis is inhibited when the dominant signal received is inhibitory.

Under normal physiologic circumstances, healthy cells express an abundance of inhibitory ligands (HLA-A, -B, -C and –G) and are resistant to NK cell attack (Lanier, 2008, Rajalingam, 2012). However, if HLA class I expression is downregulated by certain viral infections, tumour transformation processes or other forms of stress, the inhibitory signal on NK cell is attenuated and the NK cell undergoes activation with targeted elimination of the

viral-infected or tumour cell (**Figure 1.7**). This process by which NK cell receptors direct the cytolytic activity of NK cells against those cells that have lost HLA expression was originally described as the "missing-self" hypothesis (Ljunggren and Karre, 1990). In addition, NK cell attack may also be induced when there is up-regulation of stress–induced proteins, such as MHC class I chain-related molecules (MICA and MICB) due to viral infection or tumour transformation, termed "induced-self", or when HLA class I molecules present foreign peptide, termed "altered-self", or when pathogen encoded molecules trigger "non-self" recognition (Rajalingam, 2012) .

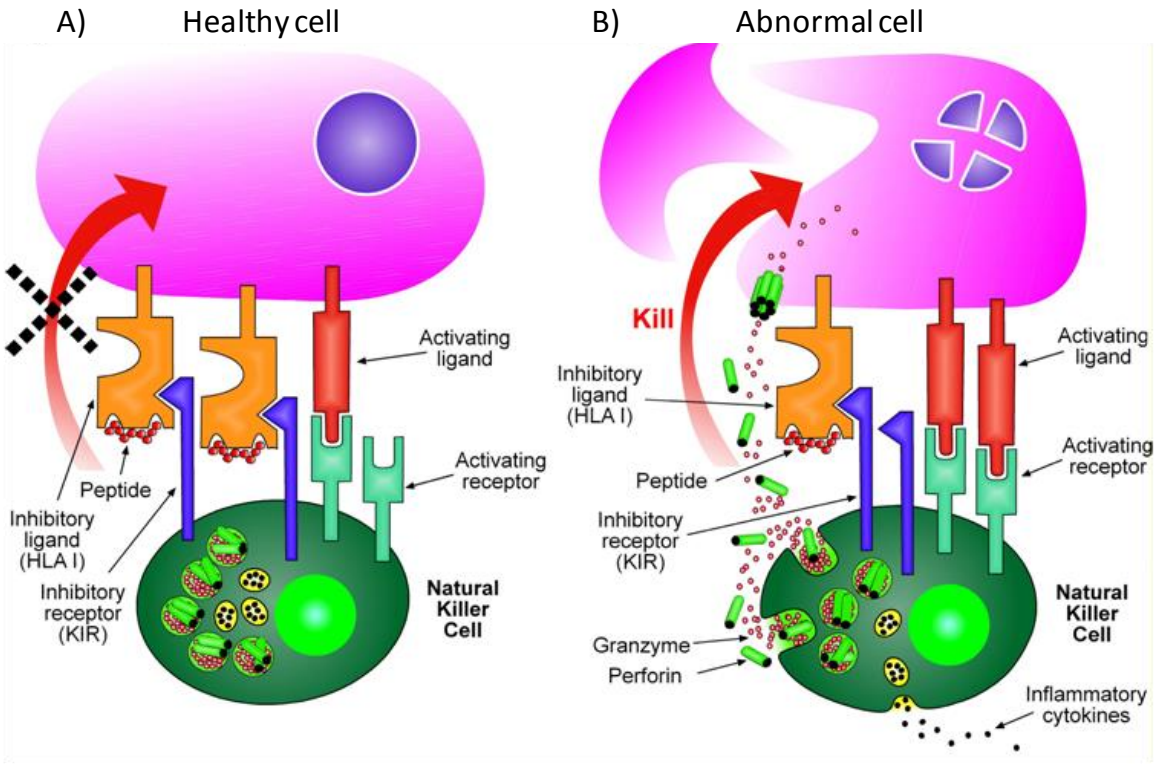


Figure 1.7 NK cell responses against healthy and abnormal cells. (A) By expressing normal levels of multiple HLA class I molecules, the healthy cells are resistant to NK cell attack and (B) down-regulation of HLA class I expression due to viral-infection or tumour transformation relieves the inhibitory influence on NK cells, permitting NK cells to lyse the unhealthy target cell, a process termed "the missing-self" hypothesis. Reproduced and modified from (www.immunopaedia.org).

However, the "missing-self" hypothesis did not fully explain the absence of NK cell autoreactivity in MHC class I deficient cells. Several studies reported that NK cells were self-tolerant and failed to kill MHC-I-deficient cells (Fernandez et al., 2005, Kim et al., 2005). Thus, the "licensing" hypothesis was introduced and proposed, in which NK cells require a process of education to obtain their full effector function (Kim et al., 2005, Raulet

and Vance, 2006). In this model, NK cells had to engage with self-MHC in order to be responsive to subsequent signals received through activating receptors, thus setting or tuning their optimal NK cell reactivity threshold. However, in the absence of self-MHC, NK cells would remain unlicensed and hyporesponsive. This mechanism was then further adapted to the "arming/disarming" model which proposed that the main factor in the adaptation of NK cell reactivity resided in the duration of stimulation. According to this model, an acute downregulation of MHC-I expression would lead to NK cell activation, whereas a chronic downregulation of MHC-I would lead to the chronic activation of NK cells and induce their hyporeactivity (Jaeger and Vivier, 2012). Nevertheless, while these models are suggestive of mechanisms controlling NK cell function, further studies are required to decipher the diversity of the mechanisms regulating NK cell reactivity and to fully understand how NK cell education and/or tolerance is achieved in physiological conditions.

It is also important to note that while every individual will inherit a full complement of NK receptors, not all are expressed on every NK cell in that individual, but every NK cell will have an inhibitory receptor for a self HLA-A, B or C molecule, which can either be a KIR or CD94/NKG2A receptor (Middleton et al., 2002). Moreover, because NK cells vary in the affinity and number of self-MHC-I inhibitory receptors they express, the strength of the educating signal varies from cell to cell (Brodin and Hoglund, 2008). In addition, compared to other NK cell receptors, the KIRs are the most polymorphic and are considered to be the key receptors that control the development and function of human NK cells (Rajalingam, 2012). KIRs recognize an array of polymorphic HLA class I molecules that evolved rapidly to mount effective adaptive immune response against rapidly evolving viruses. Furthermore, the genes encoding KIR and HLA class I ligands are located on different chromosomes (chromosome 19 and 6, respectively) and this independent segregation results in variable KIR-HLA combinations in individuals, which may to some extent explain the differential responses to viral infections across different individuals and/or population groups (Carrington et al., 2008). This thesis will focus on the role of *KIR/HLA-ligands* in MTCT of HIV-1.

1.4.3.1 Killer immunoglobulin-like receptors (KIR)

The KIRs are found on NK cells, but also on subgroups of CD4+ and CD8+ T lymphocytes (van Bergen and Koning, 2010). The *KIR* genes are arranged in a "head-to-tail" cluster encoded in a 100-200 kb region within the Leukocyte Receptor Complex (LRC) on chromosome 19 (19q13.4) (Trowsdale, 2001). Thus far, 14 functional *KIR* genes and 2 pseudogenes have been identified; these encode eight inhibitory receptors (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5A and B, KIR3DL1 and KIR3DL2), six activating molecules (KIR2DS2, KIR2DS3, KIR2DS5, KIR3DS1, KIR2DS1 and KIR2DS4) and the two pseudogenes KIR2DP1and KIR3DP1. As of 15 July 2014, 678 *KIR* alleles and 493 *KIR* genotypes were recorded on the IPD-KIR Database (release 2.5.0, http://www.ebi.ac.uk/ipd/kir/stats.html) and the Allele Frequencies KIR database (http://www.allelefrequencies.net), respectively. Together *KIR* gene content and allelic polymorphism add to the genetic diversity of the *KIR* locus.

The *KIR* genes can have between four to nine exons (**Figure 1.8A**) and based on their structure can be divided into three groups, namely: (1) Type I KIR2D genes, which encode two extra-cellular domain proteins with a D1 and D2 conformation, (2) Type II KIR2D genes which encode two extra-cellular domain proteins with a D0 and D2 conformation, and (3) KIR3D genes encoding proteins with three extra-cellular Ig-like domains (D0, D1 and D2) (Vilches and Parham, 2002). Type I KIR2D genes (*KIR2DL1-3*, *KIR2DS1-5* and *KIR2DP1*) have eight exons as well as a pseudoexon 3. Type II KIR2D genes (*KIR2DL4* and *KIR2DL5*), have a translated exon 3 but have a deleted exon 4. *KIR2DL4* is further differentiated from *KIR2DL5*, in that exon 1 of *KIR2DL4* is six nucleotides longer and possesses an initiation codon different from those present in the other *KIR* genes. KIR3D genes (such as *KIR3DL1*, *KIR3DL2* and *KIR3DS1*) possess nine exons but differ in the length of exon 9. *KIR3DL3* and *KIR3DP1* are unique in that *KIR3DL3* completely lacks exon 6, and *KIR3DP1* completely lacks exons 6 through 9, and occasionally also exon 2. Furthermore, KIRs can either have a long cytoplasmic tail (2DL or 3DL) or a short cytoplasmic tail (2DS or 3DS) (**Figure 1.8B**). Long cytoplasmic tails contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and confer an inhibitory signal when the receptors are engaged with their ligands, while short tailed receptors have a transmembrane lysine residue which allows binding to the immunoreceptor tyrosine-based activating motif (ITAM) containing adaptor molecule DAP12 allowing activation of the NK cell, leading to IFN-γ production, cytotoxicity and proliferation (Rajalingam, 2012).

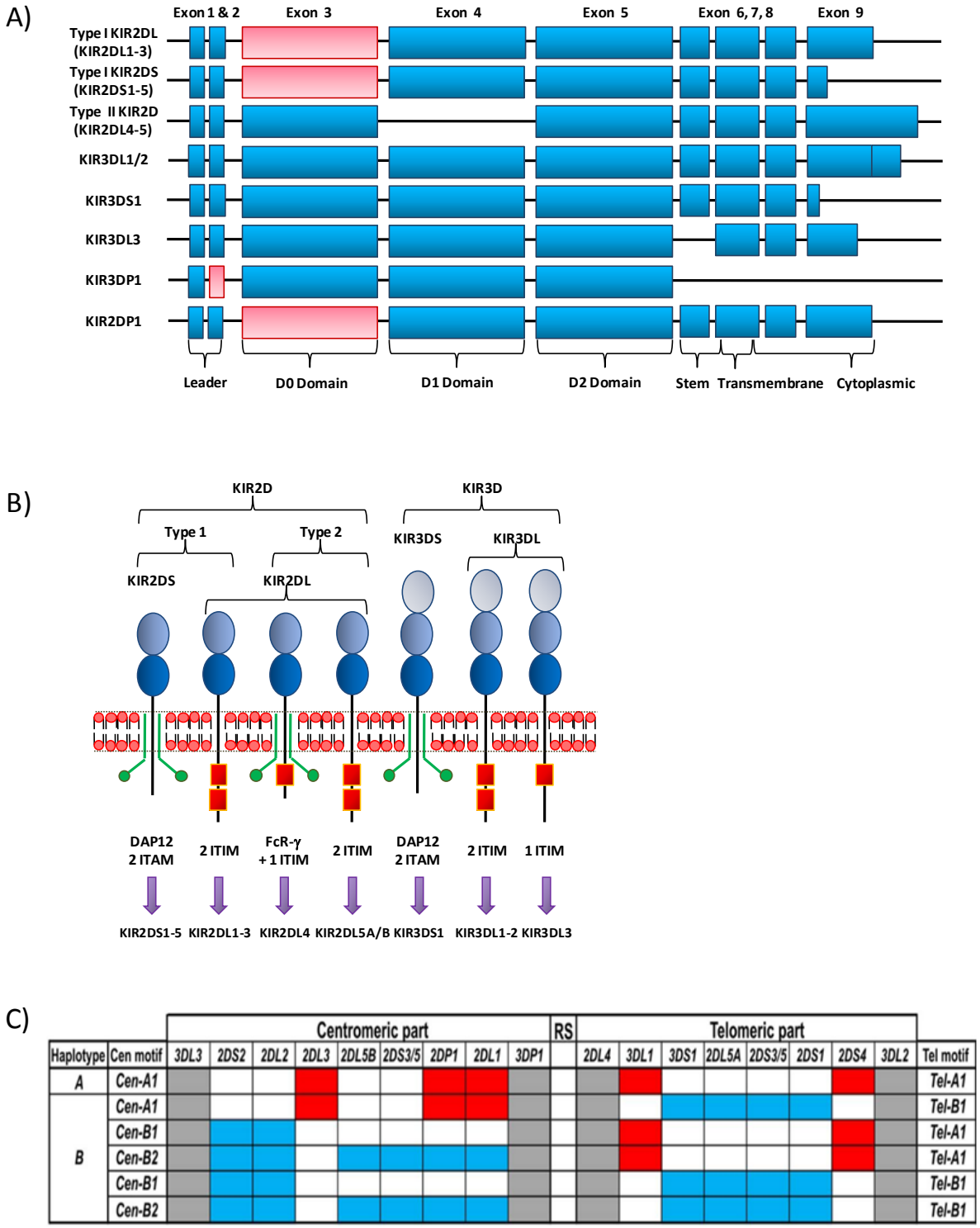


Figure 1.8 Structure of the *KIR* gene groups. (A) *KIR* gene organisation showing coding regions of the exons, represented as blue and the pseudoexon 3 and the deleted exon 2 of *KIR3DP1* are shown in pink. (B) KIR protein structures, which have either two or three Ig-like domains that associate with activating (shown in green) or inhibitory (shown in red) signals. Reproduced modified from (http://www.ebi.ac.uk/ipd/kir). (C) Organization of *KIR* genes in the *KIR* locus, showing the centromeric and telomeric regions which are separated by a unique recombination site (RS). The conserved framework genes are shaded grey, *B* haplotype genes are blue and *A* haplotype genes are red. Reproduced from (Cooley et al., 2010).

Based on *KIR* gene content two broad haplotypes, termed A and B, have been defined (Uhrberg et al., 1997). Haplotype A has a uniform set of nine *KIR* genes that are mostly inhibitory receptors (*KIR2DL1*, *KIR2DL3*, *KIR2DL4*, *KIR2DS4*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3*, *KIR2DP1* and *KIR3DP1*), whereas the haplotype B contains several activating *KIR* genes in addition to inhibitory ones. Therefore, individuals can be assigned to the *A/A* genotype (homozygous for *A* haplotypes) or the *B/x* genotype (having 1 or 2 *B* haplotypes). In addition, the whole *KIR* region can be divided into centromeric (A or B) and telomeric (A or B) halves, based on the framework *KIR* genes *KIR2DL4*, *KIR3DL2*, *KIR3DL3* and *KIR3DP1* present in all haplotypes (Cooley et al., 2010). *KIR3DL3* and *KIR3DP1* mark the centromeric region and *KIR2DL4* and *KIR3DL2* mark the telomeric region (**Figure 1.8C**).

Within the centromeric half, *KIR2DL2* and *KIR2DL3* segregate as alleles of a single locus and can be designated as *KIR2DL2/3*, while *KIR3DL1* and *KIR3DS1* (or *KIR3DL1/S1*) behave as alleles of the same locus within the telomeric half. Virtually all haplotypes will contain either *KIR2DL2* or *KIR2DL3*, and *KIR3DL1* or *KIR3DS1* within their KIR genome (Rajalingam, 2012). The *KIR* genes *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, and *KIR2DS2* are specific to the centromeric half, while *KIR3DL1*, *KIR3DS1*, *KIR2DS1*, and *KIR2DS4* are specific to the telomeric half, and *KIR2DL5*, *KIR2DS3* and *KIR2DS5* are found in both centromeric and telomeric locations. The *KIR* genes show significant linkage disequilibrium within each half, but much less for genes in the two different halves (Middleton et al., 2007b). Group A haplotype diversity is primarily associated with allelic polymorphism, while the group B haplotypes have greater diversity in gene content exhibiting only moderate allelic polymorphism (Biassoni et al., 2012).

For example, *KIR2DS4*, the only activating gene within the A haplotype, can encode functional (KIR2DS4-f) or non-functional (KIR2DS4-v) variants, with only KIR2DS4-f serving as membrane-bound receptors to activate NK cells. To date, 13 *KIR2DS4* alleles have been described (IPD-KIR: Release 2.5.0, 2013-10-11), of which only four *KIR2DS4* alleles (*KIR2DS4*001*, **011*, **014* and **015*) encode cell surface KIR2DS4-f and the remaining nine alleles (*KIR2DS4*003*, **004*, **006*, **007*, *008, **009*, **010*, **012*, **013*) encode the truncated KIR2DS4-v. *KIR2DS4-v* alleles carry a 22-bp deletion in exon 5, which causes a frame shift mutation yielding a truncated KIR2DS4 protein that lacks the transmembrane and cytoplasmic domains of the full length KIR2DS4 protein (Hsu et al., 2002, Maxwell et al.,

2002, Maxwell et al., 2004). Therefore, individuals who are homozygous for the Group-A haplotype and that harbour *KIR2DS4-v* alleles will not have a membrane-bound *KIR2DS4* protein and thus lack an activating KIR. Instead *KIR2DS4-v* alleles encode a soluble form of the protein that is potentially secreted (Middleton et al., 2007a). Genetic studies have shown that frequencies of individuals with zero activating *KIR* genotypes vary between ethnic populations, of which populations of Caucasian decent have been reported to have higher *KIR2DS4-v* frequencies than Africans and Asians. Interestingly, populations with the highest frequency of AA haplotypes show a trend to have lower prevalence of the *KIR2DS4-v* (Maxwell et al., 2004, Whang et al., 2005, Middleton et al., 2007a, Middleton et al., 2007b).

However, amongst the framework gene-encoding KIRs, KIR2DL4 is an unusual KIR because of its structure, expression, and function. Firstly, KIR2DL4 contains one N-terminus ITIM as well as a charged residue (arginine) in its transmembrane domain, allowing KIR2DL4 to have both inhibitory and activating potential (Rajagopalan and Long, 1999, Faure and Long, 2002). Secondly, KIR2DL4 is constitutively expressed by all NK cells whereas other KIRs are rather distributed clonally on some NK cells. Furthermore, in contrast to other KIRs which function as cell surface receptors, KIR2DL4 is localized in endosomes of resting NK cells (Rajagopalan and Long, 2012). Thirdly, intracellular engagement with stored soluble HLA-G ligand has been reported to result in the production of chemokines and cytokines such as IFN-γ, IL-1β, TNF-α, IL-6 and IL-8 that can either directly or indirectly act on other cell types in the local environment (Rajagopalan and Long, 1999, Rajagopalan et al., 2001, Rajagopalan et al., 2006). This intracellular feature may prove advantageous as it may escape regulation by other inhibitory receptors at the NK cell surface. Nevertheless, the exact function of KIR2DL4 remains uncertain.

In addition to allelic and haplotypic diversity, individuals may also carry more than two copies of a *KIR* gene. The copy number variation (CNV) is likely to influence mRNA transcript level and the proportion of an individual's NK cells that expresses the KIR (McErlean et al., 2010). Indeed, CNV in *KIR3DL1* and *KIR3DS1* has been shown to have immunological relevance. For example in HIV-1 infection, individuals with high *KIR3DS1* copy numbers were found to have higher KIR3DS1 expression on NK cells that correlated with more robust inhibition of HIV-1 replication (Pelak et al., 2011), and in the case of hepatitis C virus (HCV), individuals with two copies of *KIR2DL3* had increased HCV clearance compared to those with one or no copies (Khakoo et al., 2004).

1.4.3.2 Human leukocyte antigen (HLA) ligands

Thus far, only HLA class I molecules (HLA-A, B, C and G) have been identified as the ligands for some KIR (**Table 1.3**). Both the KIR and its cognate HLA ligand must be expressed in order to regulate NK cell activity (Biassoni et al., 2012). Since *KIR* and *HLA* genes are located on different chromosomes (number 19 and 6, respectively) and are inherited independently, it is possible that an individual may have KIRs with no HLA ligands and vice versa; consequently, this interaction or lack thereof can affect NK cell function and the susceptibility of an individual to disease (Gaudieri et al., 2005, Farag and Caligiuri, 2006, Kulkarni et al., 2008). The KIRs recognize specific motifs of HLA class I molecules and they bind in a nearly orthogonal orientation across the α1 and α2 helices of HLA class I molecules, which overlaps with, but is distinct from that of the TCR binding site (Boyington et al., 2000). Since the KIR binds to the polymorphic peptide binding groove of HLA class I molecules, it is constantly subjected to changes driven by rapidly evolving viruses and therefore, the KIR binding motifs are often variable between individuals and their distribution differs substantially between populations (Rajalingam, 2012).

Table 1.3 Known KIR molecules and their HLA ligands

KIR	HLA-ligand	Function
2DL1	HLA-C2 (Lys 80) allotypes	Inhibitory
2DL2 and 2DL3	HLA-C1 (Asn 80) allotypes Select HLA-C2 alleles (*C*05:01*, *C*02:02* and *C*04:01*) Select HLA-B alleles (*B*46:01* and *B*73:01*)	Inhibitory
3DL1	HLA-A and HLA-B allotypes expressing Bw4 epitope Select HLA-B alleles (*B*08*, *B*27*, *B*57* and *B*58*) Select HLA-A alleles (*A*24*, *A*23* and *A*32*)	Inhibitory
3DL2	Select HLA-A alleles (*A*03* and *A*11*)	Inhibitory
2DL4	HLA-G	Activating?
2DS1	Putatively HLA-C2 (Lys 80) allotypes	Activating
2DS2	Putatively HLA-C1 (Asn 80) allotypes	Activating
2DS4	Select HLA-C1 alleles (*C*01:02*, *C*14:02* and *C*16:01*) Select HLA-C2 alleles (*C*02:02*, *C*04:01* and *C*05:01*) Select HLA-A alleles (*A*11:01* and *A*11:02*)	Activating
3DS1	Putatively HLA-Bw4?	Activating

Ligands for KIR2DL5, KIR2DS3, KIR2DS5, and KIR3DL3 remain undefined. Reproduced and modified from (Jamil and Khakoo, 2011, Biassoni et al., 2012, Jost and Altfeld, 2013)

HLA-C molecules constitute the dominant ligands for many KIR receptors, and *HLA-C* alleles can be grouped as: C1 or C2, depending on a dimorphism present in the α1 domain at position 80 (Wagtmann et al., 1995, Mandelboim et al., 1996, Mandelboim et al., 1997). Of the HLA-C allotypes currently defined on the IMGT/HLA Database (Robinson et al., 2013b), nearly half of the HLA-C allotypes (*C*02*, *C*04*, *C*05*, *C*06* and *C*15*) carry a lysine (K) at position 80 (80K) and are termed C2, these bind to inhibitory receptor KIR2DL1; the remaining HLA-C allotypes (*C*01*, *C*03*, *C*07* and *C*08*) carry an asparagine (N) at position 80 (80N) and are termed C1, these bind inhibitory receptors KIR2DL2 and KIR2DL3 (Rajalingam, 2012). Additionally, two diverse HLA-B allotypes, B*46:01 and B*73:01 (that have the N80 motifs) as well as several C2 allotypes (notably *C*05:01* and *C*02:02*) can serve as ligands for KIR2DL2/3 (Moesta et al., 2008). Furthermore, from functional analysis and binding assays, Moesta et al. (2008) reported that KIR2DL2 bound with greater affinity to HLA-C1 than KIR2DL3, and thus mediated stronger inhibition of NK cells than KIR2DL3. However, the inhibitory signals triggered by the 2DL2/3-C1 interaction were relatively weaker compared to those triggered by the 2DL1-C2 interaction (Winter et al., 1998). The same group identified that a dimorphism at position 44 in the D1 domain of the KIR determined their ability to discriminate between the two groups of HLA-C allotypes (Winter and Long, 1997).

The activating KIR ligands have not been as thoroughly defined, but KIR2DS1 has similar Ig-like domains to inhibitory KIR2DL1 and also binds HLA-C2, but with reduced avidity (Stewart et al., 2005). The same cannot be said for KIR2DS2, although KIR2DS2 and KIR2DL2 have high homology, KIR2DS2 shows no detectable avidity for HLA-C1 (Winter et al., 1998). The only other activating KIR with defined binding specificity is KIR2DS4, wherein KIR2DS4-f variants have been reported to interact with a select group of HLA-C allotypes carrying the C1 (*C*01:02, C*14:02* or *C*16:01*) or C2 (*C*02:02, C*04:01* or *C*05:01*) epitope (Mandelboim et al., 1996), as well as two HLA-A allotypes, *A*11:01* and *A*11:02* (Graef et al., 2009).

While the binding of KIR2D receptors to HLA-C molecules has been shown to display preferences for certain peptides (Zappacosta et al., 1997), is not clear whether peptide selectivity has a role in NK receptor function. In chronically HIV-1 infected individuals, KIR-associated HIV-1 sequence polymorphisms were found to increase the binding of inhibitory KIRs to CD4+ T cells infected with HIV-1, decreasing the anti-viral activity of

KIR-positive NK cells (Alter et al., 2011). Since the peptide positions recognized by KIRs (i.e. the C-terminal residues P7 and P8 of the HLA-bound peptide) are not usually directly involved in TCR binding site (the central P5 position of the peptide), HLA molecules may be able to evolve their polymorphic regions to present diverse peptides for T cell-mediated immunity, while at the same time maintaining non-variant regions to bind self-peptides for KIR recognition and NK-cell-mediated immune defence (Li and Mariuzza, 2014).

With regards to *HLA-B* alleles, these can be classified as either Bw4 or Bw6 as designated by five variable amino acids spanning positions 77-83 on the HLA class I α1 helix (Cella et al., 1994, Gumperz et al., 1997). Bw4 epitopes have either isoleucine (80I) or threonine (80T) at position 80, and these include the HLA-B alleles: *B*13*, *B*17*, *B*27*, *B*37*, *B*38*, *B*44*, *B*47*, *B*49*, *B*51*, *B*52*, *B*53*, *B*57*, *B*58*, *B*59*, *B*63* and *B*77*, as well as several HLA-A alleles: *A*09*, *A*23*, *A*24*, *A*25* and *A*32*. In addition, Bw4:80T variants can vary with an alanine (81A) or leucine (81L) at position 81 (Muller et al., 1989, Cella et al., 1994). All other *HLA-B* alleles that lack the Bw4 motif are termed Bw6 (80N) and are not known to bind to any KIRs. KIR3DL1 recognize HLA allotypes that contain the Bw4, and have been reported to bind more strongly those with Bw4:80I than to Bw4:80T (Cella et al., 1994, Gumperz et al., 1997), furthermore, affinity seemed to be peptide dependent (Carr et al., 2007). However, certain Bw4:80T allotypes, such as *B*27:05*, have been reported to be better ligands for certain KIR3DL1 subtypes (Luque et al., 1996). As with KIR2DL2 and KIR2DS2, KIR3DL1 and KIR3DS1 share >95% similarity in their extracellular domain, but there is no direct evidence of interactions between KIR3DS1 and Bw4 allotypes; nevertheless, genetic epidemiological, functional and population genetic data strongly support such an interaction (Kulkarni et al., 2008). KIR3DL2 was reported to be specific for *HLA-A*03* and *A*11* allotypes but with a limited ability to inhibit NK-mediated lysis (Dohring et al., 1996).

Another unique KIR/HLA interaction includes KIR2DL4 with the non-classical class I HLA-G (Munz et al., 1997, Rajagopalan and Long, 1999). As mentioned previously, KIR2DL4 has both activating and inhibitory potential and it is one of the framework genes virtually present in all haplotypes and hence this receptor is constitutively expressed by all NK cells (Ivarsson et al., 2014). In addition, unlike all other KIRs, which are expressed on the surface of NK cells, KIR2DL4 as well as soluble forms of HLA-G (sHLA-G) can reside in endosomes and can signal from this intracellular site for a pro-inflammatory and

pro-angiogenic response, using a novel endosomal signalling pathway that involves the serine/threonine kinase cascade involving DNA-dependent protein kinases (DNA-PKcs), Protein kinase B (PKB) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) (Rajagopalan et al., 2001, Rajagopalan et al., 2006, Rajagopalan and Long, 2012). HLA-G differs from the classical class I molecules (HLA-A, -B and –C) in that it has limited allelic variability, several membrane-bound and soluble isoforms, restricted tissue distribution and immune modulatory properties (Donadi et al., 2011, Alegre et al., 2014). HLA-G has been found to be highly expressed on foetal trophoblasts at the maternal-foetal interface and interaction with uNK cells promotes vascularisation of the maternal decidua, implying a role for KIR2DL4 and HLA-G during implantation and placentation (Kikuchi-Maki et al., 2003, Dahl et al., 2014a). Several studies have shown that higher cell surface expression of KIR2DL4 is associated with successful pregnancy (Yan et al., 2007). However, ectopic expression of HLA-G molecules has been observed during conditions, such as viral infection, cancer, transplantation and in inflammatory diseases (Alegre et al., 2014).

Thus, if a specific KIR/HLA combination is important for recognizing a host cell infected by a specific virus, then individuals who have a repertoire with more NK cells expressing that specific receptor may have a better immune response to that specific infection; conversely, NK cells that do not express a receptor for self-MHC class I may be less responsive to pathogen (Jamil and Khakoo, 2011). Therefore, diversity within the KIR system at the level of the locus, allele, expression pattern and HLA-ligand may influence the host immune response to infection. We will now focus on the role of KIR/HLA in HIV-1.

1.5 *KIR/HLA* in HIV-1 infection

By virtue of their expression on NK cells (central components of the innate immune system), the KIR with their HLA ligand interactions are important in the surveillance of viral-infected and tumour transformed cells. In general, some *KIR* haplotypes display a tendency toward more activation and this tendency in combination with the appropriate ligands have been associated with increased risk for inflammatory diseases, but also stronger anti-viral responses (**Figure 1.9**), whilst strong inhibitory haplotypes, such as the AA1 haplotype, have been associated with disorders in pregnancy (Khakoo and Carrington, 2006). Thus, the role of KIR/HLA in disease is dependent on a fine balance between NK cell activation and inhibition.

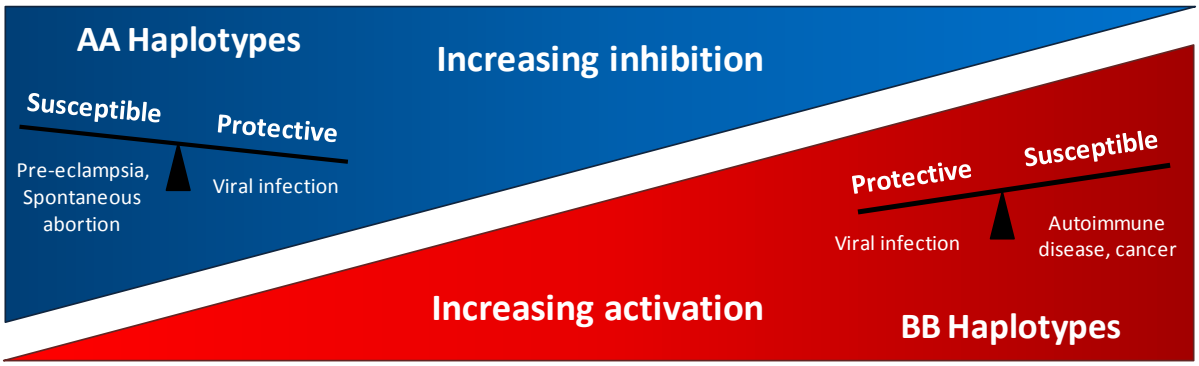


Figure 1.9 A model of KIR disease associations. In this model, *KIR/HLA* genotypes that provide either an 'activating' or 'inhibitory' potential on NK or T cells are associated with differing susceptibility to and protection against a range of diseases. Reproduced and modified from (Khakoo and Carrington, 2006).

During acute HIV-1 infection, NK cells rapidly expand in response to a variety of cytokines prior to the CD8+ T cell response (Alter et al., 2007a, Alter et al., 2009). However, in an effort to escape CD8+ T cell lysis, as well as NK cell lysis, Nef selectively down-regulates HLA-A and HLA-B by triggering their retention in the golgi apparatus, while sparing HLA-C and HLA-E, the ligands for inhibitory receptors, KIR2D and CD94/NKG2A, respectively (Martin and Carrington, 2013). Nef also down-regulates stress-induced molecules, such as MICA which serve as ligand for the activating C-type lectin NK cell receptor, NKG2D (Cerboni et al., 2007). Additionally, during the course of infection the phenotype of peripheral NK cells change, wherein the predominant $CD56^{dim}$ $CD16^{+}$ NK cell population with strong cytolytic potential starts to diminish with appearance of functionally anergic $CD56^{-}$ $CD16^{+}$ NK cells, that express significantly higher levels of inhibitory receptors and lower levels of NCR compared with that of CD56+ NK cells (Jost and Altfeld, 2013, Martin and Carrington, 2013). Similarly other cross-sectional studies of NK cells in chronically HIV-1-infected patients have shown NK cell hypofunctionality (Mavilio et al., 2003, Fauci et al., 2005), which would suggest that the NK cells are unable to lyse autologous targets.

The early events following HIV-1 infection have been reported to determine the course of disease progression in such a way that more robust control of viral replication during acute HIV-1 infection results in lower viral set-point and slower disease progression. Therefore, the early expansion of cytolytic NK cells prior to the adaptive response would suggest that NK cells play an important part in the establishing the viral set-point. In keeping with this, HIV-1 peptide-specific NK cell responses were associated with markers of less severe disease progression among chronically HIV-1-infected women (lower VL and higher CD4+ T cell

counts) as well as stronger HIV-1-specific T cell responses (Tiemessen et al., 2010). In addition, other studies have shown that early expansion of cytolytic $CD56^{dim}$ $CD16^{+}$ NK cells appears to be regulated to some extent by the individual's *KIR/HLA* genotype.

Initial reports documented that there was significant suppression of HIV-1 viraemia and slower HIV-1 disease progression associated with homozygosity for *HLA-Bw4* alleles (Flores-Villanueva et al., 2001). It was later discovered that the *HLA-Bw4* effect on viraemia was associated with NK cells expressing the KIR3DL1/S1. HIV-1-infected individuals expressing KIR3DS1 as well as HLA-Bw4 alleles with an isoleucine in position 80 (HLA Bw4-80I) experienced significantly slower progression to AIDS and lower VL (**Figure 1.10**) as well as protection against opportunistic infections compared to individuals without HLA-Bw4-80I (Martin et al., 2002, Qi et al., 2006, Martin et al., 2007). These findings suggested that KIR3DS1 may bind HLA-B Bw4-80I ligands on HIV-1-infected target cells and delay AIDS progression; thus demonstrating an epistatic interaction between *KIR* and *HLA* in disease association (Bashirova et al., 2011). *In vitro* studies demonstrated that KIR3DS1+ NK cells were able to degranulate more potently in response to HIV-1-infected CD4+ T cells bearing HLA-Bw4:80I (Alter et al., 2007a), and KIR3DS1+ NK cells were highly enriched in the peripheral blood of individuals with acute infection, but only in individuals that had *HLA-Bw4* alleles (Alter et al., 2009).

Moreover, in the context of HIV-1 acquisition, NK cell activity was found to be significantly greater in a group of EU intravenous drug users compared to HIV-1-infected patients and unexposed individuals, and this was associated with significantly higher KIR3DS1 expression in the EU individuals (Scott-Algara et al., 2003, Ravet et al., 2007). Other studies reported that *KIR3DS1* homozygosity is enriched in EU individuals (Boulet et al., 2008b, Guerini et al., 2011), suggesting that KIR3DS1 may also be involved in protection from infection. Guerini et al. (2011) reported that the frequency of the *KIR3DL1* allele and the KIR3DL1+/Bw4+ inhibitory complex was significantly lower, whereas the Bw4+/3DL1-/3DS1+ complex was significantly higher in EU individuals when compared to HIV-1-infected patients, suggesting that the reduced quantity of the KIR3DL1 in conjunction with Bw4 would reduce KIR3DL1-mediated inhibition of NK cells, thereby allowing NK cells to control acute HIV-1 infection in EU individuals. However, the KIR3DS1 protective association is not always evident: one study reported that individuals with *KIR3DS1* and *Bw4-80I* actually exhibited an accelerated progression to AIDS, as did individuals carrying

the B haplotype, of which *KIR3DS1* is associated with (Gaudieri et al., 2005). Furthermore, in a cohort of elite controllers (HIV-1 infected individuals who maintain a clinically undetectable VL without ART) HIV-1 control was not found to be associated with *KIR3DS1/HLA* genotype (O'Connell et al., 2009). Similarly, in another study, lower CD4+ T cell counts were associated with possession of group B haplotypes that lacked specific inhibitory ligands (Jennes et al., 2011).

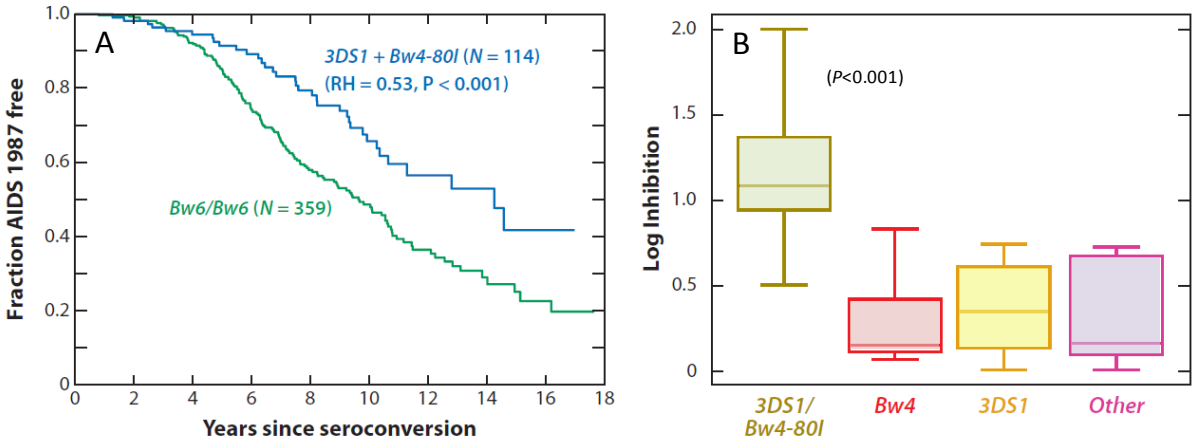


Figure 1.10 The effect of *KIR3DS1* and *HLA-B Bw4-80I* on HIV-1 infection. (A) Kaplan-Meier survival analysis comparing individuals homozygous for *HLA-B Bw6* and individuals with the *KIR3DS1/Bw4-80I* compound genotype in HIV-1 seroconverters. Modified from (Martin et al., 2002). (B) Inhibition of viral replication by NK cells derived from subjects expressing both KIR3DS1 and Bw4-80I, HLA-B Bw4 and no KIR3DS1, KIR3DS1 and no HLA-B Bw4, and neither KIR3DS1 nor HLA-B Bw4. Reproduced and modified from (Alter et al., 2007a, Bashirova et al., 2011).

In contrast, other studies identified that the inhibitory counterpart of KIR3DS1, KIR3DL1, was associated with HIV-1 disease progression. Martin et al. (2007) showed that various distinct allelic combinations of the *KIR3DL1* and *HLA-B* loci significantly and strongly influenced both AIDS progression and plasma HIV-1 RNA abundance. Individuals encoding for HLA-Bw4 and highly expressed *KIR3DL1* allotypes had significantly lower levels of HIV-1 replication as compared to individuals encoding for the same *HLA-Bw4* alleles but *KIR3DL1* allotypes associated with low expression (Martin et al., 2007). The authors suggested that during NK cell development, the higher expressing *KIR3DL1* alleles that exhibited stronger inhibitory capacity would have greater potential for NK cell activation upon interaction with aberrant target cells in the event of viral infection, given that HIV-1 infected cells downregulate cell surface expression of HLA-B. Likewise, when comparing the functional profile of NK cells from individuals carrying different *KIR3DL1* and *HLA-B* allele

combinations, NK cells from individuals with high KIR3DL1 expression and *B*57* (a Bw4:80I allele) exhibited an increased representation of polyfunctional NK cells as compared to other individuals carrying other *Bw4* or Bw6 alleles (Boulet et al., 2010, Kamya et al., 2011).

Other KIR/HLA interactions have also been found to influence HIV-1 transmission. In a cohort of commercial sex workers (CSW), Jennes et al. (2006b) showed that the EU CSW were characterised by *KIR2DL2/KIR2DL3* heterozygosity in the absence of *HLA-C1*, and *KIR3DL1* homozygosity in the absence of *HLA-Bw4*, while HIV-1 infected CSW were characterized by *KIR2DL3* homozygosity together with *HLA-C1* and a trend toward *KIR3DL1/HLA-Bw4* homozygosity (Jennes et al., 2006b). This group further reported that KIR/HLA incompatibility between sexual partners conferred protection against HIV-1 transmission and was possibly due to recipient NK cell-mediated killing of the HIV-1 infected partner's cells, i.e. allorecognition (Jennes et al., 2013). HIV-1 discordant couples were characterized by recipient partners with homozygous *KIR2DL2*, and by a mismatched recipient partner *KIR2DL1/HLA-C2* index partner *HLA-C1/C1* combination expected to allow licensed 'missing self' NK cell killing of index partners' cells. HIV-1 concordant couples on the other hand were characterized by *KIR2DL3* homozygous recipient partners with *HLA-C1/C2* bearing index partners, resulting in a matched KIR/HLA combination expected to inhibit NK cell killing.

Other evidence from Zambian serodiscordant couples showed that carriage of *KIR2DS4-f* was associated with relatively higher HIV-1 VL in index (HIV-1 seroprevalent) partners compared with index partners without the allele (Merino et al., 2011). In addition, carriage of *KIR2DS4-f* was associated with accelerated transmission of HIV-1 to cohabiting seronegative partners. This suggested that *KIR2DS4-f* might increase the likelihood of HIV-1 transmission. Furthermore, the same group observed that in chronically infected HIV-1 positive individuals with NK cells that did or did not express KIR2DS4 often differed in their functional properties, especially in terms of degranulation (production of CD107a) and/or induction of IFN-γ and MIP-1β (Merino et al., 2014). They observed that after stimulation with HLA-deficient K562 cells, NK cells that were positive for the *KIR2DS4-f* genotype (designated g+) but negative for the membrane-bound KIR2DS4 staining (designated p−) universally behaved like those that were negative for both (g−/p−), while polyfunctional NK cells co-expressing CD107a, IFN-γ and MIP-1β were highly

enriched in the g+/p+ NK cells when compared with the g+p− and g−p− NK cells. The authors postulated that prolonged or excessive NK cell activation (as seen in chronic HIV-1 infection) may fuel inflammation and promote HIV-1 pathogenesis in two possible ways: firstly, over-activation of NK cells created a pro-inflammatory environment promoting HIV-1 replication, or secondly NK cell activation promoted early senescence of NK cells with subsequent loss of viral control (Merino et al., 2014).

Regarding the ligand for KIR2DL4, several studies have shown specific *HLA-G* polymorphisms to be associated with altered risk of HIV-1 infection (Matte et al., 2004, Lajoie et al., 2010, Segat et al., 2010). In Zimbabwean women, Matte et al (2004), reported that null allele, *G*01:05N*, significantly associated with protection from HIV-1 infection, whereas, *G*01:01:08* was associated with a 2.5 fold increased risk of HIV-1 infection. Conversely, in HIV-1 infected Caucasians from North Eastern Italy, Segat et al (2010) reported that *G*01:05N* correlated with increased risk for HIV-1 infection. Other work done by Lajoie et al (2010) on HIV-1 infected and uninfected female commercial sex workers (CSW) from Benin demonstrated that HIV-1 infected CSW had significantly lower levels of plasma sHLA-G compared with those in both the HIV-1-uninfected CSW and the non-CSW groups. In addition, they also found that the presence of *G*01:01:01*, and *G*01:04:04* alleles were significantly associated with lower plasma sHLA-G levels in the HIV-1-infected CSWs. Nevertheless, while these studies seem to disagree, they do provide evidence that modulation of HLA-G expression may contribute to the risk of HIV-1 infection.

1.5 *KIR/HLA* genotypes in MTCT of HIV-1

With regards to the role of KIR and HLA ligands in MTCT of HIV-1, very little is known and to our knowledge we are the only group to have assessed *KIR/HLA* gene combinations in MTCT of HIV-1. Initial findings showed that mothers and infants who were less likely to transmit and/or acquire HIV-1 infection, respectively, had CD3-negative cells that were able to responded to HIV-1 peptides (Tiemessen et al., 2009). Using a whole blood intracellular cytokine staining (ICS) assay measuring both IFN-γ and IL-2 production, CD3-negative cells from HIV-1 non-transmitting (NT) mothers were found to respond with remarkable specificity to HIV-1 peptides from Env and Reg protein regions when compared to NK cells from the HIV-1 transmitting (TR) mothers; moreover, these cells from EU infants of NT 'responder' mothers were also able to respond to HIV-1 peptides (**Figure 1.11**).

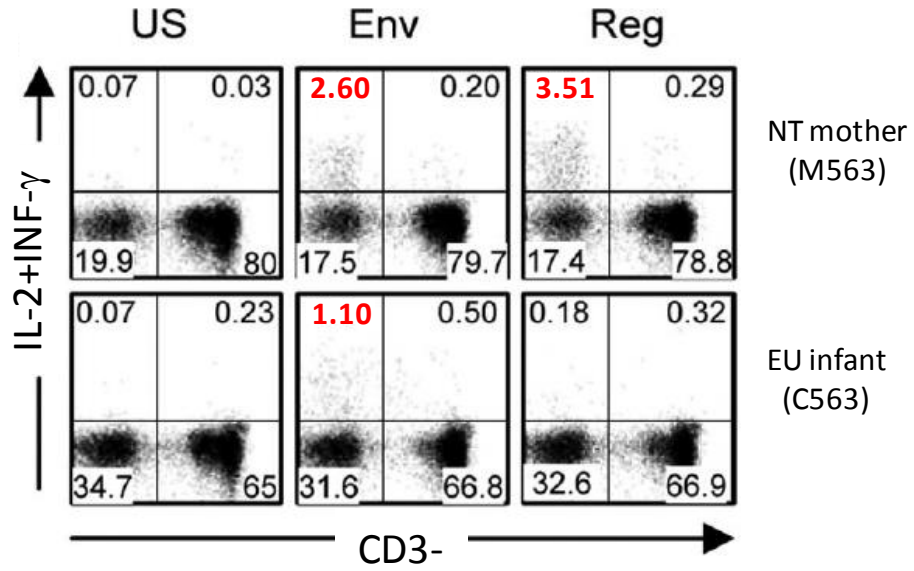


Figure 1.11 Representative flow cytometric plots showing unstimulated (US) and Env or Reg peptide-stimulated, CD3-negative response in a non-transmitting (NT) mother (top row) as well as her exposed uninfected (EU) infant's response (bottom row). The *x*-axis shows anti-CD3-allophycocyanin and the *y*-axis shows anti-IL-2-PE plus anti-IFN-γ-PE (anti-IL-2 was included for the detection of T cell responses, however, IL-2 is not produced by NK cells). Percentages in red indicate positive CD3-negative responses in the relevant quadrant. Reproduced from (Tiemessen et al., 2009).

Through further flow cytometric analyses and review of the above results, the authors inferred that these HIV-1-specific CD3-negative cells were NK cells based on the following criteria: (1) the responding CD3-negative subsets did not contain B cell (CD19) or monocyte markers (CD14) and gated monocytes did not produce IFN-γ in the ICS assay, (2) CD3-negative cell subset responses all correlate positively and significantly when comparing the same protein region responses across the CD8+ and CD8- subsets, with or without the NK cell marker (CD56), suggesting that it was highly likely these CD8+ and CD8- subsets were from the same cell type that respond similarly to specific peptides, (3) early activation marker (CD69) which correlates with cytotoxic activity was expressed on all HIV-1-specific IFN-γ expressing CD3-negative cells, (4) where anti-CD56 was included in the staining panel, CD56+ cells were $CD56^{dim}$, suggesting that they are cytotoxic NK cells and capable of perforin and granzymes production, and lastly (5) IFN-γ-producing CD3-negative cells also expressed NKp46, an NK cell receptor present on all NK cells (Tiemessen et al., 2009). Thus, the data suggested that HIV-1 exposure took place in the infants of HIV-1-infected mothers and that the immune responses mounted by NK cells in the EU infant potentially contributed to protection from infection. These specific NK cell responses to HIV-1 peptides lead the authors to further postulate that these "responders" would possess a

more activating *KIR* gene profile and/or particular KIR/HLA combinations that would explain the ability of their NK cells to overcome inhibitory signals and so be able to mount NK cell responses in the presence of HIV-1 peptides (Tiemessen et al., 2009). Therefore, Tiemessen et al. (2011) undertook to evaluate the relationship of NK cell responsiveness of the particular *KIR* gene repertoires and HLA-ligands in HIV-1 infected mothers and their infants who did/did not respond to HIV-1 peptides. It was shown that maternal NK cell responses to HIV-1 Env peptides were more likely to occur amongst individuals with at least one *C1* allele together with a *KIR* genotype supporting a more activated NK cell phenotype (Tiemessen et al., 2011). Evidence of the C1 association with HIV-1 peptide-specific NK cell responses highlighted the importance of its KIR partners KIR2DL2, KIR2DL3, and KIR2DS2. Thus, correspondingly, it was the combination of *KIR2DL3* plus *HLA-C1* (the presence of its ligand) that showed a trend towards increased representation in the responders, and homozygous *KIR2DL3+C2/C2* (the absence of its ligand) that showed a trend towards increased representation in the non-responders.

More in-depth analysis of mother and infant *KIR/HLA* genotypes revealed that *KIR2DL2* and *KIR2DL3* as well as their HLA-C ligands emerged as important factors in both maternal transmission and infant acquisition of HIV-1 (Paximadis et al., 2011). By comparing TR mothers to NT mothers, it was also shown that *KIR* haplotypes, Bx32 and Bx20, had opposing effects on maternal transmission of HIV-1, wherein Bx32 was associated with increased risk for transmission (particularly IP transmission) and Bx20 was associated with decreased risk for transmission. Examination of these two *KIR* genotypes showed that the only difference between them was the presence of *KIR2DL2* in Bx20. In addition, representation of *KIR2DL2* as well as *KIR2DL2/KIR2DL3* was higher in NT mothers compared to IP-TR mothers (P=0.053, OR=0.43 and P=0.008, OR=0.29, respectively), whilst *KIR2DL2* homozygosity in combination with C1/C1 was significantly higher in TR mothers compared to NT mothers (P=0.041, OR=5.36). Therefore, suggesting both *KIR2DL2* and *KIR2DL3* where important factors in vertical transmission of HIV-1.

Furthermore, it was found that maternal possession of *KIR2DL3* homozygosity alone and in combination with C1/C2 allotypes was associated with increased risk for IP transmission (P=0.034, OR=2.42 and P=0.010, OR=3.63, respectively). However in infants, *KIR2DL2* homozygosity was higher in EU infants compared to IP infected (P=0.052, OR=0.16) and representation of *KIR2DL3* homozygosity in combination with C1/C2 ligand was

significantly higher in EU infants compared to HIV-1 infected infants (*P*=0.038, OR=0.40). The possession of *KIR2DL3*/*KIR2DL3+C1/C2* in mothers and infants was only shown to associate with protection in infants when the genotype was absent in the mother. Comparison of concordance and discordance for *KIR2DL3*/*KIR2DL3+C1/C2* between mother and infant was noticeably different in EU infants compared to the total HIV-1-infected (INF) and IP infected infants (**Figure 1.12**). In IP infants the frequency of *KIR2DL3*/*KIR2DL3+C1/C2* genotype concordance between mother and infant was greater than that of the EU infants (Paximadis et al., 2011). These findings suggested that the risk of vertical transmission, particularly IP-transmission, is in part dependent of the *KIR/HLA* gene combination across mothers and infants. Moreover, the data also suggested that EU protection was associated with the presence of the weaker inhibitory *KIR2DL3* (Moesta et al., 2008) in combination with at least one C1 ligand which would have a lower threshold for NK cell activation than the *KIR2DL2/KIR2DL2* with C1/C1 combination, and thus homozygous *KIR2DL3* NK cells may be more competent in targeting HIV-1-infected maternal cells.

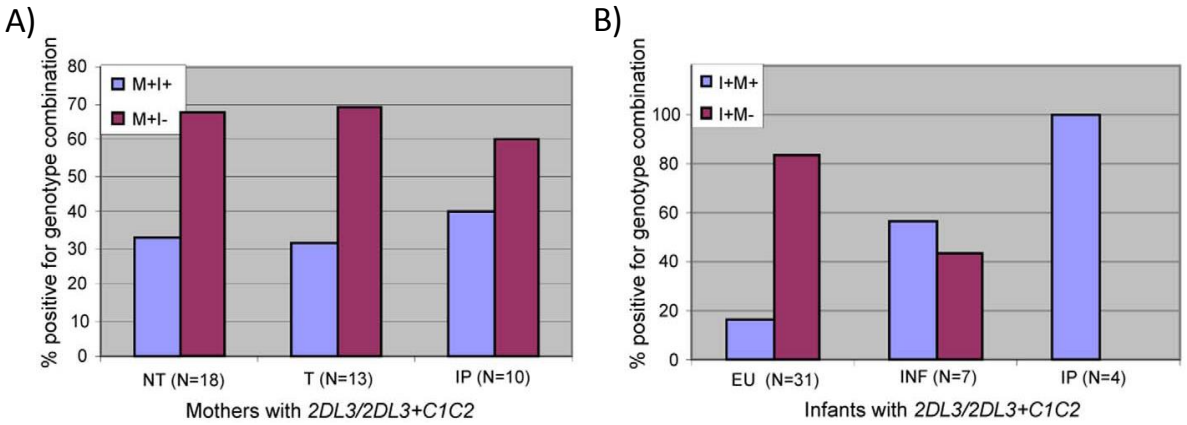


Figure 1.12 Maternal-infant concordance (M+I+) and discordance (M+I- or M-I+) with respect to *KIR2DL3/KIR2DL3+C1C2*. (A) The percentage of maternal subgroups all harbouring *KIR2DL3/KIR2DL3+C1C2* that are concordant (M+I+) and discordant (M+I-) with their respective infants for the *KIR2DL3/KIR2DL3+C1C2* genotype. (B) The percentage of infant subgroups all harbouring KIR2DL3/KIR2DL3+C1C2 that are concordant (I+M+) and discordant (I+M-) with their respective mothers for the *KIR2DL3/KIR2DL3+C1C2* genotype. M: Mother; I: Infant; +: positive; -: negative; NT: non-transmitting mothers; T: total transmitting mothers; IP: intrapartum-transmitting mothers. EU: exposed-uninfected infants; INF: total infected infants; IP: infants infected through the intrapartum route. Reproduced from (Paximadis et al., 2011).

Overall, the findings by Tiemessen et al. (2009, 2010, and 2011) and Paximadis et al. (2011) highlighted that both variation at the *KIR* locus, *KIR* genotype and ligand dosage impact on the ability of NK cells to respond to HIV-1 peptides and thus could influence both maternal transmission as well as infant acquisition of HIV-1. Moreover, *KIR/HLA* gene combinations

favouring NK cell activation over inhibition may be beneficial in the prevention of MTCT of HIV-1. However, it was emphasized by Paximadis et al. (2011) that a number areas within *KIR/HLA* analysis required further investigation to elucidate the exact role of *KIR/HLA* on MTCT of HIV-1. These included: (1) mother-infant *KIR/HLA* genotype concordance, (2) infant NK cell allorecognition of infected maternal cells, (3) *KIR* allelic variation, and (4) the role of HLA-G, the ligand for KIR2DL4, given that HLA-G is localized at the maternal–foetal interface and has an important role in regulating maternal–foetal immunity. For this reason, this thesis aimed to further explore these areas in two mother-infant cohorts that had different PMTCT therapies.

1.6 Study Rationale

It has been more than 30 years since the discovery of HIV-1 and to date there is still no preventative vaccine for HIV-1. This highlights the need to more thoroughly understand what constitutes an effective immune response against the virus. In particular, studying the immune response in those individuals who have been exposed to HIV-1 but remain uninfected (EU) may offer key insights into what might qualify as protective responses. Using the mother-to-child transmission model provides us the unique opportunity to study correlates of protection in EU infants. Importantly, having both mother (transmitter and non-transmitter) and infant (infected and uninfected) provides insight into which immunological factors contribute to HIV-1 transmission and acquisition. Evidence indicates that NK cells through their KIR and HLA-ligand interactions have a role to play in the control of adult HIV-1 infection; however, little is known about the role of *KIR/HLA* genes in vertical transmission. Using the mother-infant transmission model, Paximadis et al. (2011) suggested that certain inhibitory *KIR/HLA-ligand* gene combinations alter the risk of maternal HIV-1 transmission as well as infant susceptibility to HIV-1 acquisition. The authors emphasized the need to further explore the relationship of *KIR* and *HLA-ligands* amongst mother-infant pairs, the effect of specific *KIR* alleles and other HLA-ligands in MTCT of HIV-1. Thus, we set out to evaluate whether specific *KIR/HLA* gene combinations would equally associate with risk of transmission in two distinct mother-infant cohorts characterised by differing ART regimens and different rates of HIV-1 transmission. The overall purpose was therefore to gain better insight into the role of immunogenetic mechanisms of *KIR* and *HLA* in MTCT of HIV-1 in the context of changing PMTCT ART therapies. Knowledge of these mechanisms may ultimately assist in designing strategies for vaccination and therapy.

1.7 Study objectives

The overall purpose of this study was to identify immunogenetic factors of *KIR* and *HLA* that associate with MTCT of HIV-1. To address this, two mother-infant cohorts (MTCT1 and MTCT2) distinguished by differing ART regimens and transmission rates were studied. In MTCT1 (N=217) the overall HIV-1 transmission rate was 9.8% and approximately half the mothers received sdNVP, whilst in MTCT2 (N=79) the overall HIV-1 transmission rate was 5.2%, more than half the mothers received dual ART consisting of short course AZT as well as sdNVP at the onset of labour.

The specific objectives were:

1. To develop and validate in-house real-time PCR *KIR* and *HLA-ligand* genotyping methods and compare with conventional *KIR* and *HLA* Class 1 genotyping methods for the purpose of reducing cost, time and sample requirement, while maintaining specificity and sensitivity.

2. To determine whether *KIR/HLA-ligand* combinations of mothers and infants associate with resistance and/or susceptibility to vertical transmission of HIV-1.

3. To determine whether functional or non-functional allelic variants of *KIR2DS4* (*KIR2DS4-f* and *KIR2DS4-v*, respectively) associate with resistance or susceptibility to vertical transmission of HIV-1.

4. To determine the association between *HLA-G* (alleles and 3'UTR polymorphisms) and resistance or susceptibility to vertical transmission of HIV-1.

5. To establish if immunogenetic relationships (*KIR/HLA-ligands*, *KIR2DS4* and *HLA-G* genotypes) found in MTCT1 are maintained with increased ART exposure in MTCT2.

CHAPTER 2

Materials and Methods

2.1 Study Cohorts

This thesis describes the findings from the study of multiple mother-child cohorts, recruited from 1996 onwards (**Figure 2.1**). Selections of samples from four earlier cohorts were combined into one large cohort for analyses, and for the purpose of this thesis are termed "MTCT1". Numerous publications have resulted from studies of the individual cohorts, sub-cohorts within these as well as the greater cohort, MTCT1 (Kuhn et al., 2001, Kuhn et al., 2002, Petra_Study, 2002, Shalekoff et al., 2004, Gray et al., 2005, Meddows-Taylor et al., 2006, Schramm et al., 2006, Kuhn et al., 2007, Schramm et al., 2007, Tiemessen and Kuhn, 2007, Shalekoff et al., 2009a, Shalekoff et al., 2009b, Tiemessen et al., 2009, Schramm et al., 2010, Paximadis et al., 2011). In addition, a fifth cohort, termed "MTCT2" was recruited several years later at a time when the primary ART consisted of dual therapy regimens; likewise several publications have resulted from the study of this cohort (Lilian et al., 2012, Lilian et al., 2013, Lilian et al., 2014). Data generated from these two cohorts (MTCT1 and MTCT2) are presented in **Chapters 4, 5 and 6**.

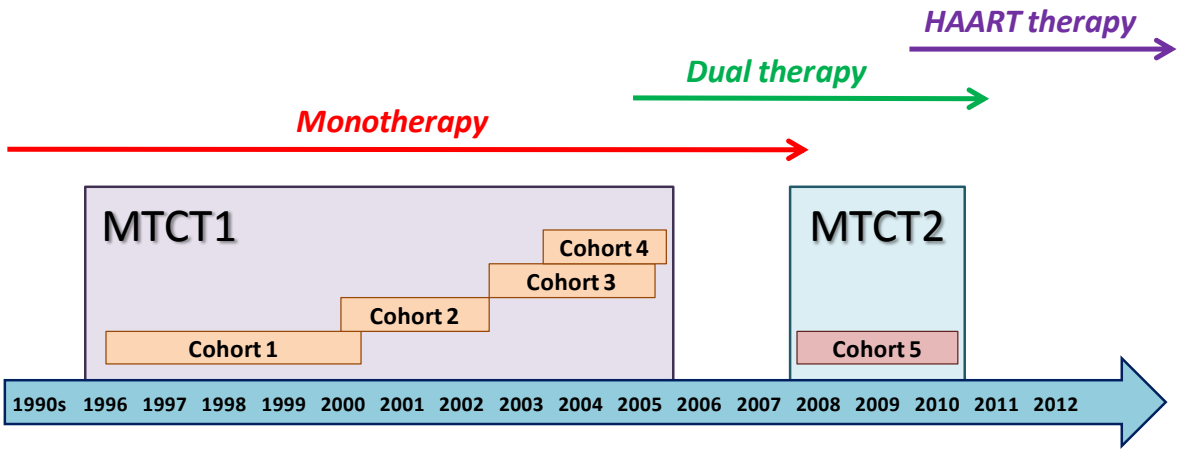


Figure 2.1 A schematic representation of the mother-infant cohorts used in this thesis designated MTCT1 (Cohorts 1 – 4 combined) and MTCT2 (Cohort 5). Monotherapy largely consisted of sdNVP given to the mother at the onset of labour and the infant within 72 hours of birth; dual therapy for the mother consisted of daily dose AZT from 28 - 37 weeks gestation as well as sdNVP at the onset of labour, while their infants received sdNVP at birth and daily AZT for a minimum of 1 week; early HAART consisted of triple drug therapy using the South African regimen 1B (d4T/3TC/NVP).

2.1.1 MTCT1

MTCT1 represents a retrospective, nested case–control study combining data from four cohorts of HIV-1 infected mothers and their infants that were enrolled between 1996 and 2005 from two hospitals in Johannesburg, South Africa. The cohorts are described in detail below:

I. **Cohort 1:** included a selection of mother-infant samples from an earlier trial that took place at Chris Hani Baragwanath Academic Hospital (CHBAH) from 1996 to 2000. The greater trial aimed to assess the efficacy of three short-course regimens of AZT and 3TC in preventing early and late transmission of HIV-1 from mother to child in Tanzania, South Africa, and Uganda (Kuhn et al., 2001, Petra_Study, 2002). Within this study, HIV-1 positive women who had not accessed antenatal HIV-1 testing were identified through a post-partum voluntary counselling and testing (PP-VCT) service that had been set up for the trial. These women had received no antiretroviral drugs before delivery; however their infants received either AZT or sdNVP as a post-exposure prophylaxis (PEP). Small aliquots (3-5 ml) of cord blood were collected from all deliveries, and if the woman enrolled, the cord blood was retained for research, otherwise it was discarded. Blood was drawn from mothers and infants at enrolment. Infants were scheduled for follow-up at 6 weeks and, if still breast-feeding, for continued follow-up for a minimum of 4 weeks after cessation of breast-feeding. Infant samples were tested for HIV-1 DNA by PCR to determine their infection status. From this cohort, 20 mother-infant pairs were selected for MTCT1, wherein all of the mothers were ART naive (**Table 2.1**).

II. **Cohort 2:** included mother-infant samples that were part of a PEP trial conducted at CHBAH from 2000 to 2002 (Gray et al., 2005). Women were eligible for the trial if they tested HIV-1-positive for the first time after delivery and were drug naive, usually because they received no prenatal care or had attended prenatal clinics where HIV-1 testing was not available at the time. However, as in cohort 1, their infants were randomized to receive either AZT or sdNVP to reduce the risk of vertical transmission. Additionally, drug-exposed, HIV-1-positive mothers (as well as their infants) were recruited amongst mothers delivering at the hospital around the same time. To qualify, these mothers needed to have attended the prenatal clinic at CHBAH, received HIV-1 counselling and testing there, and had accepted sdNVP as part of a demonstration of antiretroviral therapy (DART) initiative. NVP was given as a single maternal 200 mg oral dose at the onset of labour and

a single 0.6 mL infant dose within 72 hours of birth. Infants were followed up prospectively after birth to determine their HIV-1 status until 3 months or for a minimum of 4 weeks after cessation of breast-feeding. HIV-1 DNA PCR was performed on infant peripheral blood samples collected at birth and at 6 weeks of age. From this cohort, 61 mother-infant pairs were selected for MTCT1, wherein 14 (23%) mothers received sdNVP at the onset of labour (**Table 2.1**).

III. **Cohort 3:** mother-infant samples were collected at CHBAH but from 2003 to 2005, as part of a study to investigate immunogenetic factors involved in MTCT under antiretroviral treatment (Kuhn et al., 2001, Kuhn et al., 2002, Shalekoff et al., 2004, Meddows-Taylor et al., 2006, Schramm et al., 2006, Schramm et al., 2007, Shalekoff et al., 2009a, Shalekoff et al., 2009b, Schramm et al., 2010). Women in the maternity ward who were identified as being HIV-1 positive as part of the PP-VCT program were enrolled and maternal and infant blood was collected soon after delivery. Infants were given sdNVP as PEP and mothers were counselled about infant feeding options. In addition, women who were identified as being HIV-1 positive during pregnancy and who participated in routine PMTCT services were also enrolled. These women received sdNVP at the onset of labour and their infants received sdNVP within 72 hours of birth. As above, maternal and cord blood was collected soon after delivery. In addition, where possible a venous blood sample was collected from infants at 4 and 9 months to determine their HIV-1 status. From this cohort 75 mother-infant pairs were selected for MTCT1, wherein 52 (69%) mothers received sdNVP at the onset of labour (**Table 2.1**).

IV. **Cohort 4:** included mother-infant samples collected from mothers who were currently enrolled in PMTCT services at the former Coronation Hospital, now known as the Rahima Moosa Mother and Child Hospital (RMMCH) from 2004 to 2005. Mothers were identified at 6 weeks post-partum when they returned for postnatal follow-up services, at which time blood was drawn from mothers and infants. These mothers received either sdNVP or combination ART (lopinavir/ritonavir, stavudine (d4T), lamivudine (3TC) or efavirenz) if identified with low CD4+ T cell counts early enough during pregnancy (combination therapy would have been initiated at any stage of pregnancy but adherence in these women may not have been complete however, NVP or other ART intervention would have been taken prior to delivery). From this cohort 61 mother-infant pairs were selected for MTCT1, wherein 55 (90%) mothers received sdNVP at the onset of labour (**Table 2.1**).

Table 2.1 Distribution of the mothers across the four cohorts represented within MTCT1 with their respective PMTCT ART

MTCT1 Mothers	Cohort 1 N=20	Cohort 2 N=61	Cohort 3 N=75	Cohort 4 N=61	Total N=217
TR mothers	6	22	24	20	72
NT mothers	14	39	51	41	145
PMTCT ART (maternal)					
None	20 (100%)	47 (77%)	23 (31%)	0 (0%)	90 (41%)
sdNVP	0 (0%)	14 (23%)	52 (69%)	55 (90%)	121 (56%)
Other ART	0 (0%)	0 (0%)	0 (0%)	6 (10%)	6 (3%)

PMTCT, prevention of mother-to-child transmission; ART, antiretroviral therapy; NT, HIV-1 non-transmitting mother; TR: HIV-1 transmitting mother; sdNVP, single-dose nevirapine.

In each of these cohorts, mothers who met the eligibility criteria for these studies were counselled about the objectives and methods of the study. Eligibility criteria were: older than 18 years of age, or legal age of consent, evidence of HIV-1 infection, ability to give informed consent, estimated gestational period of less than 36 weeks at enrolment, absence of severe foetal anomalies within the limits of local diagnostic possibilities, absence of life-threatening disease, haemoglobin above 8 g/dL at enrolment and an 18 months' follow-up. Mothers interested in participating in the study were given a patients' information sheet describing the study. Women who met all eligibility criteria and signed informed consent were enrolled. In total, out of 849 HIV-1 infected mothers enrolled in the four cohorts, 83 mothers transmitted HIV-1 to their infants, thus the MTCT rate was calculated at 9.77%.

All blood specimens were collected in ethylenediaminetetraacetic acid (EDTA) tubes and samples were processed by standard procedures within 24 hours of collection. Plasma and buffy coats were stored at −70°C. Maternal plasma samples were sent for HIV-1 RNA quantitative testing using the Roche Amplicor HIV-1 RNA Monitor assay version 1.5 (Roche Diagnostic Systems, Inc., Branchburg, NJ) and CD4+ T cell counts were quantified using the commercially available FACSCount System from Becton Dickinson (San Jose, CA). Infant HIV-1 status was determined by HIV-1 DNA PCR tests (Roche Amplicor version 1.5) at birth, 6 weeks and 3 months of age. *In utero* (IU) infected infants were defined as PCR positive at birth and at 6 weeks and intrapartum (IP) infected infants were defined as PCR negative at birth but positive at 6 weeks. Infants with positive PCR results were recalled for confirmatory tests. A summary of the maternal clinical characteristics and ART regimen for the collective MTCT1 cohort (N=217) are presented in **Table 2.2**.

For MTCT1, all available HIV-1 transmitting mother-infant samples (TR, N=72) from the four transmission studies were selected as "cases" for the nested analysis. As controls, two HIV-1 non-transmitting mother-infant samples (NT, n=144) for each case were randomly selected from each of the four transmission studies giving rise to the cases (**Table 2.1**). HIV-1 infected infants were further characterized according to timing of transmission as determined by an HIV-1 DNA PCR test (Roche Amplicor version 1.5) at birth and at 6 weeks of age. In total 19 (26.4%) were IU infected, 29 (40.3%) were IP infected and the remaining 24 infants were found to be positive at 6 weeks but had no birth sample available (unknown whether IU or IP).

For analyses confined to mother-infant pairs who received maternal sdNVP (**Table 2.2**), within the total group of TR mothers (n=72), 29 mothers IP transmitted, wherein the majority of these mothers were ART naive (19/24, 65.5%) as compared to mothers that received sdNVP (10/19, 34.5%), *P*=0.035. Conversely, in IU-transmitting mothers (n=19), the majority of these mothers received sdNVP (12/19, 63.2%) as compared to mothers that were ART naive (7/19, 36.8%), *P*=0.194. We also analysed associations for an IU-enriched group called IU2. In IU2 we combined the 19 known IU-infected infants with the 24 infected infants of unknown transmission on the basis that 79% (19/24) of these mothers received sdNVP as compared to mothers that were ART naive (3/24, 12.5%), *P*<0.001 (**Table 2.2**). The rationale for this is that maternal sdNVP administration given only at the onset of labour is known to have little to no effect on reducing IU infection. Thus, we can infer that when infection does occur in an sdNVP-exposed infant, there is a greater likelihood that it is due to IU infection than due to IP infection.

Table 2.2 Clinical characteristics of MTCT1 mothers and their antiretroviral therapy (ART)

HIV+ mothers Median (IQR)	**NT (N=144)**	**TR (N=72)**	**IP-TR (N=29)**	**IU-TR (N=19)**	**Unknown-TR (N=24)**
Age (years)	27 (22-30)	29 (24-31)	29 (22-31)	29 (22-31)	28 (25-30)
$\log_{10}$ VL (copies/ml)	4.0 (3.2-4.6)	4.8 (3.7-5.4)	4.8 (3.9-5.3)	4.9 (4.2-5.5)	4.6 (2.6-5.4)
CD4 (cells/μl)	449 (319-681)	375 (253-575)	378 (262-508)	350 (182-647)	437 (301-590)
ART Naive	60 (41.7%)	29 (40.3%)	19 (65.5%)	7 (36.8%)	3 (12.5%)
sdNVP at labour	80 (55.5%)	41 (56.9%)	10 (34.5%)	12 (63.2%)	19 (79.2%)
Other ART	4 (2.8%)	2 (2.8%)	0 (0%)	0 (0%)	2 (0%)

IQR: interquartile range (25th and 75th percentile); VL: viral load; NT: HIV-1 non-transmitting mothers; TR: total group of HIV-1 transmitting mothers; IP-TR: intrapartum HIV-1 transmitting mothers; IU-TR: *in utero* HIV-1 transmitting mothers; Unknown-TR: HIV-1 transmitting mothers unknown whether IU or IP

2.1.2 MTCT2

The study involving this cohort was also a nested case–control design and utilized samples collected from a prospective, observational study that was conducted at RMMCH in Johannesburg, South Africa, from August 2008 to December 2010. HIV-1 infected women over 18 years of age, who were identified during antenatal testing and who planned to attend RMMCH for follow-up PMTCT care were eligible to participate in the study (Lilian et al., 2012). According to national PMTCT guidelines at the time, routine PMTCT care was offered to all mother-infant pairs enrolled in the study, which primarily consisted of maternal sdNVP at the onset of labour together with daily AZT from 28 weeks gestation (full term gestation considered to be 39 weeks). In addition, infants received sdNVP at birth and daily AZT for a minimum of 1 week. If maternal PMTCT care was suboptimal, defined as less than 4 weeks of AZT, the infant received 4 weeks of AZT (National Department of Health, 2008). All women with CD4+ T cell counts of ≤ 200 were eligible for HAART. Furthermore at the time of this study, most women at RMMCH elected to exclusively formula feed. Thereafter, national PMTCT guidelines were updated again in 2010, wherein maternal AZT commenced from 14 weeks gestation and all HIV-1-exposed infants provided with a daily dose of NVP for 6 weeks or for the duration of breast-feeding. Furthermore, all women with CD4+ T cell counts of ≤ 350 were eligible for HAART.

Infants underwent HIV-1 DNA PCR testing (Amplicor HIV-1 DNA PCR, version 1.5; Roche Molecular Systems Inc., Branchburg, NJ) on peripheral blood at 6 weeks, and test results were provided to the infants' caregivers at the 10 week visit. Where the PCR result was negative, the mother was counselled regarding the risk of postnatal transmission of HIV-1 and the infant discharged. Where the PCR result was positive, a confirmatory baseline VL test and a CD4+ T cell count were performed and the infant was referred for HAART initiation.

Dried blood spots (DBS) samples were obtained from the infants at each visit. At birth and 2 weeks, blood was collected by heel prick onto Schleicher & Schuell 903 (S&S 903 W-041) filter paper cards, and at the 4- and 6-week visits, blood was collected by venesection and spotted onto filter paper cards. Each DBS card was stored at RMMCH at ambient room temperature in a zip-lock plastic bag with a dessicant sachet.

Overall, the study enrolled 848 mother-infant pairs, of which 10 (1.2%) mothers withdrew during the course of the study and 128 (15.3%) HIV-1-exposed infants were excluded from all analyses because they were lost to follow-up or died before an HIV-1 status could be established. Of the remaining 710 mother-infant pairs, 557 (78.5%) received optimal maternal PMTCT care, defined as 4 or more weeks of AZT or HAART. sdNVP in combination with variable durations of AZT was administered to 653 (92.0%) infants, 56 (7.9%) infants received daily-dose NVP, and a single infant did not receive any PMTCT prophylaxis. Antenatal CD4+ T cell counts were available for 638 women, of which a CD4+ T cell count of ≤ 350 cells/μL was recorded for 316 (49.5%) women, accounting for 60.6% of vertical transmissions. Of the 710 infants, 691 (97.3%) had their HIV-1 status determined by the 6-week PCR and the remaining 19 (3%) infants had their HIV-1 status established from stored DBS samples. Out of 710 infants, 37 infants were HIV-1 infected, thus the MTCT rate was 5.21%. HIV-1 infected infants where further classified as IU-infected (N=25, 67.6%) or IP-infected (N=12, 32.4%).

For MTCT2, all available TR mother-infant samples were matched 1:1.2 with a NT mother-infant sample of similar maternal VL, maternal CD4+ T cell count, as well as mother and infant PMTCT regimen. In total, 79 mother-infant pairs were selected (**Figure 2.2**), which consisted of 36 TR mothers and their IP- or IU- infected infants as well as 43 NT mothers and their EU infants (n=44, one NT mother had twins). Clinical characteristics and ART for MTCT2 mothers is shown in **Table 2.3**.

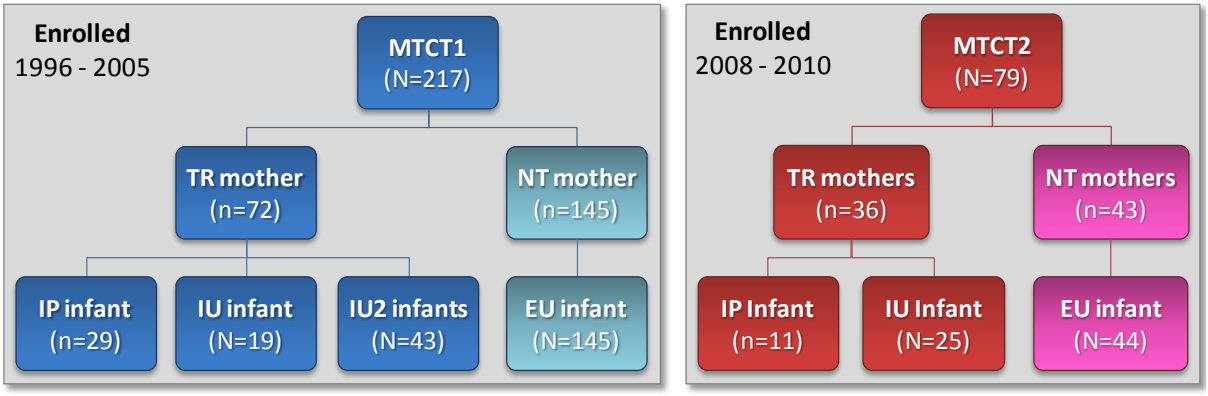


Figure 2.2 A Schematic representations of the two mother-infant cohorts used in this thesis, termed MTCT1 and MTCT2. TR, transmitting mother; NT, non-transmitting mother; IP, intrapartum infected infant; IU, *in utero* infected infant; IU2, IU-enriched group of infants; and EU, exposed uninfected infants.

Table 2.3 Clinical characteristics of MTCT2 mothers and their antiretroviral therapy (ART)

HIV+ mothers (Median, IQR)	NT (N=43)	IP-TR (N=11)	IU-TR (N=25)
Age (years)	27 (26-30)	25 (24-29)	26 (21-30)
$\log_{10}$ VL (copies/ml)	4.7 (3.9-5.1)	4.8 (4.5-5.2)	4.5 (3.2-4.9)
CD4 (cells/μl)	342 (244-447)	245 (171-406)	286 (234-424)
Maternal ART (n, %)			
ART naive	4 (9.1%)	2 (18.2%)	0 (0%)
sdNVP+AZT (< 2weeks)	14 (31.8%)	3 (27.3%)	11 (44.0%)
sdNVP+AZT (> 2weeks)	25 (56.8%)	6 (54.6%)	13 (52.0%)
HAART	1 (2.3%)	0 (0%)	1 (4.0%)
Infant ART (n, %)	**EU (N=44)**	**IP (N=11)**	**IU (N=25)**
sdNVP+1week AZT	17 (38.6%)	5 (45.4%)	13 (52.0%)
sdNVP+4week AZT	20 (45.4%	6 (54.6%)	10 (40.0%)
NVP for 6weeks or duration of BF	7 (15.9%)	0 (0%)	2 (8.0%)

IQR: interquartile range (25th and 75th percentile); VL: viral load; NT: HIV-1 non-transmitting mothers; IP-TR: intrapartum HIV-1 transmitting mothers; IU-TR: *in utero* HIV-1 transmitting mothers.

2.1.3 Comparison of clinical characteristics between MTCT1 and MTCT2

Given that the two MTCT cohorts were recruited over different years and differed with respect to overall HIV-1 transmission rate and ART regimens we compared the clinical characteristics amongst mothers from MTCT1 and MTCT2. As expected with dual therapy, the rate of HIV-1 transmission was significantly lower in MTCT2 compared to MTCT1 (**Figure 2.3A**, $P<0.001$). Moreover, since NT and TR mothers from MTCT2 were matched according to VL, CD4+ T cell count and ART regimen, it is not surprising that NT mothers from MTCT2 had significantly higher VLs and lower CD4+ T cell counts compared to NT mothers from MTCT1 (**Figure 2.3B**, $P<0.001$ and $P=0.003$, respectively). For TR mothers, while the median VL between MTCT1 and MTCT2 did not significantly differ, TR mothers from MTCT2 had significantly lower CD4+ T cell counts compared to TR mothers from MTCT1 ($P=0.015$). In MTCT1, median VL significantly differed between NT mothers and TR mothers (IP-TR, IU-TR and IU2-TR) (**Figure 2.4A**). Comparisons of median VL and CD4+ T cell counts with respect to varying ART regimens across MTCT1 and MTCT2 mothers (NT and total TR mothers) are shown (**Figure 2.4B**).

2.2 Ethical Clearance

For each of the above cohorts, written informed consent was obtained from women at the time of enrolment and ethical approval was provided by each of the principle investigators' institutional review boards. Studies specifically described in this thesis were approved by the Human Research Ethics Committee of the University of Witwatersrand, ethical clearance certificate: M130874 (Appendix A, page 193).

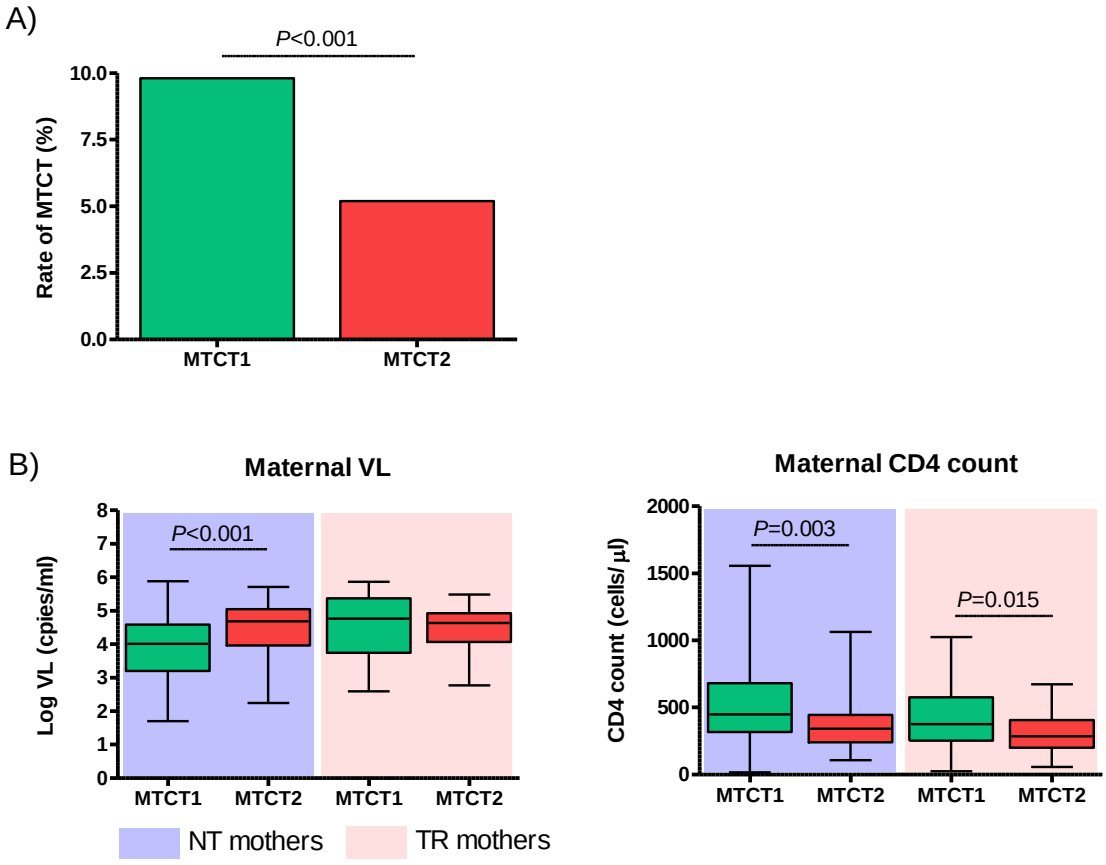


Figure 2.3 Comparison of the clinical characteristics amongst the total group of HIV-1 infected mothers from MTCT1 and MTCT2. (A) Comparison of overall rate HIV-1 transmission in MTCT1 (Green) and MTCT2 (Red), (B) Comparison of median viral load (VL, Log_{10} copies/ml) and CD4+ T cell counts (cells/µL) amongst HIV-1 non-transmitting mothers (NT, Blue) and HIV-1 transmitting mothers (TR, Pink). VL and CD4+ T cell counts were performed on maternal samples taken at the onset of birth.

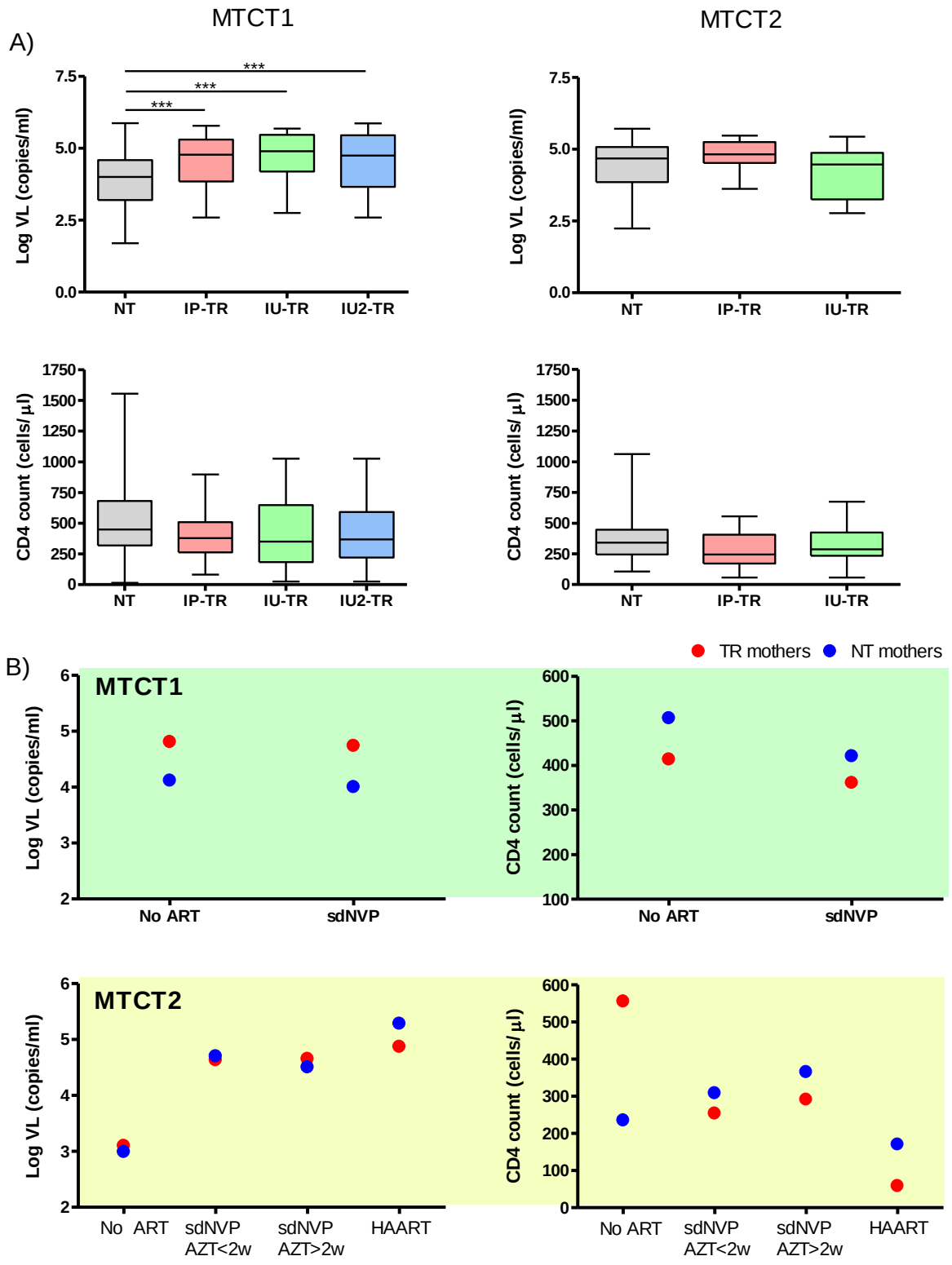


Figure 2.4 Comparison of clinical characteristics amongst HIV-1 transmitting (TR) and non-transmitting (NT) in MTCT1 and MTCT2. (A) Maternal viral load (VL, Log_{10} copies/ml) and CD4+T cell counts (cells/µL), (B) Median VL and CD4+ T cell counts in MTCT1 and MTCT2 stratified according to maternal antiretroviral therapy (ART) regimens. SdNVP (single dose Nevirapine), AZT (Azidothymidine) and HAART (a combination therapy of three or more drugs).

2.3 Genomic DNA extraction

Genomic DNA (gDNA) from 217 mother-infant samples from the MTCT1 cohort was previously extracted as described in Kuhn et al. (2007) and Paximadis et al. (2011) using the QIAamp DNA Blood Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. For the 79 mother-infant samples from MTCT2, gDNA was extracted from stored maternal and infant buffy coat samples using the QIAamp DNA Blood Mini Kit. In some cases where infants did not have buffy coat stored, gDNA was extracted from infant dried blood spots (DBS) as per the QIAamp DNA Blood Mini kit instructions. DNA concentration and quality were determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, MA). Typical DNA yield from 200 µl of buffy coat was approximately 50 µg of DNA; while three punched-out circles (each 3 mm in diameter) from the infant DBS generally yielded approximately 75 ng of DNA.

2.4 Conventional *KIR* and *HLA-ligand* genotyping

2.4.1 Olerup sequence-specific *KIR* genotyping for MTCT1

KIR genotyping on the MTCT1 samples was performed by Paximadis et al. (2011) using the commercial *Olerup* sequence-specific primers (SSP) *KIR* genotyping kit (*Olerup* SSP AB, Stockholm, Sweden) as per the manufacturer's instructions. The *KIR* SSP genotyping kit contained pre-optimized SSP for PCR amplification for 14 *KIR* genes (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3* and *KIR3DS1*) and 2 pseudogenes (*KIR2DP1* and *KIR3DP1*). In addition, the kit also allowed for the allelic discrimination of full-length (*KIR2DS4-f*: *2DS4*001:01:01-001:04*, **011:01-011:02*, **014* and **015*) from truncated (*KIR2DS4-v*: *2DS4*003*, 004, **006*, **007-010*, **012* and **013*) *KIR2DS4* alleles. PCR amplicons were visualised on a 2% agarose gel stained with ethidium bromide on a UV transilluminator and the presence/absence of specific KIR products, as well as the internal positive control bands was recorded for interpretation.

2.4.2 *HLA-B* and *HLA-C* genotyping for MTCT1

For MTCT1, 217 mother-infant samples were *HLA-B* and *HLA-C* genotyped using a previously described high resolution sequence-based typing (SBT) strategy (Cereb et al., 1995). Briefly, the method for both *HLA-B* and *HLA-C* typing involved a single PCR amplification of exon 2, intron 2 and exon 3 using gDNA as the template, followed by bi-directional sequencing of exon 2, intron 3 and exon 3 (regions that contain the defining

polymorphisms for most alleles). The PCR reaction contained 5 µl gDNA (20-100 ng/µl), dNTPs (0.2 mM), two *HLA* primers (0.2 mM each), and Taq DNA polymerase (2.5 units) in a total volume of 100 µl; thermocycling conditions: 96°C for *5* minutes, followed by 30 cycles of 94°C for 22 seconds, 65°C for 50 seconds, 72°C for 30 seconds, and 72°C for 7 minutes. Nucleotide sequencing was performed on an ABI 3730 Genetic Analyzer using Big Dye Terminator v1.1 chemistry (Applied Biosystems, Foster City, CA). Allele assignment was performed using SeqScape v2.5 software (Applied Biosystems) and a library compiled from the 2.17.0 release of the IMGT/HLA Database.

2.5 Real-time PCR assays for MTCT2

2.5.1 Validation samples used to develop real-time PCR assays

Stored gDNA was available for 270 samples that were previously genotyped using well-known assays for *KIR* and *HLA* class I. The real-time PCR *KIR* genotyping assay was validated against 50 samples that were *KIR* genotyped using the Olerup SSP KIR genotyping kit, wherein the samples consisted of 24 Black individuals and 26 Caucasian individuals from South Africa. The real-time PCR *HLA-ligand* assay was validated against 190 samples, made up of 107 Black individuals and 83 Caucasians from South Africa, that had previously been *HLA* Class I (A, B and C) genotyped using the Applied Biosystems SBT kits (Applied Biosystems, Foster City, CA) (Paximadis et al., 2012). In addition, in order to accommodate *HLA* class I allelic variation and avoid population bias, 30 reference samples obtained from the International Histocompatibility Working Group (www.ihwg.org) were also tested with the real-time *HLA-ligand* assay. Importantly, these validation samples represented all sixteen *KIR* and *HLA-ligand* variants at position 80. In addition, the *KIR* and *HLA-ligand* real-time PCR assays were used to genotype 81 Black women in the Carletonville mining area, South Africa.

2.5.2 Real-time PCR *KIR* assay for MTCT2

To date, few real-time PCR-based *KIR* genotyping and *HLA-ligand* identification assays have been described (Alves et al., 2009, Koehler et al., 2009). In our system, we wished to avoid the use of fluorescent probe chemistry and nested PCR methods used by Koehler et al. (2009) in favour of SYBR® green chemistry. We selected previously published *KIR* primer sequences (Vilches et al., 2007, Martin and Carrington, 2008, Alves et al., 2009) which we mapped to updated *KIR* gene alignments in the IPD database (Robinson et al., 2013a).

A combination of primers was used to improve detection of *KIR* allelic variants, with the exception of *KIR2DL3* and *KIR3DP1*, where a single primer set was used (**Table 2.4**). The *KIR2DL3* primer set detects all known alleles, however, the pseudogene *KIR3DP1* primer set does not detect *KIR3DP1*004* and *KIR3DP1*0090* alleles. Furthermore, an internal control gene *galactosylceramidase* (*GALC*) was included (Alves et al., 2009).

For real-time PCR, each 5 μL reaction contained 2X SYBR® Green master mix (Roche, Mannheim, Germany), 0.2 μM *KIR* primers, 0.2 μM *GALC* primers and 5 ng of gDNA. PCR cycling was performed in 96-well format on an ABI PRISM® 7500 real-time instrument (Applied Biosystems, Foster City, CA) as follows: 10 min at 95°C, 30 cycles of 15 s at 95°C and 1 min at 60°C. Melt curve analysis was performed after cycling to distinguish *GALC* (Tm = 74.89°C) from *KIR* amplicons (Tm = 78.5.5°C-85.9°C) (**Figure 2.5A**), thereby making it simple to identify the presence/absence of specific *KIR* genes. The presence of an internal control amplicon ensured that the absence of a *KIR* amplicon was a true result and not as a result of failed amplification.

2.5.3 Real-time PCR *HLA-ligand* assay for MTCT2

We designed our *HLA-ligand* primers from HLA class I alignments available at the IMGT/HLA database (Robinson et al., 2013a). Briefly, *HLA-ligand* primers were designed to detect HLA-C group 1 (C1) allotypes, which have an asparagine at position 80, designated as C1:80N, HLA-C group 2 (C2) allotypes, which have a lysine at position 80, designated as C2:80K, HLA-B allotypes with the Bw4 motif as designated by five variable amino acids spanning positions 77–83, were classified as either Bw4:80I (with an isoleucine at position 80) or Bw4:80T (with a threonine at position 80) (Cella et al., 1994, Gumperz et al., 1995). In addition, Bw4:80T variants can vary with alanine (Bw4:80T81A) or leucine (Bw4:80T81L) at position 81 (Muller et al., 1989, Cella et al., 1994, Gumperz et al., 1995). Some HLA-A allotypes also have the Bw4 motif (Bw4:80I) and bind KIR3DL1 and putatively KIR3DS1, whereas HLA-A allotypes lacking the Bw4 motif (non-Bw4:80T) and HLA-B Bw6 allotypes (Bw6:80N) are not known to bind any KIRs. All primers were synthesised with 3' locked nucleic acid (LNA) bases (Exiqon, Vedbaek, Denmark) in order to increase template specificity (**Table 2.5**). Each PCR reaction included an internal positive control amplification of the *albumin* (*ALB*) gene (Douek et al., 2002).

For real-time PCR, each 5 µL reaction contained 2X SYBR® Green master mix (Roche, Mannheim, Germany), 0.2 µM *HLA-ligand* primers, 0.2 µM *ALB* primers and 5 ng of gDNA. PCR cycling was performed in 96-well format on an ABI PRISM® 7500 real-time instrument (Applied Biosystems, Foster City, USA) as follows: 10 min at 95°C, 30 cycles of 15 s at 95°C, 5s at 60°C and 1 min at 72°C. Melt curve analysis was performed after cycling, and as with the *KIR* assay, differences in melting temperature could differentiate *ALB* (Tm=75.62°C) from *HLA-ligand* amplicons (Tm=87.3°C-88.3°C) (**Figure 2.5B**).

Table 2.4 PCR primer sets used for real-time *KIR* genotyping assay

KIR	Primers	Sequences (5' – 3')	Binding site	Product Tm, °C	Alleles not detected
2DL1	F1[a]	GTTGGTCAGATGTCATGTTTGAA	Exon 4	80.36	
	R1[a]	GGTCCCTGCCAGGTCTTGCG	Exon 4		
	F1[a]	TGGACCAAGAGTCTGCAGGA	Exon 8	82.12	*KIR2DL1*005*
	R1[a]	TGTTGTCTCCCTAGAAGACG	3'UTR		
2DL2	F1[a]	CTGGCCCACCCAGGTCG	Exon 4	80.68	*KIR2DL2*004, *00601, *00602, *00303, *004*
	R1[a]	GGACCGATGGAGAAGTTGGCT	Exon 4		
	F1[b]	AAACCTTCTCTCTCAGCCCA	Exon 5	83.30	*KIR2DL2*009*
	R1[b]	GCCCTGCAGAGAACCTACA	Exon 5		
2DL3	F[b]	AGACCCTCAGGAGGTGA	Exon 9	79.47	
	R[b]	CAGGAGACAACTTTGGATCA	Exon 9		
2DL4	F1[a]	CAGGACAAGCCCTTCTGC	Exon 3	78.08	
	R1[a]	CTGGGTGCCGACCACT	Exon 3		
	F1[a]	ACCTTCGCTTACAGCCCG	Exon 5	84.21	
	R1[a]	CCTCACCTGTGACAGAAACAG	Exon 5		
2DL5	F1[a]	GCGCTGTGGTGCCTCG	Exon 3	82.19	
	R1[a]	GACCACTCAATGGGGGAGC	Exon 3		
	F1[a]	TGCAGCTCCAGGAGCTCA	Exon 5	83.09	
	R1[a]	GGGTCTGACCACTCATAGGGT	Exon 5		
2DS1	F1[b]	TCTCCATCAGTCGCATGAG	Exon 4	79.34	
	F2[b]	TCTCCATCAGTCGCATGAA	Exon 4		
	R[b]	GGTCACTGGGAGCTGAC	Exon 4		
2DS2	F1[a]	TTCTGCACAGAGAGGGGAAGTA	Exon 4	80.55	
	R1[a]	GGGTCACTGGGAGCTGACAA	Exon 4		
	F1[a]	CGGGCCCCACGGTTT	Exon 5	83.47	*KIR2DS2*00104*
	R1[a]	GGTCACTCGAGTTTGACCACTCA	Exon 5		
2DS3	F1[c]	AAACCTTCTCTCTCAGCCCA	Exon 5	84.07	
	R1[b]	GCATCTGTAGGTTCCTCCT	Exon 5		
	F1[a]	CTATGACATGTACCATCTATCCAC	Exon 5	83.07	
	R1[a]	AAGCAGTGGGTCACTTGAC	Exon 5		

Table 2.4 *Continued*

KIR	Primers	Sequences (5' – 3')	Binding site	Product Tm, °C	Alleles not detected
2DS4	F1[a]	CTGGCCCTCCCAGGTCA	Exon 4	80.83	
	R1[a]	TCTGTAGGTTCCTGCAAGGACAG	Exon 4		
	F1[a]	GTTCAGGCAGGAGAGAAT	Exon 5	84.35	
	R1[a]	GTTTGACCACTCGTAGGGAGC	Exon 5		
2DS5	F1[a]	TGATGGGGTCTCCAAGGG	Exon 4	81.54	*KIR2DS5*003*
	R1[a]	TCCAGAGGGTCACTGGGC	Exon 4		
	F1[a]	ACAGAGAGGGGACGTTTAACC	Exon 4	82.18	
	R1[a]	ATGTCCAGAGGGTCACTGGG	Exon 4		
2DP1	F1[a]	GTCTGCCTGGCCCAGCT	Exon 3	80.67	
	R1[a]	GTGTGAACCCCGACATCTGTAC	Exon 3		
	F1[a]	CCATCGGTCCCATGATGG	Exon 4	79.28	
	R1[a]	CACTGGGAGCTGACAACTGATG	Exon 4		
3DL1	F1[a]	CGCTGTGGTGCCTCGA	Exon 3	79.62	*KIR3DL1*009, *042, *057*
	R1[a]	GGTGTGAACCCCGACATG	Exon 3		
	F1[b]	CCATCGGTCCCATGATGCT	Exon 4	79.13	
	F2[b]	CCATT GGTCCCATGATGCT	Exon 4		
	F3[b]	TCCATCGGTCCCATGATGTT	Exon 4		
	R[b]	CCACGATGTCCAGGGGA	Exon 4		
3DL2	F1[a]	CAAACCCTTCCTGTCTGCCC	Exon 3	82.94	*KIR3DL2*013, *014*
	R1[a]	GTGCCGACCACCCAGTGA	Exon 3		
	F1[a]	CCCATGAACGTAGGCTCCG	Exon 5	83.70	*KIR3DL2*018*
	R1[a]	CACACGCAGGGCAGGG	Exon 5		
3DL3	F1[a]	GTCAGGACAAGCCCTTCCTC	Exon 3	82.32	
	R1[a]	GAGTGTGGGTGTGAACTGCA	Exon 3		
	F1[a]	TTCTGCACAGAGAGGGGATCA	Exon 4	82.47	
	R1[a]	GAGCCGACAACTCATAGGGTA	Exon 4		
3DS1	F1[a]	AGCCTGCAGGGAACAGAAG	Exon 8	81.41	
	R1[a]	GCCTGACTGTGGTGCTCG	3'UTR		
	F1[b]	CATCGGTTCCATGATGCG	Exon 4	80.38	
	F2[b]	CATCAGTTCCATGATGCG	Exon 4		
	R[b]	CCACGATGTCCAGGGGA	Exon 4		
3DP1	F[b]	GTACGTCACCCTCCCATGATGTA	5'UTR	85.97	*KIR3DP1*004, *009002*
	R[b]	GAAAACGGTGTTTCGGAATAC	Exon 3		
GALC	F[c]	TTACCCAGAGCCCTATCGTTCT		74.89	
	R[c]	GTCTGCCCATCACCACCTATT			

GALC, galactosylceramidase; KIR, killer immunoglobulin-like receptor; PCR, polymerase chain reaction; UTR, untranslated region. [a]primers designed by (Martin and Carrington, 2008); [b]primers designed by (Vilches et al., 2007); [c]primers designed by (Alves et al., 2009). Melting temperature (Tm) for each PCR product was determined automatically following melt curve analysis.

Table 2.5 PCR primer sets used for real-time PCR *HLA-ligand* genotyping assay

HLA-ligand		Primers	Sequences (5' – 3') [a]	Product Tm, °C
HLA-A	Bw4:80I	F	CCATTGGGTGTCGGGTTTC[C]	87.39
		R [b]	CTCTGGTTGTAGTAGCGGAGCGCG[A]	
	Non-Bw4:80T	F	AATCAGTGTCGTCGCGGTC[G]	87.65
		R	TGTAGTAGCCGCGCAGG[G]	
HLA-B	Bw6:80N	F	TCAGCGTCGCCGGGGTCCC[A]	87.73
		R	TGTAGTAGCCGCGCAGG[T]	
	Bw4:80I	F	ACCCGGACTCAGAATCTCC[T]	87.96
		R [b]	CTCTGGTTGTAGTAGCGGAGCGCG[A]	
	Bw4:80T81A	F	ACCCGGACTCAGAATCTCC[T]	88.08
		R	TGTAGTAGCGGAGCGCG[G]	
	Bw4:80T81L	F	ACCCGGACTCAGAATCTCC[T]	87.73
		R	CTCTGGTTGTAGTAGCGGAGCAGG[G]	
HLA-C	C1:80N	F	AGCCAATCAGCGTCTCCGC[A]	88.34
		R	GCTCTGGTTGTAGTAGCCGCGCAG[G]	
	C2:80K	F	CCATTGGGTGTCGGGTTCT[A]	88.06
		R	GCTCTGGTTGTAGTAGCCGCGCAG[T]	
Albumin [c]		F	TCGATGAGAAAACGCCAGTAA	75.62
		R	ATGGTCGCCTGTTCACCAA	

KIR (killer immunoglobulin–like receptor); HLA (human leukocyte antigen); PCR (polymerase chain reaction); [a] 3' Locked nucleic acid bases are indicated in square brackets. [b] The same reverse primer is used for detection of HLA-A and -B Bw4:80I alleles. [c] Albumin primer design was adapted from (Douek et al., 2002)

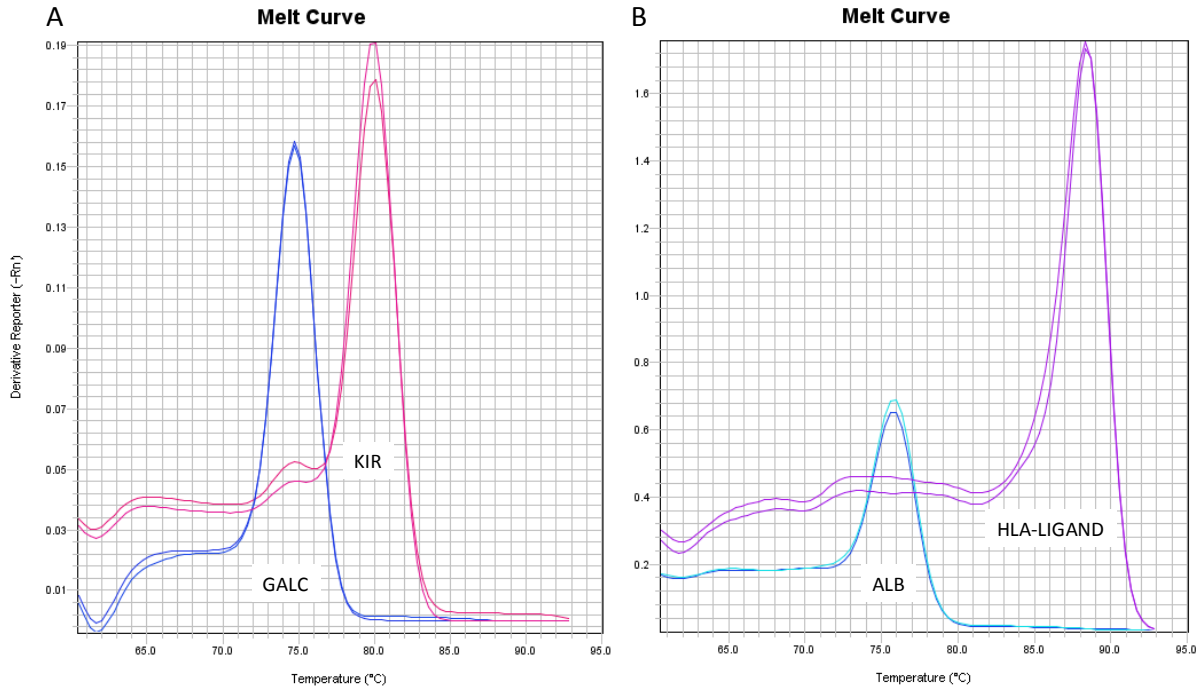


Figure 2.5 Typical melt curve analysis of real-time PCR *KIR* and *HLA-ligand* products using SYBER green chemistry, (A) Melt curve analysis of real-time PCR KIR products melting to the right of the internal control *galactosylceramidase* (*GALC*) (74.89°C), and (B) *HLA-ligand* PCR amplicons melting to the right of the internal control *albumin* (*ALB*) (75.62°C).

2.5.4 Real-time PCR *KIR2DS4* variant assay for MTCT2

Previously published primers and probes specific for *KIR2DS4-f* and *KIR2DS4-v* (Jiang et al., 2012) were used in a real-time PCR assay to detect the presence or absence of allelic variants of KIR2DS4 (**Table 2.6** and **Figure 2.6**). Control DNA for the *KIR2DS4-f/v* assay was derived from a haplotype AA1 donor with one copy each of *KIR2DS4-f* and *KIR2DS4-v*. Briefly, real-time PCR was performed in 96-well microtitre plates using an ABI 7500 real-time PCR instrument (Life Technologies, Carlsbad, USA). Reaction volumes were 5 µl containing 5 ng of gDNA, 2X Lightcycler 480 probes Mastermix (Roche, Mannheim, Germany), 0.5 µM *KIR2DS4-f/v* forward and reverse primers, and 0.1 µM VIC-labelled, 3'-MGB *KIR2DS4-f/v* probe (Life Technologies, Carlsbad, USA). Cycling conditions were an initial incubation of 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s, 60˚C for 60 s. All samples were run in duplicate and repeated if the values were discordant.

Table 2.6 Real-time *KIR2DS4* primers and probes

Name	Primer sequences (5' – 3')
KIR2DS4-f f1	CCGGAGCTCCTATGACATG
KIR2DS4-f/v r1	TGACGGAAACAAGCAGTGG[A]
KIR2DS4-f/v probe	VIC-AACATTCCAGGCCGACTTTCCTCTG-MGB
KIR2DS4-v f1	CCTTGTCCTGCAGCTCCAT
KIR2DS4-f/v r1	TGACGGAAACAAGCAGTGG[A]
KIR2DS4-f/v probe	VIC-AACATTCCAGGCCGACTTTCCTCTG-MGB

VIC, probe reporter fluorescent dyes; MGB, minor groove binding protein (non-fluorescent quencher); 3' locked nucleic acid (LNA) base modifications are indicated in square brackets.

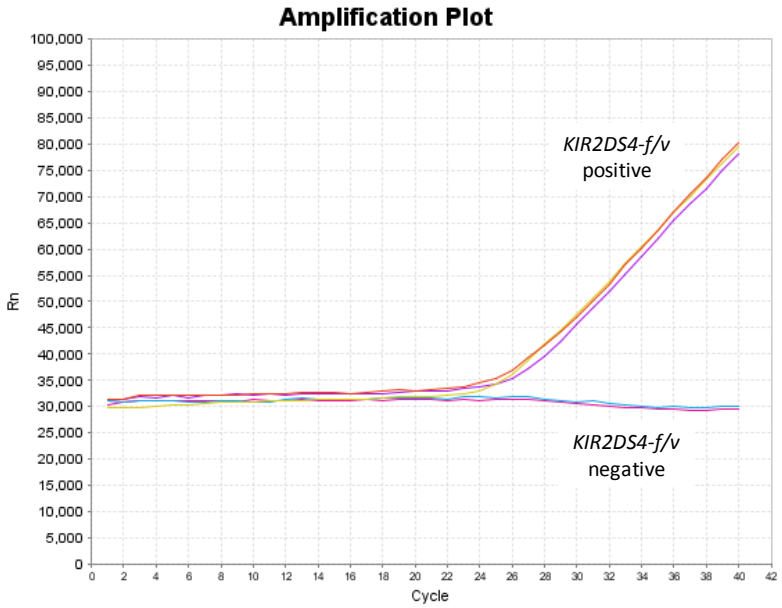


Figure 2.6 An amplification plot of the real-time PCR *KIR2DS4* assay

2.6 *HLA-G* genotyping for MTCT1 and MTCT2

2.6.1 Sequence-based typing of *HLA-G* alleles

Exons 2, 3 and 4 of *HLA-G* were amplified from gDNA and sequenced using previously published primers (Hviid et al., 1997, Turk et al., 2013) and are shown in **Table 2.7**. Briefly, a 994-bp fragment encompassing exons 2 and 3 was amplified using primers HLAG_2/3PCRF and HLAG_2/3PCRR. Primers HLAG_4PCRF and HLAG_4PCRR were used to amplify a 463-bp fragment spanning exon 4. Thermocycling conditions were: 94˚C for 2 min followed by 35 cycles of 94˚C for 30 sec, 61.5˚C/60˚C for 30 sec (for exons 2-3 and exon 4, respectively) and 72˚C for 1 min followed by a final extension step of 72˚C for 7 min. Purified PCR amplicons were sequenced in both directions by capillary electrophoresis using an ABI 3100 DNA analyzer (Applied Biosystems, Foster City, California, USA) using the sequencing primers listed in **Table 2.7**. HLAG_2SeqF was designed using HLA_G intron 1 alignments on Sequencher, version 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and synthesised with a LNA modified 3' end (indicated in square brackets). Sequence analysis and allele assignment were performed using Assign™ SBT version 4.7 software (Conexio Genomics, Fremantle, Western Australia) with the IMGT/HLA-G 2013 reference library compiled and supplied by Conexio Genomics (personal request).

2.6.2 *HLA-G* 3'UTR genotyping

Nucleotide sequence variation of the *HLA-G* 3'UTR region was evaluated by direct sequencing of a 343-bp fragment encompassing the genomic positions +2885 through to +3228 using published primers (Sizzano et al., 2012). Briefly, the 3'UTR region was amplified using HLAG_3UTRF and HLAG_3UTRR primers (**Table 2.7**) with the following thermocycling conditions: 95˚C for 15 min followed by 30 cycles of 93˚C for 1 min, 58˚C for 1 min, 72˚C for 1 min. A final extension step was carried out at 72˚C for 10 min. Purified amplicons were sequenced in both directions by capillary electrophoresis using an ABI 3100 DNA analyzer (Applied Biosystems, Foster City, California, USA) using the same PCR primers. The chromatograms obtained were analysed using Sequencher version 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and sequences were aligned with an available reference sequence (GenBank Accession number NG_029039.1) to identify known polymorphic positions (Castelli et al., 2010) and any other polymorphism that had not been previously described.

Table 2.7 *HLA-G* allele and 3'UTR PCR and sequencing primers

Name	Primer sequences (5' – 3')
HLAG_2/3PCRF	CGGCCCCTGCGCGGAGGAGGGAGGGG
HLAG_2/3PCRR	TCAGGACCAGAGGGAGGGCGATATTC
HLAG_4PCRF	AGGTGCTGCTGGAGTGTC
HLAG_4PCRR	TCTGGGAAAGGAGGTGAAG
HLAG_2SeqF	CTCCATGAGGTATTTCAGC[G]
HLAG_2SeqR	TCGTGATCTGCGCCCTG
HLAG_3SeqF	TGGGCGGGGCTGACCGAGGGGGTGGG
HLAG_3SeqR	TCAGGACCAGAGGGAGGGCGATATTC
HLAG_4SeqF	GTGCTTGAATTTTCTGACTCTT
HLAG_4SeqR	TGCTTTCCCTAACAGACATGAT
HLAG_3'UTRF	TCACCCCTCACTGTGACTGA
HLAG_3'UTRR	CCCATCAAT CTCTCTTGGAAA

3' locked nucleic acid (LNA) base modifications are indicated in square brackets

2.6.3 *HLA-G* 3'UTR haplotypes

Arrangement of variations in the *HLA-G* 3'UTR into haplotypes was predicted both by visual examination of the genotypic data as well as using the Bayesian algorithm through the HAPLOTYPER software (Niu et al., 2002). Observed haplotypes were compared to published 3'UTR haplotypes and assigned the published nomenclature when the haplotypes corresponded in sequence/structure (Sabbagh et al., 2014). The frequencies of haplotypes were calculated by counting the number of alleles harbouring the haplotypes and dividing by the total number of alleles. Counting of the haplotypes was irrespective of the presence of additional SNPs not forming part of the haplotypes in question.

2.7 Data analysis

2.7.1 *KIR* descriptions

In this study, to explore any possible associations with MTCT of HIV-1 we first examined the presence or absence of all 16 *KIR* genes in mothers and their infants. Then, based on results of single *KIR* gene content, we assessed haplotypes A and B. Group A Haplotypes were defined by the presence of 9 *KIR* genes: *KIR3DL3*, *KIR2DL3*, *KIR2DL1*, *KIR2DP1*, *KIR3DP1*, *KIR2DL4*, *KIR3DL1*, *KIR2DS4* and *KIR3DL2* only, while Group B haplotypes were defined by the presence of one or more of the following genes: *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5*, and *KIR3DS1*. The group B haplotypes were collectively termed Bx, since they represent a mixture of AB and BB haplotypes. Furthermore, KIR genotype profiles were assigned to the AA and Bx haplotype groups using the online Allele Frequency Net Database (Gonzalez-Galarza et al., 2011).

For *KIR2DS4* variants, we grouped *KIR2DS4* carriers into *KIR2DS4-f* (one or two copies of the functional alleles: *KIR2DS4*0010101-00103*), *KIR2DS4-v* (one or two copies of deleted alleles: *KIR2DS4*003*, **004*, **006*, **007*, **009*) or *KIR2DS4-fv* (one copy of each). This comparison allows one to look at the presence (but not dose) of one or the other, given one may expect opposing functional effects of full length and soluble protein versions. Consequently, AA genotypes carrying *KIR2DS4-f*, *KIR2DS4-fv* or *KIR2DS4-v* were classified as *AA-f*, *AA-fv* and *AA-v*, respectively.

2.7.2 HLA-ligand allotypes

HLA-B and *HLA-C* alleles genotyped through the SBT method (for MTCT1) were further grouped as *HLA-Bw4* (*Bw4*) and *Bw6* allotypes based on five variable amino acids spanning residues 77-83 at the carboxyl-terminal end of the α1 helix. The *Bw4* allotype subset Bw4:80Ile contains an isoleucine at position 80 as opposed to the Bw4:80Thr subset that contains a threonine at the same position and serves as the better ligand for KIR3DL1, and is a putative ligand for KIR3DS1 based on epidemiologic data. *HLA-C* alleles were grouped as C1 allotypes (asparagine at residue 80 and are known ligands for KIR2DL2, KIR2DL3, and KIR2DS2) or C2 allotypes (lysine at residue 80, known ligands for KIR2DL1 and KIR2DS1). Data were analyzed between groups as presence of at least one allele of a particular allotype (*C1* or *C2*, *Bw4* or *Bw6*) and total allelic representation or allelic dose (*C1/C1*, *C2/C2*, *C1/C2* and *Bw4/Bw4*, *Bw6/Bw6*, *Bw4/Bw6*).

2.7.3 Classification of mother-infant gene concordance

To assess the effect of specific gene/allele concordance on MTCT of HIV-1, we scored the number of matching mother-infant pairs following the previously reported method (MacDonald et al., 1998, Polycarpou et al., 2002, Biggar et al., 2006a, Mackelprang et al., 2008). Since an infant will inherit one allele from the mother and one from the father, the infant will match at least 50% of the mother's alleles. Therefore, when the mother was homozygous for a given genotype which matched one of the child's alleles this was also considered as concordant, likewise, if the two alleles of the child matched two alleles of the mother, they were considered concordant and were classified as M+I+. Discordance was defined as either the presence (+) of the given genotype that was absent (−) in either the mother or the infant, i.e. M+I− or M−I+, respectively.

2.7.4 Computational and statistical analysis

2.7.4.1 Power analysis

Given that the MTCT cohorts were recruited prior to the initiation of this PhD thesis and the ratio of cases to controls were defined on the basis of sample availability, 1:2 for MTCT1 and 1:1.2 for MTCT2, we performed a retrospective power analysis for each MTCT cohort. Power analysis is the probability of correctly rejecting the null hypothesis that the sample estimates (Suresh and Chandrashekara, 2012). Here, the actual sample size and alpha-level are known, and the variance observed in the sample provides an estimate of variance of population. Having a high power means that the study has a high chance of detecting a difference between groups if one exists. Consequently, if the study demonstrates no difference between groups, the study can confidently conclude that none exists.

For MTCT1: using the example of the *KIR* AA1 haplotype (the most common *KIR* haplotype in the NT group) having a frequency of 26.9% and based on our 1:2 case-control design (cases=72), assuming an alpha of 5% and a minimum odds ratio of 2.5 to detect a difference, the overall power of the MTCT1 cohort was calculated at 86.4%, using the online statistical program (http://sampsize.sourceforge.net). However, within the IP- and IU- TR groups overall power decreased (IP-TR vs. NT: 60.6% and IU-TR vs. NT: 47.5%) and we acknowledge this as a limitation. Nevertheless, upon evaluation of the IU-enriched group (IU2-TR, n=43) power within this comparison increased to 73.1%. Thus, we feel that our data can be accurately interpreted.

For MTCT2: since cases and controls were matched on mVL, CD4+ T cell count and ART, the power of MTCT2 was less stringent. Therefore, using the example of the Bx21 haplotype (the most common *KIR* haplotype in the NT group) having a having a frequency of 16.3% and based on our 1:1 matched case-control design (cases=36), assuming an alpha of 5% and a minimum odds ratio of 2.5 to detect a difference, the overall power of the MTCT1 cohort was calculated at 40.2%. Additionally for power across IP-TR and IU-TR was calculated at 25.8% and 35.4%, respectively. As in MTCT1, we acknowledge this is a limitation but given the MTCT transmission rates have fallen from ~32% in the early 1990s to <2.5% in 2012 with current ART regimens (Ramkisoon and Coovadia, 2014), the data obtained from MTCT2 has relevance to understanding the role of *KIR/HLA* gene combinations in HIV-1 transmission.

2.7.4.2 Hardy-Weinberg Equilibrium (HWE)

HWE is the theorem that states that allele and genotype frequencies in a population will remain constant from generation to generation in the absence of other evolutionary influences, such as mate choice, mutation, selection, genetic drift, gene flow and meiotic drive (Ryckman and Williams, 2008). Because one or more of these influences are typically present in real populations, the HWE principle describes an ideal condition against which the effects of these influences can be analyzed. Thus, for this study, deviations from HWE were assessed using the conventional Monte Carlo exact test (Guo and Thompson, 1992) using the computer program TFPGA (Tools for Population Genetic Analyses version 1.3; 1997: author Mark. P. Miller).

2.7.4.3 Linkage Disequilibrium (LD)

LD, the non-random association of alleles at two loci, finds applications in diverse contexts, including the inference of demographic events in human evolutionary history, the fine-mapping of disease genes after localization via linkage analysis, and the modelling, selection, and evaluation of sets of informative SNPs for use in detecting disease susceptibility alleles in genetic association studies (VanLiere and Rosenberg, 2008). In our MTCT cohorts, LD was estimated and visualised using the method described by Lewontin (1964) as well as Haploview bioinformatics software, version 4.2 (Barrett et al., 2005).

For the Lewontin calculation, the LD coefficient D was calculated ($D_{ij} = HF_{ij} - p_i p_j$) and to account for differing allele frequencies at the loci, D was normalized (D') or standardized by the maximum value it can take (D_{max}) using the formula $D'_{ij} = Dij/D_{max}$, where HF_{ij} is the frequency of the haplotype carrying alleles i and j, p_i and p_j are the frequencies of alleles i and j, respectively. D_{max} is either $\min[p_i p_j, (1 - p_i)(1 - p_j)]$ if $D_{ij} < 0$; or $\min[(1 - pi) p_j, p_i(1 - p_j)]$ if $Dij > 0$ (Lewontin, 1964b, Lewontin, 1964a) The statistical significance of the LD between each of the allele pairs was evaluated by the approximate chi-square test (χ^2, below) described by (Liau et al., 1984); where GF refers to the genotypic frequency of the particular allele, i.e., i or j (equivalent to pi or pj described above).

$$\chi^2 = \left\{ \frac{4n[HF(ij)-GF(i)GF(j)]^2}{GF(i)GF(j)[2-GF(i)][2-GF(j)]} \right\} \; 1+ \; \left\{ \frac{[HF(ij)-GF(i)GF(j)]}{2[1-GF(i)][1-GF(j)]} \right\}^2$$

2.7.4.3 Statistical analysis

Chapter 3: *KIR and HLA-ligand identification by real-time PCR*

For both sets of data (SSP-PCR, SBT and real-time PCR), carrier frequencies for the observed *KIR* and *HLA-ligands* were determined by direct counting (individuals positive for the gene divided by the individuals tested per population ×100). Real-time PCR *KIR* and *HLA-ligand* assays were validated by direct comparison with SBT- and SSP-PCR based typing results of the same samples.

Chapter 4, 5 and 6: *KIR genes and HLA-ligands in MTCT*

Gene, genotype and haplotype frequencies of *KIR* and *HLA-ligands* were determined by direct counting. Their fit to Hardy–Weinberg equilibrium proportions was examined and established, using the Monte Carlo exact test. Differences in the frequency distributions between maternal, as well as infant groups were compared using 2x2 contingency tables and a Fisher's exact test at 95% confidence interval (CI) limit using the online statistical program VassarStats (http://www.vassarstats.net), which was also used to estimate the odds ratio (OR). Two-sided *P* values less than 0.05 were considered significant. Graphical representation and Mann-Whitney *U* tests were performed with GraphPad Prism Version 4.02 software (GraphPad Software, San Diego CA).

For all MTCT1 analyses Bonferroni correction was not applied, as adjustment for multiple comparisons correct for type 1 errors but increase the risk of type 2 errors. Thus, given the complexity and multifactorial nature of maternal-infant HIV-1 transmission, we considered it more important to identify potential factors that may play a role in this route of infection rather than simply dismissing these leads as due to chance variations brought about by multiple comparisons. Thus, to analyse maternal and infant *KIR/HLA* associations with HIV-transmission, we used logistic regression with infant infection as the outcome. Unconditional logistic regression (Pezzullo, 2005) was used to adjust for the effects of the following variables: maternal viral load (mVL), maternal sdNVP, as well as infant possession of *KIR2DS4-v* which we recently reported to impact on IU MTCT in the same cohort (Hong et al., 2013). With regards to MTCT2, NT and TR mother-infant samples were matched according to maternal VL, CD4+ T cell count as well PMTCT regiments, which negated the need to adjust for these factors.

CHAPTER 3

KIR and *HLA-ligand* Identification by Real-Time PCR

The following section has been published: Hong et al. (2011) *Tissue Antigens* 78, 185 – 194.

3.1 Introduction

It is well recognised that NK cells are one of the critical components of the innate immune system and have great potential to mediate antiviral activity in the early stages of HIV-1 infection (Chavan et al., 2014). To date, 14 functional *KIR* genes and two pseudogenes have been described on human chromosome 19q13.4, 10 of which encode receptors with two immunoglobulin domains (2D) and four encoding receptors with three domains (3D). The KIRs can be further classified as inhibitory (2DL1, 2DL2, 2DL3, 2DL5A and B, 3DL1, 3DL2 and 3DL3) or activating (2DL4, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 2DS5 and 3DS1) based on the length of their cytoplasmic tail. KIRs with long tails (L) have an immunoreceptor tyrosine-based inhibitory motif (ITIM) and are able to suppress NK cell activation, whereas short-tail (S) receptors are characterized by a charged residue in the transmembrane portion that allows their association with the adaptor molecule (DAP12) required to generate activating signals that induce the secretion of IFN-γ and the release of perforin and granzymes.

Despite the framework *KIR* genes (*KIR2DL3, KIR2DL4, KIR3DL2*, and *KIR3DP1*) that are virtually present in all individuals, other *KIR* genes display a high degree of variability at the level of gene presence or absence. Moreover the frequency of these genes has been found to vary in different population groups with different ethnic-history, linguistic and geographic backgrounds. Therefore, based on *KIR* gene content, *KIR* haplotypes can be divided into two broad groups, namely group A and B. The group A haplotype contains one activating *KIR* gene, *KIR2DS4*, and six inhibitory *KIR* genes: *KIR2DL1*, *KIR2DL3*, *KIR2DL4*, *KIR3DL1*, *KIR3DL2*, and *KIR3DL3*. Group B haplotypes are more diverse as they have more activating receptors and are characterized by *KIR2DL2*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, and *KIR2DS5* genes (Rajalingam, 2012). Several KIRs bind to HLA class I molecules as their ligands. KIR2DL1/S1 recognise HLA-C group 2 (C2) allotypes which have a lysine at position 80 (80K), whereas KIR2DL2/L3 recognise HLA-C group 1 (C1) allotypes that have an asparagine at position 80 (80N) (Mandelboim et al., 1996). KIR2DS4 recognise a select group of C1 and C2 allotypes (*C*01:02, C*14:02, C*16:01, C*02:02, C*04:01* and

*C*05:01*) as well as two *HLA-A* alleles (*A*11:01* and *A*11:02*) (Graef et al., 2009). Whilst KIR3DL1, and putatively KIR3DS1, bind to HLA-B allotypes with the Bw4 motif which either have an isoleucine or a threonine at position 80 (Bw4:80I and Bw4:80T, respectively) (Gumperz et al., 1995). The HLA-B Bw4:80T variants further differentiate at position 81 with an alanine or leucine (Bw4:81A and Bw4:81L, respectively). HLA-A and -B allotypes that lack the Bw4 motif are not known to bind any KIRs, which we have been termed non-Bw4:80T and Bw6:80N, respectively.

As NK cell receptors, the KIR and their HLA-ligand interactions have an important role in modulating outcomes of infectious diseases, transplantation as well as pregnancy, and have become subjects of intense research (Roberts et al., 2014). Furthermore, with regards to HIV-1 infection, NK cell function and KIR have not only been linked to HIV-1 disease progression, but also with resistance and susceptibility to HIV-1 infection (Ballan et al., 2007, Carrington et al., 2008, Tiemessen et al., 2009, Tiemessen et al., 2010, Paximadis et al., 2011, Jost and Altfeld, 2013). However, their genotyping and subsequent use in association studies and histocompatibility matching are made difficult by the underlying structural and sequence complexity in the KIR region. Conventionally both *KIR* and *HLA* genotyping is routinely performed using SSP-PCR and SBT assays, respectively. These methods are often cumbersome, expensive and have low throughput, but moreover require large quantities of high-quality genomic DNA. In addition, amplified products are visualized by agarose gel electrophoresis, which is time-consuming as well as hazardous if using ethidium bromide staining. Thus, we developed and validated two real-time PCR assays for *KIR* and *HLA-ligand* genotyping using samples that have been previously characterized by SSP-PCR and SBT, respectively. The use of a real-time PCR assay allows for the quick, easy and cost effective detection of sixteen *KIR* genes and the presence/absence of *HLA-ligands* based on allelic discrimination at codon 80 in *HLA-A*, *-B* and *-C* genes.

3.2 Results

3.2.1 Validation of real-time PCR *KIR* assay

Using samples that represented the full spectrum of the *KIR* genes, we tested 50 validation samples that were *KIR* genotyped with the conventional Olerup SSP *KIR* Genotyping kit (Chapter 2, section 2.4.1) and compared these results with our real-time PCR *KIR* assay (Chapter 2, section 2.5.2). We found the real-time PCR *KIR* assay was 100% concordant with conventional SSP-PCR methods (**Table 3.1**).

Table 3.1 Validation of real-time PCR *KIR* genotyping assay (*n*=50)

***KIR* genes**	**Carriers / Non-carriers**		**Concordant** (%)
	SSP KIR PCR	**Real-time PCR KIR assay**	
2DL1	48 / 2	48 / 2	100
2DL2	27 / 23	27 / 23	100
2DL3	41 / 9	41 / 9	100
2DL4	50 / 0	50 / 0	100
2DL5	26 / 24	26 / 24	100
2DS1	15 / 35	15 / 35	100
2DS2	27 / 23	27 / 23	100
2DS3	10 / 40	10 / 40	100
2DS4	47 / 7	47 / 7	100
2DS5	21 / 29	21 / 29	100
2DP1	48 / 2	48 / 2	100
3DL1	47 / 3	47 / 3	100
3DL2	50 / 0	50 / 0	100
3DL3	50 / 0	50 / 0	100
3DS1	13 / 37	13 / 37	100
3DP1	50 / 0	50 / 0	100

SSP KIR PCR (*Olerup* SSP AB, Stockholm, Sweden) and real-time PCR *KIR* genotyping concordance was established by directly comparing the number of individuals who did/did not carry the *KIR* gene divided by the *KIR* genes present/absent ×100.

3.2.2 Validation of real-time PCR *HLA-ligand* assay

On comparison with the commercial high-resolution SBT method, the real-time *HLA-ligand* assay correctly genotyped 99.1 % (218/220) of individuals tested. All HLA-ligand primer sets, with the exception of HLA-B Bw4:80T 81A, were 100% concordant with SBT results (**Table 3.2**). In our real-time PCR *HLA-ligand* assay, the HLA-B Bw4:80T 81A primer failed to correctly assign *HLA-B*38:02* to the Bw4:80T 81A allotype in two of the DNA samples obtained from the IHWG database. These two samples were re-typed using the SBT method and confirmed to be correctly assigned based on exon 2, 3 and 4 sequences. Additionally, the genomic sequences for *HLA-B*38:02:01*, *B*38:02:02* and *B*38:02:03* were available to confirm that the reverse primer binding site had no changes. SBT typing did not include the 5'UTR, however, genomic sequences were available for *HLA-B*38:01:01*, *B*38:02:01* and *B*38:14* and confirmed that the forward primer was conserved except for one mismatch seven bases from the 3' end. It is unlikely that this was the cause of a failed real-time PCR amplification since the forward primer was found to successfully amplify other *HLA-B* alleles with the same mismatch. It is therefore possible that these two samples are un-described

*B*38:02* alleles with changes in the 5'UTR. Nevertheless, the *HLA-B*38:02* allele is prevalent at a low frequency in people of Taiwanese/Chinese descent (0.11% and 0.08%, respectively) and to our knowledge has not been reported within the African continent (Gonzalez-Galarza et al., 2011).

Table 3.2 Validation of real-time PCR *HLA-ligand* genotyping assay (*n*=220)

HLA-ligand		Carriers / Non-carriers		Concordant (%)
		Sequence based typing	Real-Time PCR Assay	
HLA-A	Bw4:80I	62 / 158	62 / 158	100
	Non-Bw4:80T	215 / 5	215 / 5	100
HLA-B	Bw6:80N	180 / 40	180 / 40	100
	Bw4:80I	93 / 127	93 / 127	100
	Bw4:80T81A	49 / 171	47 / 171	95.9
	Bw4:80T81L	11 / 209	11 / 209	100
HLA-C	C1:80N	173 / 47	173 / 47	100
	C2:80K	161 / 59	161 / 59	100

Concordance between HLA sequence based typing (SBT) and real-time PCR assay for *HLA-ligand* genotyping was established by directly comparing the number of individuals who did/did not carry the *KIR* gene divided by the *KIR* genes present/absent ×100.

3.2.3 Characterising *KIR* and *HLA-ligand* genes in a small South African population

The real-time PCR *KIR* and *HLA-ligand* assays were used to genotype a small group of 81 Black South African women from the Carletonville mining district in Johannesburg, South Africa. We identified twenty different *KIR* genotype profiles (**Table 3.3**), the three most common *KIR* genotypes were AA1 (24.7%), Bx21 (13.6%) and Bx5 (8.6%) which account for 47% of the total *KIR* genotypes. In addition, we found two *KIR* genotypes (*Bx46* and *Bx70*) that had not been previously reported in other South Africa studies (Wong et al., 2010, Paximadis et al., 2011), as well as one unique *KIR* Bx* genotype with all *KIR* genes present with the exception of *KIR2DL2*, *KIR2DL3*, *KIR2DS1*, *KIR2DS2* and *KIR3DS1* which had not been described at the time of manuscript publication (Hong et al., 2011), but has since been classified as Bx555 and was reported in low frequency (<1%) in a Northeast Indian Adivasi population (Dutta et al., 2013).

Table 3.3 Real-time PCR *KIR* genotyping of a South African cohort (N=81) showing *KIR* gene and genotype frequencies

KIR **genotype**	***KIR*** **genes**																**# *KIR* genes**	**Genotype N (%)**
	2DL1	**2DL2**	**2DL3**	**2DL4**	**2DL5**	**2DS1**	**2DS2**	**2DS3**	**2DS4**	**2DS5**	**2DP1**	**3DL1**	**3DL2**	**3DL3**	**3DS1**	**3DP1**		
AA1	■		■	■					■		■	■	■	■		■	9	20 (24.7)
Bx21	■	■	■	■	■		■		■	■	■	■	■	■		■	13	11 (13.6)
Bx5	■	■	■	■	■		■	■	■		■	■	■	■		■	13	7 (8.6)
Bx10	■	■	■	■			■		■		■	■	■	■		■	11	6 (7.4)
Bx20	■	■	■	■	■				■	■	■	■	■	■		■	12	6 (7.4)
Bx112	■	■		■	■		■	■	■	■	■	■	■	■		■	13	5 (6.2)
Bx73	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	15	5 (6.2)
Bx92	■	■		■	■	■	■		■	■	■	■	■	■		■	13	4 (4.9)
Bx228	■	■		■	■		■		■	■	■	■	■	■	■	■	12	3 (3.7)
Bx6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	16	2 (2.5)
Bx9	■	■	■	■	■	■	■		■	■	■	■	■	■		■	14	2 (2.5)
Bx91	■	■		■	■	■	■	■	■	■	■	■	■	■		■	14	2 (2.5)
Bx118	■	■		■	■	■	■		■	■	■	■	■	■	■	■	14	1 (1.2)
Bx27	■		■	■	■			■	■		■	■	■	■	■	■	12	1 (1.2)
Bx32	■		■	■	■				■	■	■	■	■	■		■	11	1 (1.2)
Bx35	■		■	■	■	■			■	■	■	■	■	■		■	12	1 (1.2)
Bx4	■	■	■	■			■		■		■	■	■	■		■	11	1 (1.2)
Bx46	■	■	■	■	■	■			■	■	■	■	■	■		■	13	1 (1.2)
Bx70	■	■	■	■	■	■	■	■		■	■		■	■	■	■	14	1 (1.2)
Bx*	■			■	■			■	■	■	■	■	■	■		■	11	1 (1.2)
Gene N (%)	81 (100)	56 (69)	60 (74)	81 (100)	54 (67)	19 (23)	50 (62)	24 (30)	80 (99)	46 (57)	81 (100)	80 (99)	81 (100)	81 (100)	10 (12)	81 (100)		

The purple boxes indicate the presence of the gene; white boxes indicate the absence of the gene. #, Number of *KIR* genes present in the *KIR* genotype. Frequency of each *KIR* gene is defined as the number of individuals having the gene divided by the number of individuals in the cohort expressed as a percentage. *KIR* genotype frequency is defined by the number of individuals having a particular genotype divided by the number of individuals within the cohort expressed as a percentage. Based on *KIR* gene content, genotypes (AA and Bx) were grouped according to (Gonzalez-Galarza et al., 2011). Bx* is a new *KIR* genotype that has not been previously identified. KIR (killer immunoglobulin–like receptor).

With regards to *HLA-ligand* typing, we found 2 (2.5%) women were homozygous for Bw4:80I (the ligand for KIR3DL1/S1), 56 (69.1%) were homozygous for non-Bw4:80T and the remaining 23 (28.4%) were heterozygotes at the *HLA-A* locus (**Figure 3.1**). At the *HLA-B* locus, more than half (48/81) the women carried a Bw4 allele, the ligand for KIR3DL1/S1; of which, 39 (48.1%) were heterozygous for Bw6:80N/Bw4:80I, 3 (3.7%) were heterozygous for Bw4:80I/80T and 6 (7.4%) were Bw4:80I homozygous. Bw4:80I/80T allotypes were further divided into Bw4:80I/80T81A and Bw4:80I/80T81L which were present in 2.5% and 1.2% of the women, respectively. Bw4:80T81L homozygotes or Bw4:80T81L/Bw6:80N heterozygotes were not detected in this population. At the *HLA-C* locus, 13 (16.0%) women were homozygous for C1:80N, 30 (37.1%) were homozygous for C2:80K and the remaining 38 (46.9%) were C1/C2 heterozygous.

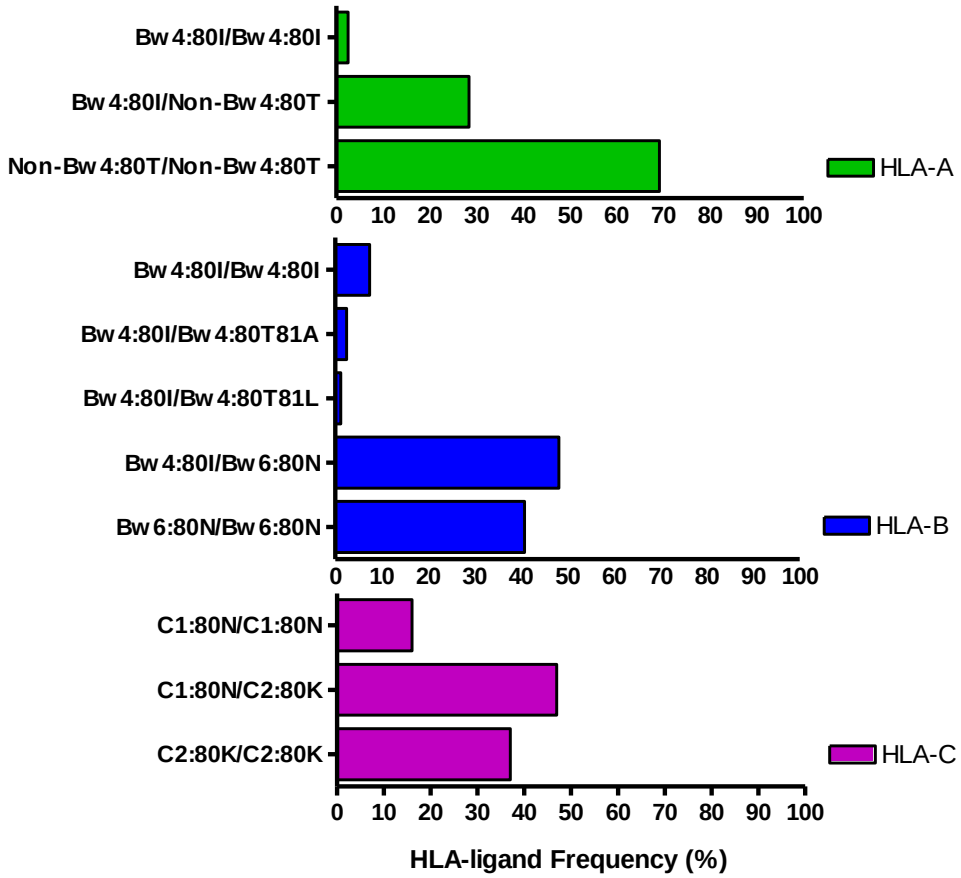


Figure 3.1 Frequencies of various allotype combinations of the HLA-ligands in a South African Cohort (n=81). On the basis of allelic discrimination at codon 80 the HLA-ligand assay can identify the presence/absence of the HLA-ligands, HLA-A (Bw4:80I and non-Bw4:80T) and HLA-B (Bw4:80I, Bw4:80T81A, Bw4:80T81L and Bw6:80N) and HLA-C (C1:80N and C2:80K).

Furthermore when analysing *KIR* and *HLA-ligand* data together (**Table 3.4**), the frequency of activating *KIR/HLA-ligand* pairs was lower than that of the inhibitory *KIR/HLA-ligand* pairs. Activating *KIR2DS1* paired with HLA-C C2 ligand was found at a frequency of 16% and *KIR3DS1* paired with the putative HLA-B Bw4 ligand was present in 6.2% of individuals. Inhibitory *KIR* genes and their ligands: *KIR2DL1*+HLA-C C2, *KIR2DL2/3*+HLA-C C1 and *KIR3DL1*+HLA-A/B Bw4 had frequencies of 84%, 44%, 44% and 58%, respectively.

Table 3.4 *KIR/HLA-ligand* frequencies in a South African cohort (N=81)

***KIR* Gene**		***KIR* Gene N (%)**	***HLA-ligand***	***HLA-ligand* N (%)**	***KIR+HLA-ligand* N (%)**
Inhibitory *KIR*	2DL1	81 (100)	C2	68 (84)	68 (84)
	2DL2	56 (69)	C1	51 (63)	36 (44)
	2DL3	6 (74)	C1	51 (63)	36 (44)
	3DL1	80 (99)	Bw4	58 (72)	47 (58)
Activating *KIR*	2DS1	19 (23)	C2	68 (84)	13 (16)
	3DS1	10 (12)	Bw4*	58 (72)	5 (6)

Frequency of each *KIR+HLA-ligand* combination is defined as the number of individuals having the paired *KIR/HLA-ligand* (N) divided by the number of individuals within the cohort (N=81) expressed as a percentage. *Putative HLA-ligand for KIR3DS1 based on homology to KIR3DL1.

3.3 Discussion

Conventional *KIR* and *HLA* genotyping methodologies involve gel-based SSP-PCR and SBT methods, respectively. These methods can be expensive, labour-intensive and time-consuming. We developed a fast and effective tool to determine *KIR* and *HLA-ligand* profiles using real-time PCR and SYBR® Green chemistry. Our *KIR* and *HLA-ligand* assays were validated against commercial and published methods and were found to be 100% and 99.1% concordant, respectively.

The advantage of our real-time PCR assays (Chapter 2, section 2.5.2) compared to commercial gel-based *KIR* SSP-PCR and *HLA* SBT methods as well as other published KIR/HLA-ligand typing methods included low DNA input, simple technique and ease of result interpretation. Melt curve analysis offers straightforward determination of the presence or absence of *KIRs* or known *HLA-ligands* for KIRs, thus generating results much faster than conventional methods. Furthermore, to enhance the detection of *KIR* genes that may contain sequence variations in the primer binding sites, multiple primers were used for all except three *KIR* genes (**Chapter 2, Table 2.1**). In addition, each of the real-time PCR assays

utilised a single internal positive control gene with a melting peak significantly lower than the *KIR* and *HLA-ligand* amplicon melting peak (**Chapter 2, Figure 2.3**). This enabled accurate discrimination between the absence of a target gene and a failed PCR reaction. Most importantly, provided that the users have access to a real-time PCR instrument, the *KIR* and *HLA-ligand* assays proved to be very cost effective; costing as little as 15 – 20% of the price of commercial *KIR* and SBT *HLA* typing kits, and even in comparison to other real-time PCR methods that made use of fluorescent probes (Koehler et al., 2009). Nevertheless, with respect to the HLA *SBT* genotyping one must keep in mind the level of allele-specific detail that sequencing provides as opposed to the real-time HLA-ligand allotype designations (*HLA-A/B Bw4* and *HLA-C C1/C2*).

A limitation of the KIR and HLA-ligand assay design was that due to the large number of *KIR* and *HLA* genes described and allelic variation within these, certain alleles can be either undetected or misclassified. The KIR primers used in our KIR assay were able to detect all known allelic variants, with the exception of two *KIR3DP1* alleles (*KIR3DP1*004* and *KIR3DP1*009:02*). With regards to the HLA-ligand assay, the HLA-ligand primers were designed to discriminate Bw4 from Bw6 at codon 80, of which amino-acid residues at position 77 and 80-83 are important in defining the Bw4 (NXXIALR, NXXTALR, SXXIALR, DXXTLLR and SXXTLLR) and Bw6 (SXXNLRG and GXXNLRG) amino-acid motifs (Gumperz et al., 1995, Gumperz et al., 1997). Therefore, the limitation of our approach is that unclassified *HLA-B* alleles such as *HLA-B*44:11*, *B*51:50*, *B*57:08* and *HLA-A*24:61* that contain amino-acid changes within the Bw4 motif will be classified as Bw4. Likewise, unclassified *HLA-B* alleles such as *B*07:11*, *B*07:57*, *B*08:17*, *B*35:56*, *B*39:20*, *B*40:37*, *B*55:12*, *B*78:06*, *B*95:35*, *B*08:29*, *B*27:42*, *B*37:05*, *B*25:52*, *B*37:14*, *B*47:03*, *B*40:76* and *B*41:05* that have amino-acid changes that are divergent from the Bw6 motifs will be classified as Bw6. Moreover a number of *HLA-B* alleles that encode the C1 amino-acid motif (*B*07:13*, *B*07:15*, *B*08:15*, *B*15:57*, *B*18:06*, *B*35:74*, *B*39:27*, *B*40:73*, *B*46:01-18*, *B*55:03*, *B*67:02* and *B*73:01*) and will be misclassified as Bw6 allotypes by our assay. Therefore, complementary *HLA-A* SBT genotyping would be necessary in the geographic regions where these genes have high frequencies, for example *HLA-B46*01* in Asian populations (Gonzalez-Galarza et al., 2011).

Thus following validation of the real-time PCR *KIR* and *HLA-ligand* assays, these assays were applied to a small South African sample set of 81 Black individuals. We found similar

KIR gene frequencies as compared to other published South African cohorts (Wong et al., 2010, Paximadis et al., 2011), wherein the AA1 and Bx21 genotype (25% and 14%, respectively) were the most frequent *KIR* genotypes. In addition, we also identified one unique Bx genotype (*Bx**, that had all *KIR* genes present with the exception of *KIR2DL2*, *KIR2DL3*, *KIR2DS1*, *KIR2DS2* and *KIR3DS1*) that at the time of publication had not been reported (Hong et al., 2011). This genotype has since been classified as Bx555 and was also reported in low frequency (<1%) in a Northeast Indian Adivasi population (Dutta et al., 2013).

To conclude, several interactions between KIRs and their HLA-ligands have been found to be associated with a variety of outcomes in autoimmune and infectious diseases (Qi et al., 2006, Kulkarni et al., 2008, Martin and Carrington, 2008, Rajalingam, 2012), highlighting the importance of their systematic characterization in disease association studies. We describe two easy and inexpensive real-time PCR assays for the detection of *KIR* and *HLA-ligands* that were optimised and validated against commercial and published methods. These two assays allow for high throughput data generation with straightforward determination of the presence/absence of *KIRs* and their known *HLA-ligands*. Furthermore, using real-time PCR input DNA is less stringent requiring as little as 5ng of genomic DNA and allowing typing of samples with limited genomic DNA. Therefore, given the availability of small volumes of buffy coat samples and/or blood spots for some infants on MTCT2, these methods were therefore used to obtain *KIR* and *HLA-ligand* genotype data for this particular cohort.

CHAPTER 4

KIR and their *HLA-ligands* in risk of vertical transmission of HIV-1

4.1 Introduction

There are many factors contributing to MTCT of HIV-1 and a better understanding of these mechanisms is crucial for the design of interventions other than ART for prevention of HIV-1 transmission. Current evidence strongly suggests that NK cells and their KIR/HLA-ligand interactions have an important role in modulating HIV-1 disease susceptibility as well as disease progression (Khakoo and Carrington, 2006, Qi et al., 2006, Carrington et al., 2008, Jamil and Khakoo, 2011, Carrington and Alter, 2012, Jost and Altfeld, 2013, Martin and Carrington, 2013).

In the context of HIV-1 disease progression, Martin et al. (2002) showed that HIV-1 infected individuals who possessed the activating *KIR3DS1* gene in combination with *HLA-Bw4* with an isoleucine at position 80 (*HLA-Bw4:80I*) progressed significantly more slowly to AIDS. However, in the absence of *HLA-Bw4:80I* alleles, *KIR3DS1* significantly associated with more rapid progression to AIDS (Martin et al., 2002). This protective effective effect of *KIR3DS1+HLA-Bw4* was further strengthened by evidence that individuals carrying both *KIR3DS1* and *HLA-Bw4:80I* suppress HIV-1 replication *in vitro* to a greater extent than NK cells from individuals carrying *KIR3DS1* in the absence of *HLA-Bw4:80I*, or vice versa, i.e. carrying *HLA-Bw4:80I* in the absence of *KIR3DS1* (Alter et al., 2007a). In addition, an increased *KIR3DS1* count generated by copy number variants of *KIR3DL1/S1* associated with a lower viral set point in the presence of *HLA-Bw4:80I* alleles (Pelak et al., 2011). Taken together, these data support a role for KIR3DS1+ve NK cells in conjunction with their putative ligand Bw4:80I in restricting HIV-1 infection (Carrington and Alter, 2012, Korner and Altfeld, 2012). Likewise, the inhibitory counterpart, *KIR3DL1*, also associated with delayed HIV-disease progression. Co-carriage of high expression *KIR3DL1* genotypes with HLA-B*57 (a *Bw4:80I* allele) were found to associate with both slow HIV disease progression and protection from infection (Martin et al., 2007, Boulet et al., 2008a).

Other data supporting the fact that KIR/HLA combinations have a major role in HIV-1 infection are that HIV-1 has devised strategies to evade recognition by NK cells (Carrington and Alter, 2012). Although the HIV-1 Nef protein has been shown to be able to downregulate

HLA-A and HLA-B expression on the surface of infected cells in order to escape CD8+ T cell recognition and lysis, HLA-C and HLA-E expression were not significantly affected (Cohen et al., 1999). It was proposed that the selective downregulation allows HIV-1-infected cells to avoid NK cell-mediated lysis and may represent, for HIV-1, a balance between escape from CTL and maintenance of protection from NK cells since HLA-C molecules represent the ligands for the inhibitory KIRs and HLA-E is the ligand for the other inhibitory NK cell receptor CD94/NKG2A (Martin and Carrington, 2013). In addition, it was also reported that KIR-associated HIV-1 sequence polymorphisms can enhance the binding of inhibitory KIRs to HIV-1-infected CD4+ T cells, leading to reduced antiviral activity of KIR+ve NK cells (Alter et al., 2011). It was shown that KIR+ve NK cells can place immunological pressure on HIV-1, and that the virus can evade such NK cell mediated immune pressure by selecting for such sequence polymorphisms.

With regards to resistance to HIV-1 transmission, Jennes et al. (2006) reported that certain inhibitory *KIR* genes in the absence of their matching ligand were associated with the HIV-1 negative status of female sex workers (FSW) from Abidjan, Côte d'Ivoire. HIV-1-exposed seronegative FSW had increased frequencies of *KIR2DL2/KIR2DL3* heterozygosity in the absence of the HLA-C1 ligand (i.e., *C2/C2* homozygous), *KIR3DL1* homozygosity in the absence of HLA-Bw4 (i.e., *Bw6* homozygous) and also possessed a greater proportion of *Bx* genotypes (which meant a higher number of activating *KIR*); whilst HIV-1-seropositive FSW were characterized by *KIR2DL3* homozygosity together with *HLA-C1* and a trend toward *KIR3DL1/HLA-Bw4* homozygosity (Jennes et al., 2006b). The authors postulated that the absence of HLA ligands for inhibitory KIR may lower the threshold for NK cell activation via activating KIR, resulting in NK cytotoxic activity and early elimination of HIV-1-infected cells.

Moreover, *KIR/HLA* gene combinations between index and recipient partners have also been found to influence HIV-1 transmission. In Senegalese HIV-1 discordant and concordant couples, HIV-1 acquisition was associated with the *KIR/HLA* genotype of the recipient partners and transmission correlated with recipient/index allogeneic *KIR/HLA* combinations predictive of recipient NK cell alloreactivity towards index target cells (Jennes et al., 2013). In particular, it was noted that HIV-1-discordant couples were characterized by recipient partners with homozygous *KIR2DL2*, and by a mismatched recipient partner *KIR2DL1/HLA-C2* with index partner *HLA-C1/C1* combination, expected to allow licensed

‘missing self’ NK cell killing of index partners' cells. However, HIV-1-concordant couples were characterized by *KIR2DL3* homozygous recipient partners with *HLA-C1/C2* bearing index partners, resulting in a matched KIR/HLA combination expected to inhibit NK cell killing. Jennes et al. (2013) concluded that HIV-1 transmission and/or the lack thereof in the HIV-1 concordant and discordant couples was associated with mismatched KIR/HLA ligand combinations predictive of alloreactive NK cells targeting incoming HIV-1 infected index cells. Similarly, along these lines, NK cell alloreactivity has been used in the treatment of leukaemia (Ruggeri et al., 2005). In this setting, donor-versus-recipient NK cell alloreactivity was induced when there was a mismatch between donor NK clones, carrying specific inhibitory receptors for self MHC class I molecules, and MHC class I ligands on recipient cells, which resulted in donor NK clones recognizing ‘missing self HLA’ on recipient cells and inducing alloreactivity towards residual leukaemic cells. In this manner haematopoietic transplantation from NK alloreactive donors cells were associated with clearance of leukaemia, reduction in rate of relapse as well as improved engraftment without causing graft-versus-host disease (Ruggeri et al., 2002, Ruggeri et al., 2005).

Overall a number of studies have associated KIR/HLA with the outcome of HIV-1 infection (mainly concerning heterosexual transmission), however only one study thus far has associated *KIR/HLA* genotypes with mother-to-child transmission (MTCT) of HIV-1 (Paximadis et al., 2011), wherein *KIR2DL2* and *KIR2DL3* (and their HLA-C ligands) emerged as important factors in both maternal transmission and infant acquisition of HIV-1. Representation of *KIR2DL3* homozygosity alone and in combination with C1/C2 allotypes was significantly higher in intrapartum transmitting (IP-TR) mothers compared to non-transmitting (NT) mothers (P=0.034, OR=2.42 and P=0.01, OR=3.63 respectively) and after adjustment for maternal VL (P=0.033, OR=2.70 and P=0.027, OR=3.26, respectively), suggesting that maternal possession of *KIR2DL3* homozygosity increased the risk of IP transmission of HIV-1 (Paximadis et al., 2011). Yet conversely in infants, possession of *KIR2DL3* in combination with its C1 ligand as well as homozygosity for *KIR2DL3* with *C1C2*, were both found to be significantly higher in exposed uninfected (EU) infants compared to HIV-1-infected infants (P=0.06, OR=0.41 and P=0.038, OR=0.40 respectively) and these associations were stronger after correction for maternal VL (P=0.02, OR=0.47 and P=0.009, OR=0.25, respectively), suggesting that in infants *KIR2DL3* homozygosity in combination with at least one copy of the C1 ligand was protective against HIV-1 acquisition (Paximadis et al., 2011).

Although this study thoroughly assessed the role of *KIR* genes, *KIR* genotypes, *KIR/HLA* ligand associations, and the effect of *KIR2DL3/KIR2DL3+C1/C2* concordance/discordance amongst mother-infant pairs in MTCT of HIV-1, the authors stated that there were a number of areas that required further investigation to elucidate the exact role of *KIR/HLA* on MTCT of HIV-1 (Paximadis et al., 2011). Firstly, since concordance for *KIR3DL3/KIR2DL3+C1/C2* genotype amongst mother and infant showed an association with increased risk for IP transmission, there was a need to further explore the influence of other *KIR/HLA* gene combinations amongst mother-infant pairs. Moreover, this association also suggests that infant allorecognition of maternal cells might play a role in HIV-1 acquisition, similar to the associations reported by Jennes et al. (2011) regarding *KIR* and *HLA* discordance amongst HIV-1 serodiscordant adults. Secondly, Paximadis et al. (2011) also emphasized the need to explore the role of *KIR* allelic variation, as the detection of a particular *KIR* gene does not equate with expression of that gene nor the presence of that gene on the NK cell surface, and thirdly to determine the role of the non-classical HLA-G molecule, the putative ligand for KIR2DL4, a framework *KIR* gene that has both activating and inhibitory potential.

Thus in this chapter, analyses build on the *KIR/HLA* associations with mother-to-child HIV-1 transmission first reported by Paximadis et al. (2011) in the MTCT1 cohort, and establish if the same and/or other *KIR/HLA* gene combinations are associated with vertical transmission in mother-infant pairs from another MTCT cohort (MTCT2). The roles of mother-infant *KIR/HLA* concordance, as well as mother-infant *KIR/HLA* gene combinations that may indicate the potential for infant allorecognition of infected maternal cells were evaluated.

4.2 Results

4.2.1 Mother-infant concordance for *KIR/HLA* in MTCT1

A total of 217 mother-infant pairs from MTCT1 were *KIR* and *HLA-B* and *-C* genotyped using the conventional methods described in Chapter 2, section 2.4 and the findings have been published (Paximadis et al., 2011). However with regards to mother-infant *KIR/HLA* concordance, only one *KIR/HLA* genotype (*KIR2DL3/KIR3DL3+C1/C2*) was evaluated with respect to HIV-1 transmission. Paximadis et al. (2011) reported that two thirds of the mothers, irrespective of transmissibility (i.e. TR or NT), harbouring the *KIR2DL3/KIR3DL3* in combination with *C1/C2* ligand genotype were discordant with their infants (i.e. mother positive for genotype and corresponding infant negative for genotype) compared to a third that were concordant (i.e. the genotype was present in both mother and infant). Here we evaluated if mother-infant concordance for other known inhibitory and activating *KIR/HLA* ligand combinations are associated with MTCT.

We found that mother-infant concordance for the inhibitory *KIR* combination, *KIR2DL1+C1/C2* was significantly over-represented in NT/EU mother-infant pairs compared to IU2-TR/IU mother-infant pairs (**Table 4.1**, *P*=0.041, OR=0.34), which maintained significance post adjustment for mVL (*P*=0.011, OR=0.23) but not after adjustments for sdNVP or CD4+ T cell count (**Table 4.3**). In addition, concordant representation of *2DL3* homozygosity in the absence of C1 ligand (i.e. *C2/C2*) was significantly higher in IP-TR/IP mother-infant pairs compared to NT/EU mother-infant pairs (*P*=0.043, OR=4.48) and this association remained significant after adjusting for mVL as well as maternal sdNVP administration (**Table 4.3**). Of note, concordance for the *KIR2DL3/KIR3DL3+C1/*C2 genotype reported by Paximadis et al. (2011) showed a trend towards increased representation in IP-TR/IP mother-infant pairs compared to NT/EU mother-infant pairs (*P*=0.064, OR=3.71), which gained significance post adjustment for maternal CD4+ T cell count (*P*=0.021, OR=5.62, **Table 4.3**). With regards to the activating *KIR* (*KIR2DS1* and *KIR2DS2*) and their putative HLA ligands (C2 and C1, respectively), we found no significant associations (**Table 4.1**).

4.2.2 Allogeneic *KIR/HLA* combinations in MTCT1

It has been hypothesized that HIV-1 transmission in heterosexual couples is in part dependant on the *KIR/HLA* genotype of the recipient partner, in such a way that resistance to HIV-1 acquisition may be characterized by a mismatched allogeneic KIR/HLA combination and HIV-1 infected cells present in genital secretions of the index partner could be efficiently targeted by the recipients allogeneic NK cell responses (Jennes et al., 2013). Here we evaluated whether the level of allogeneic *KIR/HLA* incompatibility between mothers and infants could determine HIV-1 transmission. Thus we compared frequencies of 'matched' and 'missing self' *KIR/HLA* combinations between NT and TR mother-infant pairs. We found that IU2 infants had significantly greater representation of homozygous *iKIR2DL2+C1/C2* with mothers bearing the *mC2/C2* combination (**Table 4.2**, *P*=0.011, OR=ND), which would theoretically allow licensed 'missing self' NK cell killing of maternal cells through KIR2DL2. However, this association was not maintained post adjustment for mVL, maternal sdNVP or CD4+ T cell count (**Table 4.3**). Nevertheless, when infants were *iKIR2DL2/KIR2DL3* heterozygous (irrespective of their HLA-C ligands) and mothers possessed the *mC1/C1* combination, resulting in a matched KIR/HLA combination, there was increased risk for IU acquisition of HIV-1 (IU: *P*=0.047, OR=4.02 and IU2: *P*=0.019, OR=3.45). Furthermore, these two IU associations maintained significance after adjusting for mVL as well as sdNVP (**Table 4.3**). With regards to allogeneic combinations of *KIR3DL1* and *KIR3DS1* with *HLA-Bw4* between TR (IP, IU and IU2) and NT mother-infant pairs, no significant differences were seen.

Table 4.1 Representation of mother-infant concordance with known *KIR/HLA* ligand combinations in MTCT1, N (%)

***KIR/HLA* ligand**	**NT/EU (N=145)**	**IP-TR/IP (N=29)**	**IU-TR/IU (N=19)**	**IU2-TR/IU2 (N=43)**	**IP-TR/IP vs. NT**		**IU-TR/IU vs. NT**		**IU2-TR/IU2 vs. NT**	
					OR (95% CI)	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
2DL1+C1/C1	10 (6.90)	1 (3.45)	3 (15.79)	5 (11.63)	0.48 (0.06-3.92)	0.694	2.53 (0.63-10.2)	0.367	1.78 (0.57-5.51)	0.340
2DL1+C1/C2	40 (27.59)	7 (24.14)	3 (15.79)	5 (11.63)	0.83 (0.33-2.11)	0.821	0.49 (0.14-1.78)	0.406	0.34 (0.12-0.93)	**0.041**
2DL1+C2/C2	24 (16.55)	6 (20.69)	2 (10.53)	7 (16.28)	1.31 (0.48-3.57)	0.789	0.59 (0.13-2.73)	0.547	0.98 (0.39-2.46)	1.000
2DL2/2DL2+C1/C1	1 (0.69)	0 (0.00)	0 (0.00)	1 (2.33)	-	1.000	-	1.000	3.42 (0.21-55.9)	0.406
2DL2/2DL2+C1/C2	7 (4.83)	0 (0.00)	1 (5.26)	1 (2.33)	-	0.364	1.09 (0.13-9.42)	1.000	0.47 (0.06-3.93)	0.685
2DL2/2DL2+C2/C2	2 (1.38)	0 (0.00)	0 (0.00)	1 (2.33)	-	1.000	-	1.000	1.70 (0.15-19.2)	1.000
2DL2/2DL3+C1/C1	5 (3.45)	1 (3.45)	1 (5.26)	1 (2.33)	1.00 (0.11-8.89)	1.000	1.56 (0.17-14.1)	1.000	0.67 (0.07-5.86)	1.000
2DL2/2DL3+C1/C2	13 (8.97)	2 (6.90)	0 (0.00)	0 (0.00)	0.75 (0.16-3.53)	1.000	-	0.236	-	0.078
2DL2/2DL3+C2/C2	6 (4.14)	1 (3.45)	0 (0.00)	0 (0.00)	0.83 (0.09-7.14)	1.000	-	0.616	-	0.339
2DL3/2DL3+C1/C1	3 (2.07)	0 (0.00)	1 (5.26)	1 (2.33)	-	1.000	2.62 (0.26-26.6)	0.392	1.13 (0.11-11.1)	1.000
2DL3/2DL3+C1/C2	6 (4.14)	4 (13.79)	0 (0.00)	0 (0.00)	3.71 (0.97-14.1)	**0.064**	-	0.616		0.339
2DL3/2DL3+C2/C2	5 (3.45)	4 (13.79)	1 (5.26)	3 (6.98)	4.48 (1.12-17.8)	**0.043**	1.56 (0.17-14.1)	1.000	2.10 (0.48-9.17)	0.386
3DL1/3DL1+Bw4/Bw4	8 (5.52)	2 (6.90)	2 (10.53)	4 (9.30)	1.26 (0.25-6.30)	1.000	2.01 (0.39-10.3)	0.607	1.75 (0.50-6.14)	0.475
3DL1/3DL1+Bw4/Bw6	25 (17.24)	6 (20.69)	4 (21.05)	7 (16.28)	1.25 (0.46-3.39)	0.790	1.28 (0.39-4.18)	0.749	0.93 (0.37-2.33)	1.000
3DL1/3DL1+Bw6/Bw6	27 (18.62)	8 (27.59)	2 (10.53)	8 (18.60)	1.60 (0.66-4.15)	0.311	0.51 (0.11-2.35)	0.531	0.99 (0.42-2.39)	1.000
3DL1/3DS1+Bw4/Bw4	1 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
3DL1/3DS1+Bw4/Bw6	1 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
3DL1/3DS1+Bw6/Bw6	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
2SD1+C1/C1	1 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
2SD1+C1/C2	1 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
2SD1+C2/C2	1 (0.69)	1 (3.45)	0 (0.00)	0 (0.00)	5.14 (0.31-84.7)	0.306	-	1.000	-	1.000
2SD2+C1/C1	6 (4.14)	1 (3.45)	1 (5.26)	3 (6.98)	0.82 (0.09-7.14)	1.000	1.29 (0.15-11.3)	1.000	1.73 (0.41-7.26)	0.687
2SD2+C1/C2	21 (14.48)	2 (6.90)	2 (10.53)	3 (6.98)	0.44 (0.09-1.98)	0.375	0.69 (0.15-3.23)	0.746	0.44 (0.12-1.56)	0.297
2SD2+C2/C2	12 (8.28)	3 (10.34)	0 (0.00)	1 (2.33)	1.27 (0.34-4.85)	1.000	-	0.363	0.26 (0.03-2.09)	0.304

NT/EU, non-transmitting mother and her exposed uninfected infant; IP-TR/IP, intrapartum transmitting mother and her infant; IU-TR/IU, *in utero* transmitting mother and her infant; IU2-TR/IU2, *in utero* enriched transmitting mother and her infant. Bold and highlighted *P* values indicate significant differences ($P<0.05$).

Table 4.2 Frequencies of allogeneic combinations of *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR3DL1* and *KIR3DS1* in recipient infants with *HLA-ligands* (*C1/C2* or *Bw4/Bw6*, respectively) in index mothers in MTCT1, N (%)

Type*	Recipient (Infant)		Index (Mother)	EU	IP	IU	IU2	IP vs. EU		IU vs. EU		IU2 vs. EU	
	KIR	*HLA*	*HLA*	(N=145)	(N=29)	(N=19)	(N=43)	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Matched	*2DL1*	Any	*C1/C2*	71 (48.97)	18 (62.07)	7 (36.84)	15 (34.88)	1.71 (0.75-3.86)	0.226	0.61 (0.23-1.63)	0.342	0.56 (0.28-1.13)	0.119
Matched	*2DL1*	Any	*C2/C2*	53 (36.55)	10 (34.48)	6 (31.58)	16 (37.21)	0.91 (0.39-2.11)	1.000	0.80 (0.29-2.23)	0.802	1.03 (0.51-2.08)	1.000
Missing self	*2DL1*	*C1/C2*	*C1/C1*	10 (6.90)	0 (0.00)	2 (10.53)	6 (13.95)	-	0.217	1.58 (0.32-7.86)	0.633	2.19 (0.75-6.42)	0.208
Missing self	*2DL1*	*C2/C2*	*C1/C1*	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
Matched	*2DL2/2DL2*	Any	*C1/C1*	3 (2.07)	0 (0.00)	0 (0.00)	2 (4.65)	-	1.000	-	1.000	2.30 (0.37-14.2)	0.590
Matched	*2DL2/2DL2*	Any	*C1/C2*	19 (13.10)	1 (3.45)	5 (26.32)	7 (16.28)	0.24 (0.03-1.84)	0.204	2.36 (0.76-7.32)	0.161	1.28 (0.50-3.31)	0.618
Missing self	*2DL2/2DL2*	*C1/C1*	*C2/C2*	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
Missing self	*2DL2/2DL2*	*C1/C2*	*C2/C2*	0 (0.00)	0 (0.00)	1 (5.26)	3 (6.98)	-	1.000	-	0.116	-	**0.011**
Matched	*2DL2/2DL3*	Any	*C1/C1*	9 (6.21)	1 (3.45)	4 (21.05)	8 (18.60)	0.54 (0.06-4.43)	0.700	4.02 (1.11-14.7)	**0.047**	3.45 (1.24-9.60)	**0.019**
Matched	*2DL2/2DL3*	Any	*C1/C2*	33 (22.76)	8 (27.59)	2 (10.53)	6 (13.95)	1.29 (0.52-3.19)	0.633	0.40 (0.09-1.81)	0.257	0.55 (0.21-1.42)	0.285
Missing self	*2DL2/2DL3*	*C1/C1*	*C2/C2*	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
Missing self	*2DL2/2DL3*	*C1/C2*	*C2/C2*	12 (8.28)	3 (10.34)	0 (0.00)	3 (6.98)	1.28 (0.34-4.85)	1.000		0.363	0.83 (0.22-3.09)	1.000
Matched	*2DL3/2DL3*	Any	*C1/C1*	8 (5.52)	0 (0.00)	1 (5.26)	1 (2.33)	-	0.355	0.95 (0.11-8.05)	1.000	0.41 (0.05-3.35)	0.471
Matched	*2DL3/2DL3*	Any	*C1/C2*	20 (13.79)	8 (27.59)	0 (0.00)	2 (4.65)	2.38 (0.92-6.10)	0.093	-	0.133	0.30 (0.07-1.36)	0.114
Missing self	*2DL3/2DL3*	*C1/C1*	*C2/C2*	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
Missing self	*2DL3/2DL3*	*C1/C2*	*C2/C2*	17 (11.72)	0 (0.00)	3 (15.79)	3 (6.98)	-	0.080	1.41 (0.37-5.35)	0.707	0.56 (0.16-2.02)	0.422
Matched	*3DL1/3DL1*	Any	*Bw4/Bw4*	21 (14.48)	4 (13.79)	4 (21.05)	7 (16.28)	0.94 (0.29-2.99)	1.000	1.57 (0.47-5.21)	0.496	1.14 (0.45-2.92)	0.808
Matched	*3DL1/3DL1*	Any	*Bw4/Bw6*	60 (41.38)	12 (41.38)	6 (31.58)	14 (32.56)	1.00 (0.44-2.24)	1.000	0.65 (0.23-1.82)	0.466	0.68 (0.33-1.40)	0.375
Missing self	*3DL1/3DL1*	*Bw4/Bw4*	*Bw6/Bw6*	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
Missing self	*3DL1/3DL1*	*Bw4/Bw6*	*Bw6/Bw6*	25 (17.24)	4 (13.79)	4 (21.05)	7 (16.28)	0.77 (0.24-2.40)	0.789	1.28 (0.39-4.18)	0.749	0.93 (0.37-2.33)	1.000
Matched	*3DL1/3DS1*	Any	*Bw4/Bw4*	2 (1.38)	0 (0.00)	0 (0.00)	2 (4.65)	-	1.000	-	1.000	3.48 (0.47-25.5)	0.225
Matched	*3DL1/3DS1*	Any	*Bw4/Bw6*	6 (4.14)	0 (0.00)	1 (5.26)	2 (4.65)	-	0.591	1.28 (0.15-11.3)	1.000	1.13 (0.22-5.81)	1.000
Missing self	*3DL1/3DS1*	*Bw4/Bw4*	*Bw6/Bw6*	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
Missing self	*3DL1/3DS1*	*Bw4/Bw6*	*Bw6/Bw6*	2 (1.38)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000

EU, exposed uninfected infants; IP, intrapartum infected infants; IU, *in utero* infected infants; IU2, IU-enriched group of infants. *According to models described by Moretta et al. (2011) and Jennes et al. (2013): 'Matched' occurs when the specified inhibitory *KIR* of the recipient recognizes an *HLA* allele present in the index (regardless of recipient *HLA*) and 'Missing self' occurs when the specified inhibitory *KIR* of the recipient recognizes an *HLA* allele present in the recipient that is lacking in the index. Bold and highlighted *P* values indicate significant differences ($P<0.05$).

Table 4.3 Logistic regression analysis, adjustments made for maternal viral load (mVL), single dose nevirapine (sdNVP) and CD4+ T cell count

Mother-infant	*KIR/HLA* combination	Association	Adjusted mVL		Adjusted sdNVP		Adjusted CD4+ T cell count	
			OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95%CI)	*P*
Concordance	*2DL3/2DL3+C2/C2*	IP-TR/IP vs. NT/EU	8.81 (1.56-49.5)	**0.014**	5.74 (1.29-25.5)	**0.022**	3.30 (0.71-15.2)	0.125
	2DL1+C1/C2	IU2-TR/IU2 vs. NT/EU	0.23 (0.07-0.71)	**0.011**	0.41 (0.15-1.14)	0.088	0.39 (0.14-1.11)	0.077
	2DL3/2DL3+C1/C2	IP-TR/IP vs. NT/EU	2.25 (0.46-11.1)	0.316	3.05 (0.78-11.9)	0.108	5.62 (1.29-24.5)	**0.021**
Allorecognition	*i2DL2/2DL2(C1/C2)+mC2/C2*	IU2 vs. EU	-	1.000	-	1.000	-	1.000
	i2DL2/2DL3(Any)+*mC1/C1*	IU vs. EU	4.64 (1.10-19.5)	**0.036**	4.30 (1.14-16.2)	**0.031**	4.40 (0.99-19.5)	0.051
	i2DL2/2DL3(Any)+*mC1/C1*	IU2 vs. EU	3.26 (1.02-10.3)	**0.045**	3.86 (1.26-11.8)	**0.018**	3.46 (1.16-10.3)	**0.026**

NT/EU, HIV-1 non-transmitting mothers and exposed uninfected infants; IP-TR/IP, intrapartum HIV-1 transmitting mothers and their infants, IU-TR/IU, *in utero* HIV-1 transmitting mothers and their infants; IU2-TR/IU2, *in utero* enriched HIV-1 transmitting mothers and their infants. Bold and highlighted *P* values indicate significant differences ($P<0.05$).

4.2.3 Diversity of *KIR* genes in MTCT2

To determine if *KIR* genes were associated with MTCT of HIV-1 in mother and infants from MTCT2 we used the real-time PCR method described in **Chapter 2, section 2.5**. All 16 *KIR* genes were detected in the MTCT2 cohort and their observed frequencies are shown in **Table 4.4**. The framework genes were found in all individuals whereas all other *KIR* genes were present in varying percentages. Overall, inhibitory *KIR* genes had higher frequencies than activating *KIR* genes, however, comparison of individual *KIR* gene frequencies between TR (IP-TR and IU-TR) and NT mothers, as well as their infants, showed no significant differences (**Table 4.4**). With regards to *KIR* gene numbers, comparison of the total *KIR*, inhibitory *KIR* and activating *KIR* genes between TR and NT mothers as well as their infants showed no significant differences (**Table 4.5**).

Based on *KIR* gene presence or absence, and using the *KIR* allele frequencies database (http://www.allelefrequencies.net), we identified 29 distinct *KIR* genotypes in MTCT2 mothers, of which the three most common *KIR* genotypes were Bx21 (16.3%), AA1 (13.9%) and Bx32 (8.9%) which accounted for 40.5% of the total *KIR* genotypes (**Figure 4.1**). In addition, we found one unique Bx genotype (Bx?) that had not been described in the *KIR* allele frequencies database. This 'Bx?' genotype had a total of 12 *KIR* genes (*2DL1*, *2DL2*, *2DL4*, *2DL5*, *2DS1*, *2DS2*, *2DS5*, *2DP1*, *3DL1*, *3DL2*, *3DL3* and *3DP1*) and was identified in one NT mother. While in the infant group, out of 23 distinct *KIR* genotypes, AA1 was the most common *KIR* genotype with 33.7% representation followed by Bx21 and Bx5 (**Figure 4.1**). Comparison of the total AA1 haplotype frequency between mothers and infants showed that the AA1 haplotype was significantly under-represented in mothers (13.9% vs. 33.7%, *P*=0.005), while the other common *KIR* haplotypes were similar amongst mothers and infants (**Figure 4.1A**). Moreover, within the maternal group, both IP-TR and IU-TR mothers were found to have significantly higher representation of Bx32 genotype compared to NT mothers (**Figure 4.1B**, *P*=0.038 and *P*=0.005, respectively). This finding was similar to the findings reported by Paximadis et al. (2011) where TR mothers had significantly higher representation of Bx32 compared to NT mothers (5.4% vs. 0.7%; *P*=0.04; OR=8.5). Comparison of the Bx32 genotype in relation to Bx21, the other most common Bx genotype in MTCT2 mothers, showed that two genotypes were similar except for the absence of *KIR2DL2* and *KIR2DS2* in Bx32.

Table 4.4 *KIR* gene frequencies in mothers and their infants in MTCT2, N (%)

Maternal *KIR*	NT (N=43)	IP-TR (N=11)	IU-TR (N=25)	IP-TR vs. NT		IU-TR vs. NT	
				OR (95% CI)	*P*	OR (95% CI)	*P*
2DL1	43 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
2DL2	31 (72.09)	7 (63.64)	13 (52.00)	0.68 (0.17-2.74)	0.714	0.42 (0.15-1.17)	0.118
2DL3	34 (79.07)	9 (81.82)	21 (84.00)	1.19 (0.22-6.51)	1.000	1.38 (0.37-5.09)	0.754
2DL4	43 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
2DL5	28 (65.12)	8 (72.73)	18 (72.00)	1.42 (0.33-6.19)	0.733	1.38 (0.47-4.04)	0.602
2DP1	42 (97.67)	10 (90.91)	25 (100.00)	0.24 (0.01-4.14)	0.369	-	1.000
2DS1	8 (18.60)	2 (18.18)	4 (16.00)	0.97 (0.17-5.39)	1.000	0.83 (0.22-3.11)	1.000
2DS2	25 (58.14)	6 (54.55)	12 (48.00)	0.86 (0.23-3.27)	1.000	0.66 (0.24-1.79)	0.458
2DS3	8 (18.60)	3 (27.27)	5 (20.00)	1.64 (0.35-7.60)	0.676	1.09 (0.31-3.80)	1.000
2DS4	40 (93.02)	11 (100.00)	24 (96.00)	-	0.600	1.80 (0.18-18.2)	1.000
2DS5	34 (79.07)	7 (63.64)	15(60.00)	0.46 (0.11-1.93)	0.429	0.39 (0.13-1.17)	0.103
3DL1	43 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
3DL2	43 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
3DL3	43 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
3DP1	43 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
3DS1	4 (9.30)	1 (9.09)	1 (4.00)	0.97 (0.09-9.71)	1.000	0.41 (0.04-3.85)	0.645
Infant *KIR*	**EU (N=44)**	**IP (N=11)**	**IU (N=25)**	**IP vs. EU**		**IU vs. EU**	
				OR (95% CI)	***P***	**OR (95% CI)**	***P***
2DL1	44 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
2DL2	25 (56.82)	8 (72.73)	12 (48.00)	2.03 (0.47-8.68)	0.495	0.70 (0.26-1.87)	0.616
2DL3	40 (90.91)	8 (72.73)	19 (76.00)	0.27 (0.05-1.42)	0.134	0.32 (0.08-1.25)	0.152
2DL4	44 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
2DL5	26 (59.09)	8 (72.73)	15 (60.00)	1.80 (0.43-7.92)	0.502	1.04 (0.38-2.82)	1.000
2DP1	43 (97.73)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
2DS1	6 (13.64)	3 (27.27)	3 (12.00)	2.37 (0.49-11.6)	0.362	0.86 (0.19-3.80)	1.000
2DS2	23 (52.27)	8 (72.73)	2 (48.00)	2.43 (0.57-10.4)	0.314	0.84 (0.31-2.25)	0.805
2DS3	6 (13.64)	3 (27.27)	7 (28.00)	2.37 (0.49-11.6)	0.362	2.46 (0.72-8.39)	0.201
2DS4	43 (97.73)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
2DS5	22 (50.00)	7 (63.64)	12 (48.00)	1.75 (0.45-6.84)	0.510	0.92 (0.34-2.46)	1.000
3DL1	44 (100.00)	11 (100.00)	24 (96.00)	-	1.000	-	0.362
3DL2	44 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
3DL3	44 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
3DP1	44 (100.00)	11 (100.00)	25 (100.00)	-	1.000	-	1.000
3DS1	5 (11.36)	3 (27.27)	2 (8.00)	2.92 (0.58-14.7)	0.335	0.67 (0.12-3.78)	1.000

NT, non-transmitting mother; IP-TR, intrapartum transmitting mother; IU, *in utero* transmitting mother; EU, exposed uninfected infants; IP, intrapartum infected infants; IU, *in utero* infected infants.

Table 4.5 Mann-Whitney U tests of *KIR* gene numbers in mothers and infants from MTCT2

KIR gene number	**Median (range)**			***Mann-Whitney U test, P value***	
Mothers	**NT**	**IP-TR**	**IU-TR**	***IP-TR vs. NT***	***IU-TR vs. NT***
Total	12 (9-15)	12 (9-16)	12 (8-16)	0.855	0.314
Inhibitory	7 (6-8)	7 (6-8)	7 (6-8)	0.923	0.673
Activating	3 (1-5)	2 (1-6)	2 (0-6)	0.554	0.213
Infants	**EU**	**IP**	**IU**	***IP vs. EU***	***IU vs. EU***
Total	12 (9-16)	13 (9-16)	11 (9-15)	0.230	0.793
Inhibitory	7 (6-8)	7 (6-8)	7 (6-8)	0.728	0.243
Activating	2 (1-6)	3 (1-6)	2 (1-6)	0.155	0.920

NT, HIV-1 non-transmitting mother; IP-TR, intrapartum transmitting mother; IU, *in utero* transmitting mother; EU, exposed uninfected infants; IP, intrapartum infected infants; IU, *in utero* infected infants.

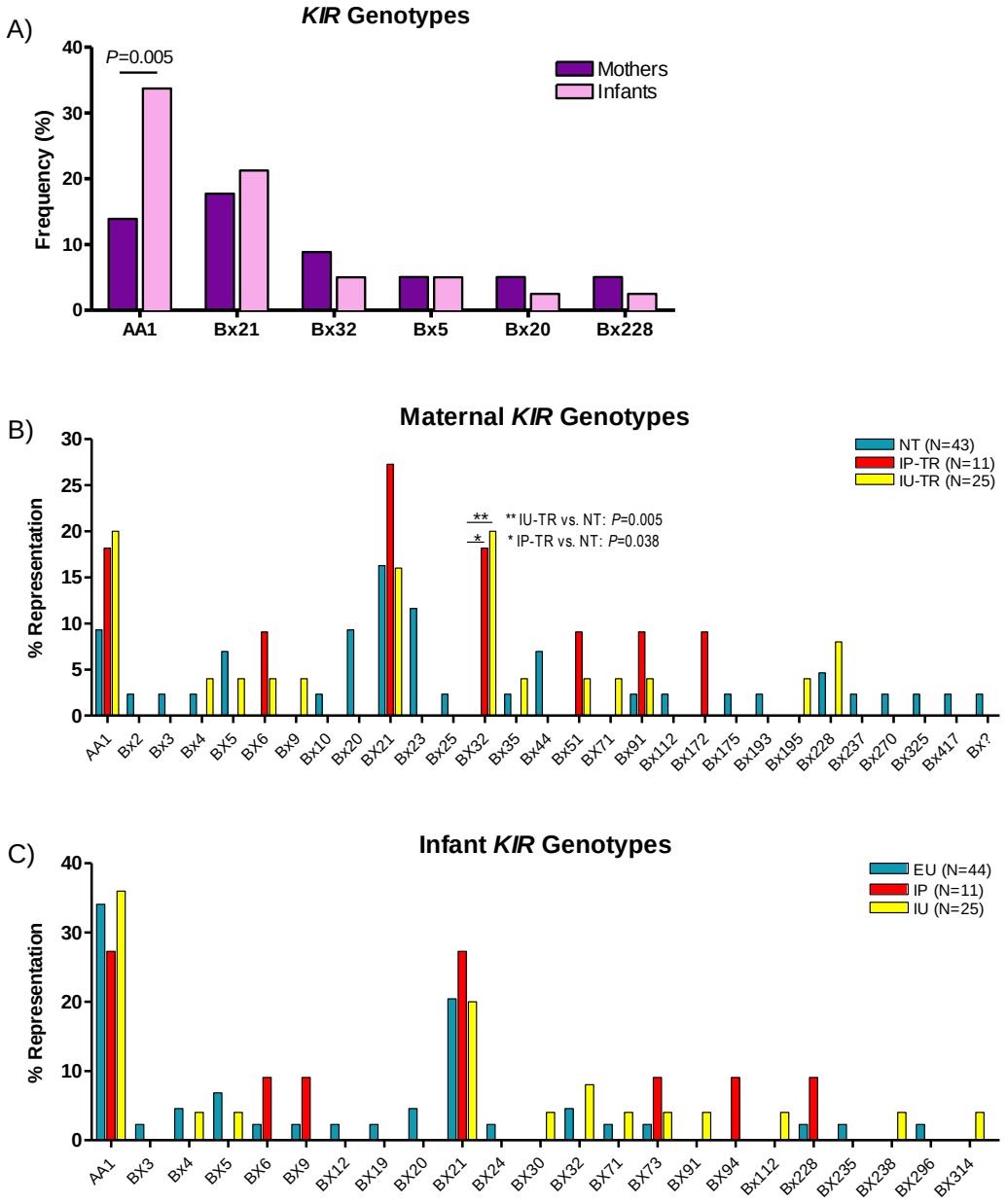


Figure 4.1 *KIR* genotypes in MTCT2. (A) Representation of the most common *KIR* genotypes in mothers and infants from MTCT2, (B) individual *KIR* genotypes in MTCT2 mothers, and (C) individual *KIR* genotypes in MTCT2 infants.

4.2.4 Characterization of HLA-ligands in MTCT2

Given that the majority of KIR function is reliant on their interaction with HLA-ligands, we evaluated the frequency *HLA-ligands* in MTCT2 using the real-time PCR method described in Chapter 2, section 2.5. The observed allele and genotype frequencies for MTCT2 mothers are shown in **Figure 4.2** and **Table 4.6**, respectively. With regards to the major inhibitory HLA ligand, *HLA-C*, mothers were predominantly *C1/C2* heterozygous (43.0%) while the remaining were *C1/C1* homozygous (25.3%) or *C2/C2* homozygous (31.6%). Similarly for *HLA-A* and *-B*, representation of *Bw4/Bw6* heterozygosity was higher compared to *Bw4* and *Bw6* homozygosity (54.4%, 13.9% and 31.6%, respectively). Comparison of *HLA-ligand* frequencies amongst TR and NT mothers showed that maternal possession of H*LA-A Bw4:80I* was associated with increased risk for IU HIV-1 transmission (**Figure 4.2**). IU-TR mothers had significantly higher representation of *HLA-A Bw4:80I* than NT mothers (*P*=0.005, OR=3.67) and conversely, possession of *non-Bw4 HLA-A* alleles was found to be protective in NT mothers (*P*=0.005, OR=0.27). Furthermore, analysis of *HLA-A* ligand genotypes showed representation of *Bw4:80I/Non-Bw4* heterozygosity was significantly higher in IP-TR and IU-TR mothers compared to NT mothers (*P*=0.025, OR=7.61 and *P*=0.031, OR=5.18, respectively), whilst *non-Bw4* homozygosity was elevated in NT mothers (*P*=0.009, OR=0.21). Comparison of *HLA-B* ligand alleles showed an association with reduced risk for HIV-1 transmission; *HLA-B Bw4:80T* allele frequency was significantly higher in NT mothers compared to IU-TR mothers (*P*=0.030, OR=0.20), but *HLA-B* genotypes did not significantly differ amongst NT and TR mothers. In contrast, *HLA-C* genotype frequencies showed no differences amongst MTCT2 mothers (**Figure 4.2**).

Similarly within the infant group (**Figure 4.3**), representation of *HLA-A Bw4:80I* allele was significantly higher in IU infants compared to EU infants (*P*=0.001, OR=7.99) of which the heterozygous *HLA-A Bw4:80I/Non-Bw4* genotype was also significantly higher in IU infants compared to EU infants (**Table 4.6**: *P*=0.003, OR=7.69), whereas the absence of HLA-A Bw4 ligand (*Non-Bw4/Non-Bw4*) was associated with protection from IU acquisition of HIV-1 (*P*=0.001, OR=0.11). However, no other significant differences were detected when comparing infant *HLA-B* and *HLA-C* alleles' and/or genotypes in those infants that did or did not acquire HIV-1.

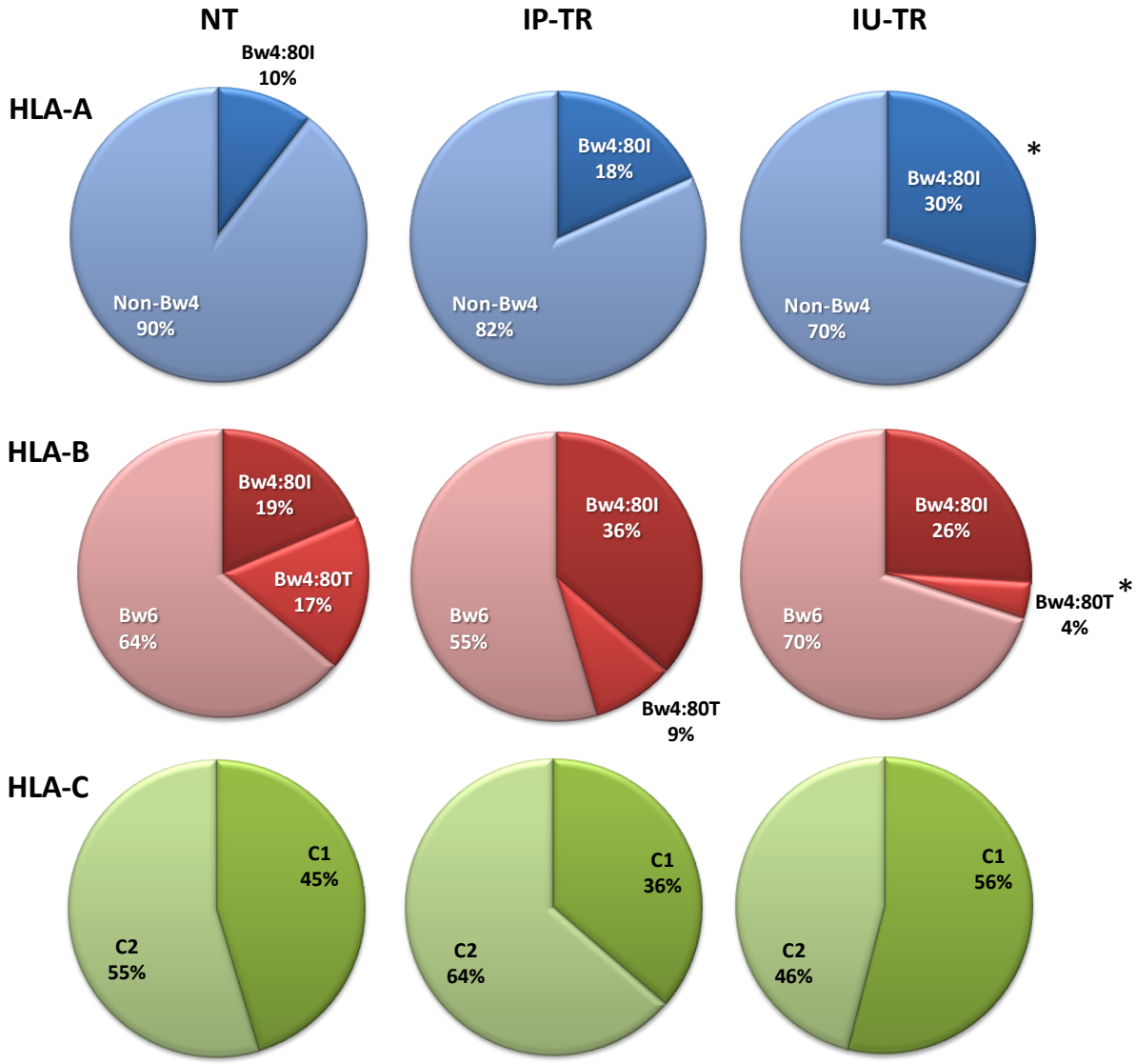


Figure 4.2 Maternal allele frequencies for HLA-A, -B and –C ligands in non-transmitting (NT), intrapartum transmitting (IP-TR) and *in utero* transmitting (IU-TR) mothers from MTCT2. *The frequency of HLA-A Bw4:80I was significantly higher in IU-TR mothers compared to NT mothers (*P*=0.005, OR=3.67, CI: 1.46-9.18); whereas the frequency of HLA-B Bw4:80T was significantly lower in IU-TR mothers compared to NT mothers (*P*=0.030, OR=0.20, CI: 0.04-0.90).

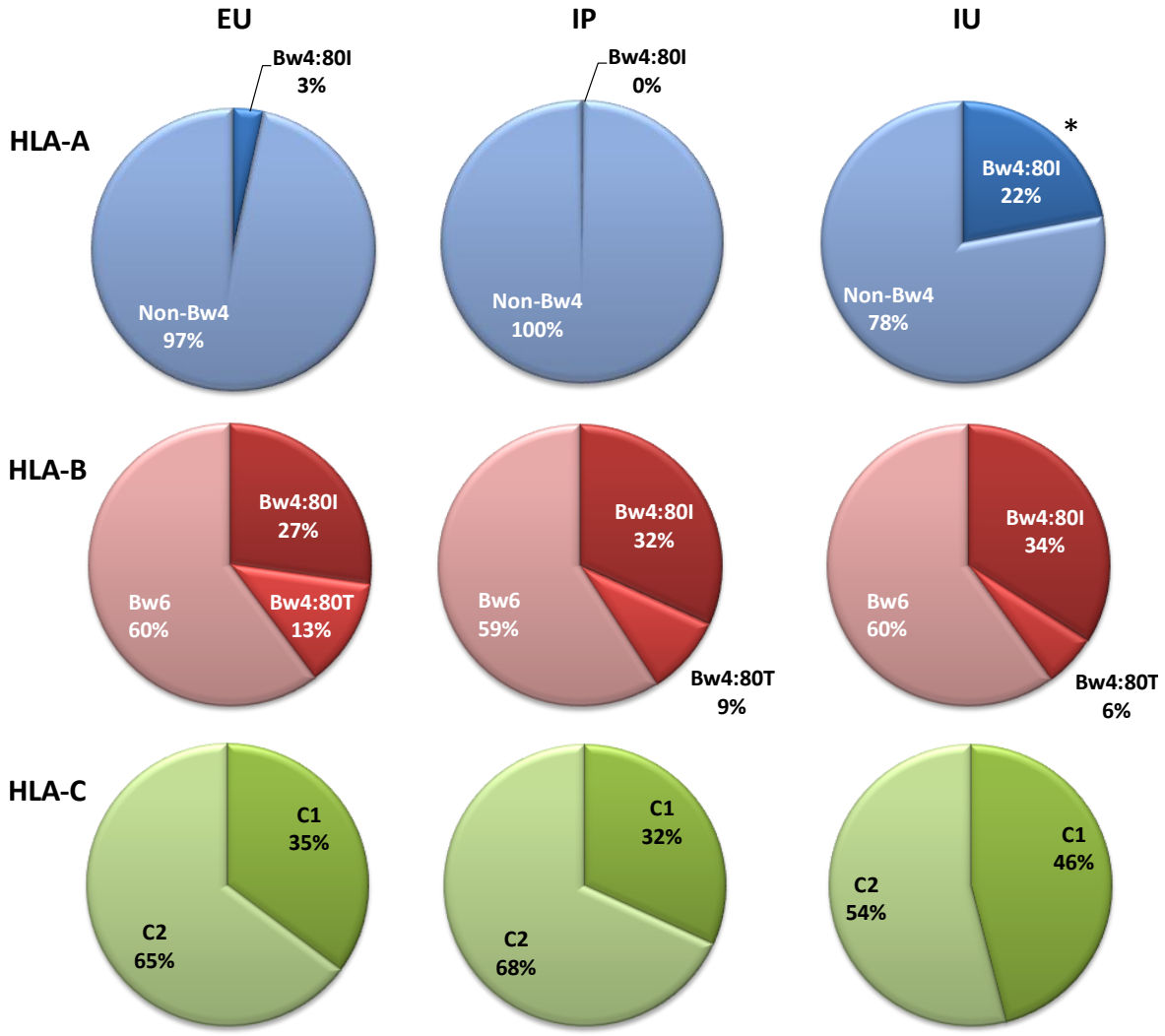


Figure 4.3 Infant allele frequencies for HLA-A, -B and -C ligands in exposed uninfected (EU), intrapartum (IP) and *in utero* (IU) infected infants from MTCT2. *The frequency of HLA-A Bw4:80I was significantly higher in IU infants compared to EU infants (*P*=0.001, OR=7.99, CI: 2.11-30.3)

Table 4.6 Representation of mother and infant genotype frequencies for HLA-A, -B and –C ligands in MTCT2

Mothers		NT (N=43)	IP-TR (N=11)	IU-TR (N=25)	IP-TR vs. NT OR (95% CI)	*P*	IU-TR vs. NT OR (95% CI)	*P*
HLA-A	*Bw4/Bw4*	3 (6.98)	0 (0.00)	4 (16.00)	-	0.600	2.53 (0.52-12.4)	0.409
	Bw4/Non	3 (6.98)	4 (36.36)	7 (28.00)	7.61 (1.39-41.7)	**0.025**	5.18 (1.20-22.3)	**0.031**
	Non/Non	37 (86.05)	7 (63.64)	14 (56.00)	0.28 (0.06-1.27)	0.185	0.21 (0.06-0.66)	**0.009**
HLA-B	*Bw4/Bw4*	6 (13.95)	3 (27.27)	2 (8.00)	2.31 (0.47-11.2)	0.367	0.54 (0.10-2.88)	0.700
	Bw4/Bw6	19 (44.19)	4 (36.36)	11 (44.00)	0.72 (0.18-2.83)	0.741	0.99 (0.36-2.67)	1.000
	Bw6/Bw6	18 (41.86)	4 (36.36)	12 (48.00)	0.79 (0.20-3.12)	1.000	1.28 (0.47-3.45)	0.800
HLA-A/B	*Bw4/Bw4*	8 (18.60)	3 (27.27)	5 (20.00)	1.64 (0.35-7.60)	0.676	1.09 (0.31-3.80)	1.000
	Bw4/Non	20 (46.51)	6 (54.55)	15 (60.00)	1.38 (0.36-5.21)	0.741	1.72 (0.63-4.68)	0.323
HLA-C	*C1/C1*	11 (25.58)	2 (18.18)	7 (28.00)	0.65 (0.12-3.46)	0.715	1.13 (0.37-3.43)	1.000
	C1/C2	17 (39.53)	4 (36.36)	13 (52.00)	0.87 (0.22-3.44)	1.000	1.65 (0.61-4.47)	0.448
	C2/C2	15 (34.88)	5 (45.45)	5 (20.00)	1.56 (0.41-5.95)	0.728	0.47 (0.15-1.49)	0.272
Infants		**EU (N=44)**	**IP (N=11)**	**IU (N=25)**	**IP vs. EU OR (95% CI)**	***P***	**IU vs. EU OR (95% CI)**	***P***
HLA-A	*Bw4/Bw4*	0 (0.00)	0 (0.00)	1 (4.00)	-	1.000	-	0.362
	Bw4/Non	3 (6.82)	0 (0.00)	9 (36.00)	-	1.000	7.69 (1.84-32.1)	**0.003**
	Non/Non	41 (93.18)	11 (100.00)	15 (60.00)	-	1.000	0.11 (0.03-0.45)	**0.001**
HLA-B	*Bw4/Bw4*	7 (15.91)	1 (9.09)	4 (16.00)	0.53 (0.06-4.81)	0.681	1.01 (0.26-3.84)	1.000
	Bw4/Bw6	21 (47.73)	7 (63.64)	12 (48.00)	1.91 (0.49-7.49)	0.503	1.01 (0.37-2.70)	1.000
	Bw6/Bw6	16 (36.36)	3 (27.27)	9 (36.00)	0.65 (0.15-2.83)	0.730	0.98 (0.35-2.73)	1.000
HLA-A/B	*Bw4/Bw4*	7 (15.91)	1 (9.09)	5 (20.00)	0.52 (0.06-4.81)	0.681	1.32 (0.37-4.71)	0.746
	Bw4/Non	22 (50.00)	7 (63.64)	16 (64.00)	1.75 (0.45-6.84)	0.510	1.78 (0.65-4.87)	0.319
HLA-C	*C1/C1*	5 (11.36)	1 (9.09)	4 (16.00)	0.78 (0.08-7.45)	1.000	1.48 (0.36-6.13)	0.714
	C1/C2	21 (47.73)	5 (45.45)	15 (60.00)	0.91 (0.24-3.43)	1.000	1.64 (0.61-4.44)	0.453
	C2/C2	18 (40.91)	5 (45.45)	16 (64.00)	1.20 (0.32-4.560	1.000	0.46 (0.15-1.37)	0.194

NT, non-transmitting mother; IP-TR, intrapartum transmitting mother; IU, *in utero* transmitting mother; EU, exposed uninfected infants; IP, intrapartum infected infants; IU, *in utero* infected infants

4.2.4 Characterization of *KIR/HLA* genotypes in MTCT2

Analysis of the interactions between inhibitory *KIR* and their known *HLA-ligands* (*KIR2DL1* with *C2, KIR2DL2* and *KIR2DL3* with *C1* and *KIR3DL1* with *Bw4*) as well as the putative activating *KIR/HLA* combinations (*2DS1*, *2DS2* and *3DS1*) are shown in **Table 4.7**. Unlike the NT mothers from MTCT1 that had significantly higher representation of *KIR2DL2/KIR2DL3* compared to TR mothers (*P*=0.044; OR=0.53) and IP-TR mothers (*P*=0.008; OR=0.29), in MTCT2, neither *KIR2DL2/KIR2DL3* heterozygosity nor homozygosity for *KIR2DL2* or *KIR3DL3* showed any significant differences amongst the TR and NT mothers. In addition, when paired with *HLA-C* ligands (*C1/C1*, *C1/C2* and *C2/C2*), *KIR/HLA* frequencies did not significantly differ amongst TR and NT mothers from MTCT2. Next, we analyzed frequencies of inhibitory *KIR3DL1* and activating *KIR3DS1* (also alleles of the same gene locus) together with the *HLA-Bw4* ligand. Whilst the *KIR3DL1+Bw4* representation was similar across TR and NT mothers, stratification of Bw4 alleles into those

with an isoleucine or threonine at position 80 showed an association with risk of HIV-1 transmission. *KIR3DL1* in combination with *Bw4:80I* was significantly higher in IP-TR and IU-TR mothers compared to NT mothers (*P*=0.039, OR=6.25 and *P*=0.047, OR=2.95, respectively), whilst *KIR3DL1+Bw4:80T* was over-represented in NT mothers compared to IU-TR mothers (*P*=0.038, OR=0.20). This finding was not evident when comparing *KIR3DL1/S1* genotypes, or *KIR3DL1* homozygosity in combination with *Bw4/Bw4* or *Bw4/Bw6* (**Table 4.6**), suggesting that these findings were dependant on the HLA alone. In MTCT2 infants, no significant differences were detected in *KIR* alleles (*KIR2DL2/L3* and *KIR3DL1/S1*), HLA ligands (*C1/C2* and *Bw4/Bw6*) and *KIR/HLA* combinations amongst HIV-1-infected and EU infants (data not shown).

Table 4.7 Representation of maternal *KIR/HLA* combinations in MTCT2, N (%)

Maternal *KIR/HLA*	NT (N=43)	IP-TR (N=11)	IU-TR (N=25)	IP-TR vs. NT		IU-TR vs. NT	
				OR (95% CI)	*P*	OR (95% CI)	*P*
2DL1+C2	32 (74.42)	9 (81.82)	18 (72.00)	1.54 (0.29-8.29)	0.715	0.88 (0.29-2.68)	1.000
2DL1+C2/C2	15 (34.88)	5 (45.45)	5 (20.00)	1.56 (0.41-5.95)	0.728	0.47 (0.15-1.49)	0.272
2DL2+C1	20 (46.51)	4 (36.36)	11 (44.00)	0.66 (0.17-2.57)	0.736	0.90 (0.33-2.43)	1.000
2DL2+C1/C1	7 (16.28)	1 (9.09)	4 (16.00)	0.51 (0.06-4.68)	0.678	0.97 (0.26-3.75)	1.000
2DL3+C1	24 (55.81)	5 (45.45)	17 (68.00)	0.65 (0.17-2.49)	0.736	1.68 (0.59-4.73)	0.442
2DL3+C1/C1	10 (23.26)	2 (18.18)	6 (24.00)	0.73 (0.13-3.96)	1.000	1.04 (0.32-3.32)	1.000
2DS1+C2	7 (16.28)	2 (18.18)	1 (4.00)	1.14 (0.20-6.45)	1.000	0.21 (0.02-1.85)	0.242
2DS1+C2/C2	7 (16.28)	2 (18.18)	1 (4.00)	1.14 (0.20-6.46)	1.000	0.21 (0.02-1.85)	0.242
2DS2+C1	17 (39.53)	3 (27.27)	10 (40.00)	0.57 (0.13-2.47)	0.510	1.02 (0.37-2.79)	1.000
2DS2+C1/C1	6 (13.95)	1 (9.09)	4 (16.00)	0.62 (0.07-5.73)	1.000	1.17 (0.29-4.64)	1.000
2DL2/2DL2+C1C1	1 (2.33)	0 (0.00)	1 (4.00)	-	1.000	1.75 (0.10-29.3)	1.000
2DL2/2DL2+C1C2	3 (6.98)	1 (9.09)	2 (8.00)	1.33 (0.12-14.2)	1.000	1.15 (0.18-7.45)	1.000
2DL2/2DL2+C2C2	5 (11.63)	1 (9.09)	1 (4.00)	0.76 (0.08-7.26)	1.000	0.32 (0.03-2.87)	0.402
2DL2/2DL3+C1C1	6 (13.95)	1 (9.09)	3 (12.00)	0.62 (0.07-5.73)	1.000	0.84 (0.19-3.71)	1.000
2DL2/2DL3+C1C2	10 (23.26)	2 (18.18)	5 (20.00)	0.73 (0.13-3.96)	1.000	1.28 (0.42-3.95)	0.773
2DL2/2DL3+C2C2	6 (13.95)	2 (18.18)	1 (4.00)	1.37 (0.24-7.95)	1.000	0.26 (0.03-2.69)	0.248
2DL3/2DL3+C1C1	4 (9.30)	1 (9.09)	3 (12.00)	0.97 (0.10-9.71)	1.000	1.32 (0.27-6.49)	1.000
2DL3/2DL3+C1C2	4 (9.30)	1 (9.09)	6 (24.00)	0.97 (0.10-9.71)	1.000	3.07 (0.77-12.2)	0.154
2DL3/2DL3+C2C2	4 (9.30)	2 (18.18)	3 (12.00)	2.17 (0.34-13.7)	0.590	1.32 (0.27-6.49)	1.000
3DL1+A/B Bw4	28 (65.12)	9 (81.82)	17 (68.00)	2.41 (0.46-12.6)	0.470	1.14 (0.39-3.24)	1.000
3DL1+A/B Bw4:80I	18 (41.86)	9 (81.82)	17 (68.00)	6.25 (1.20-32.5)	**0.039**	2.95 (1.05-8.32)	**0.047**
3DL1+B Bw4:80T	13 (30.23)	2 (18.18)	2 (8.00)	0.51 (0.10-2.71)	0.488	0.20 (0.04-0.97)	**0.038**
3DS1+A/B Bw4	3 (6.98)	1 (9.09)	0 (0.00)	1.33 (0.12-14.2)	1.000	-	0.292
3DS1+A/B Bw4:80I	1 (2.33)	1 (9.09)	0 (0.00)	4.20 (0.24-73.1)	0.369	-	1.000
3DS1+B Bw4:80T	2 (4.65)	1 (9.09)	0 (0.00)	2.05 (0.17-24.9)	1.000	-	0.528
3DL1/3DL1+A/B Bw4/Bw4	7 (16.28)	2 (18.18)	2 (20.00)	1.14 (0.20-6.46)	1.000	1.28 (0.36-4.58)	0.748
3DL1/3DL1+A/B Bw4/Bw6	18 (41.86)	6 (54.55)	15 (60.00)	1.67 (0.44-6.31)	0.510	2.08 (0.76-5.68)	0.209
3DL1/3DS1+A/B Bw4/Bw4	1 (2.33)	1 (9.09)	0 (0.00)	4.20 (0.24-73.1)	0.369	-	1.000
3DL1/3DS1+A/B Bw4/Bw6	2 (4.65)	0 (0.00)	0 (0.00)	-	1.000	-	0.528

NT, non-transmitting mothers; IP-TR, intrapartum transmitting mothers; IU, *in utero* transmitting mothers.

4.2.5 Mother-infant concordance for *KIR/HLA* in MTCT2

Given that mother-infant concordance for *KIR/HLA* in MTCT1 showed two significant associations with risk of transmission, we analysed *KIR/HLA* concordance in MTCT2. Concordant comparisons for both inhibitory and activating *KIR/HLA* genotypes are shown in **Table 4.8**. We found that concordant *KIR/HLA* frequencies were similar across TR and NT mother-infant pairs and did not show an association with MTCT of HIV-1.

Table 4.8 Representation of mother-infant *KIR/HLA* concordance in MTCT2, N (%)

KIR/HLA	NT/EU (N=43)	IP-TR/IP (N=11)	IU-TR/IU (N=25)	IP-TR/IP vs. NT/EU		IU-TR/IU vs. NT/EU	
				OR (95% CI)	*P*	OR (95% CI)	*P*
2DL1+C1/C1	2 (4.55)	0 (0.00)	3 (12.00)	-	1.000	2.86 (0.44-18.4)	0.344
2DL1+C1/C2	9 (20.45)	3 (27.27)	10 (40.00)	1.45 (0.32-6.63)	0.689	2.59 (0.87-7.67)	0.098
2DL1+C2/C2	13 (29.55)	3 (27.27)	4 (16.00)	0.89 (0.20-3.91)	1.000	-	1.000
2DL2/2DL2+C1C1	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
2DL2/2DL2+C1C2	1 (2.27)	1 (9.09)	1 (4.00)	4.30 (0.24-74.8)	0.363	1.79 (0.11-29.9)	1.000
2DL2/2DL2+C2C2	0 (0.00)	1 (9.09)	1 (4.00)	-	0.200	-	0.362
2DL2/2DL3+C1C1	1 (2.27)	0 (0.00)	1 (4.00)	-	1.000	1.79 (0.11-29.9)	1.000
2DL2/2DL3+C1C2	3 (6.82)	0 (0.00)	2 (8.00)	-	1.000	1.19 (0.18-7.64)	1.000
2DL2/2DL3+C2C2	2 (4.55)	0 (0.00)	1 (4.00)	-	1.000	0.87 (0.08-10.2)	1.000
2DL3/2DL3+C1C1	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
2DL3/2DL3+C1C2	1 (2.27)	0 (0.00)	3 (12.00)	-	1.000	5.86 (0.57-59.7)	0.132
2DL3/2DL3+C2C2	3 (6.82)	1 (9.09)	2 (8.00)	1.37 (0.13-14.6)	1.000	1.19 (0.18-7.64)	1.000
3DL1/3DL1+Bw4Bw4	4 (9.09)	1 (9.09)	2 (8.00)	1.00 (0.10-9.95)	1.000	0.87 (0.15-5.12)	1.000
3DL1/3DL1+Bw4Bw6	10 (22.73)	3 (27.27)	11 (44.00)	1.27 (0.28-5.72)	1.000	2.67 (0.92-7.70)	0.101
3DL1/3DL1+Bw6Bw6	7 (15.91)	1 (9.09)	1 (4.00)	0.53 (0.06-4.81)	0.681	0.22 (0.02-1.90)	0.243
3DL1/3DS1+Bw4Bw4	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
3DL1/3DS1+Bw4Bw6	1 (2.27)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
3DL1/3DS1+Bw6Bw6	0 (0.00)	0 (0.00)	1 (4.00)	-	1.000	-	0.362
2SD1+C1/C1	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
2SD1+C1/C2	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
2SD1+C2/C2	1 (2.27)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
2SD2+C1/C1	0 (0.00)	0 (0.00)	2 (8.00)	-	1.000	-	0.128
2SD2+C1/C2	5 (11.36)	1 (9.09)	5 (20.00)	0.78 (0.08-7.45)	1.000	1.95 (0.50-7.53)	0.478
2SD2+C2/C2	5 (11.36)	1 (9.09)	2 (8.00)	0.78 (0.08-7.45)	1.000	0.67 (0.12-3.78)	1.000

NT, non-transmitting mothers; IP-TR, intrapartum transmitting mothers; IU, *in utero* transmitting mothers.

4.2.6 Allogeneic *KIR/HLA ligand* combinations in MTCT2

As in MTCT1, allogeneic *KIR/HLA* gene combinations between mother and infant may influence susceptibility to HIV-1 acquisition, thus we compared frequencies of 'matched' and 'missing self' *KIR/HLA* combinations between TR and NT and mother-infant pairs from MTCT2 (**Table 4.9**). However, in MTCT2 we found no significant differences in allogeneic combinations of *KIR2DL1*, *KIR2DL2*/*L3* or *KIR3DL1/S1* with *HLA-C* or *HLA-B* ligands between TR and NT mother-infant pairs.

Table 4.9 Frequencies of allogeneic combinations of *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR3DL1* and *KIR3DS1* in recipient infants with *HLA-ligands* (*C1/C2* or *Bw4/Bw6*, respectively) in index mothers in MTCT2, N (%)

Type*	Recipient (Infant)		Index (Mother)	EU	IP	IU	IP vs. EU		IU vs. EU	
	KIR	*HLA*	*HLA*	(N=44)	(N=11)	(N=25)	OR (95% CI)	*P*	OR (95% CI)	*P*
Matched	*2DL1*	any	*C1/C2*	17 (38.64)	4 (36.36)	13 (52.00)	0.91 (0.23-3.57)	1.000	1.72 (0.64-4.64)	0.320
Matched	*2DL1*	any	*C2/C2*	16 (36.36)	5 (45.45)	5 (20.00)	1.45 (0.38-5.54)	0.731	0.44 (0.14-1.39)	0.184
Missing self	*2DL1*	*C1/C2*	*C1/C1*	9 (20.45)	1 (9.09)	4 (16.00)	0.38 (0.04-3.45)	0.667	0.74 (0.20-2.71)	0.756
Missing self	*2DL1*	*C2/C2*	*C1/C1*	0 (0.00)	1 (9.09)	0 (0.00)	-	0.200	-	1.000
Matched	*2DL2/2DL2*	any	*C1/C1*	1 (2.27)	1 (9.09)	2 (8.00)	4.30 (0.25-74.8)	0.363	3.73 (0.32-43.5)	0.549
Matched	*2DL2/2DL2*	any	*C1/C2*	2 (4.55)	1 (9.09)	2 (8.00)	2.10 (0.17-25.5)	1.000	1.82 (0.24-13.8)	0.617
Missing self	*2DL2/2DL2*	*C1/C1*	*C2/C2*	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
Missing self	*2DL2/2DL2*	*C1/C2*	*C2/C2*	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
Matched	*2DL2/2DL3*	any	*C1/C1*	6 (13.64)	1 (9.09)	2 (8.00)	0.63 (0.07-5.88)	1.000	0.55 (0.10-2.96)	0.701
Matched	*2DL2/2DL3*	any	*C1/C2*	8 (18.18)	1 (9.09)	4 (16.00)	0.45 (0.05-4.03)	0.669	0.85 (0.23-3.19)	1.000
Missing self	*2DL2/2DL3*	*C1/C1*	*C2/C2*	0 (0.00)	1 (9.09)	0 (0.00)	-	0.200	-	1.000
Missing self	*2DL2/2DL3*	*C1/C2*	*C2/C2*	3 (6.82)	1 (9.09)	0 (0.00)	1.37 (0.13-14.6)	1.000	-	0.297
Matched	*2DL3/2DL3*	any	*C1/C1*	4 (9.09)	0 (0.00)	2 (8.00)	-	0.573	0.86 (0.15-5.12)	1.000
Matched	*2DL3/2DL3*	any	*C1/C2*	7 (15.91)	2 (18.18)	7 (28.00)	1.17 (0.21-6.63)	1.000	2.06 (0.62-6.75)	0.350
Missing self	*2DL3/2DL3*	*C1/C1*	*C2/C2*	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
Missing self	*2DL3/2DL3*	*C1/C2*	*C2/C2*	0 (0.00)	0 (0.00)	1 (4.00)	-	1.000	-	0.362
Matched	*3DL1/3DL1*	any	*Bw4/Bw4*	5 (11.36)	2 (18.18)	2 (8.00)	1.73 (0.29-10.4)	0.617	0.68 (0.12-3.78)	1.000
Matched	*3DL1/3DL1*	any	*Bw4/Bw6*	19 (43.18)	5 (45.45)	14 (56.00)	1.09 (0.29-4.13)	1.000	1.67 (0.62-4.50)	0.328
Missing self	*3DL1/3DL1*	*Bw4/Bw4*	*Bw6/Bw6*	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
Missing self	*3DL1/3DL1*	*Bw4/Bw6*	*Bw6/Bw6*	7 (15.91)	0 (0.00)	5 (20.00)	-	0.323	1.32 (0.37-4.71)	0.746
Matched	*3DL1/3DS1*	any	*Bw4/Bw4*	1 (2.27)	1 (9.09)	0 (0.00)	4.30 (0.25-74.7)	0.363	-	1.000
Matched	*3DL1/3DS1*	any	*Bw4/Bw6*	3 (6.82)	1 (9.09)	0 (0.00)	1.37 (0.13-14.6)	1.000	-	0.297
Missing self	*3DL1/3DS1*	*Bw4/Bw4*	*Bw6/Bw6*	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
Missing self	*3DL1/3DS1*	*Bw4/Bw6*	*Bw6/Bw6*	0 (0.00)	1 (9.09)	0 (0.00)	-	0.200	-	1.000

EU, exposed uninfected infants; IP, intrapartum infected infants; IU, *in utero* infected infants; IU2. *According to models described by Moretta et al. (2011) and Jennes et al. (2013): 'Matched' occurs when the specified inhibitory *KIR* of the recipient recognizes an *HLA* allele present in the index (regardless of recipient *HLA*) and 'missing self' occurs when the specified inhibitory *KIR* of the recipient recognizes an *HLA* allele present in the recipient that is lacking in the index. Bold and highlighted *P* values indicate significant differences ($P<0.05$).

4.3 Discussion

In this chapter, we evaluated two mother-infant cohorts (MTCT1 and MTCT2, discussed in Chapter 2, Section 2.1.1, that were recruited from 1996 to 2010, and as such mothers and infants from MTCT1 and MTCT2 differed in their PMTCT therapy as well as their overall HIV-1 transmission rates (9.8% and 5.2%, $P<0.001$). In MTCT1 approximately half (56%) the mothers received sdNVP at the onset of labour, whereas the majority (89.9%) of mothers from MTCT2 received daily AZT ranging from 28 – 37 weeks gestation as well as sdNVP at the onset of labour. While all infants from MTCT1 received sdNVP within 72 hours of birth and all infants from MTCT2 received sdNVP within 72 hours of birth and a minimum 1 week course of daily AZT.

In the initial findings reported on the MTCT1 cohort Paximadis et al. (2011) identified the *KIR* genes, *KIR2DL2* and *KIR2DL3*, strongly associated with risk of vertical transmission. Importantly, (1) maternal *KIR2DL2/KIR2DL3* heterozygosity associated with decreased risk for IP transmission ($P=0.008$, OR=0.29), (2) maternal *KIR2DL3* homozygosity (alone and in combination with C1/C2) associated with increased risk for IP transmission ($P=0.034$, OR=2.42 and $P=0.010$, OR=3.63, respectively), and (3) infant *KIR2DL3* homozygosity in combination with C1/C2 associated with protection from HIV-1 acquisition ($P=0.038$, OR=0.40). Moreover, because the *KIR/HLA* gene combination, *KIR2DL3/KIR2DL3+C1/C2*, appeared to have conflicting associations in mothers and infants, the influence of gene concordance between mother and infant was only determined with respect to *KIR2DL3/KIR2DL3+C1/C2* and not the other *KIR/HLA* gene combinations. Expanding on these analyses we found two additional *KIR/HLA* gene combinations that influenced the risk of HIV-1 transmission when mother and infant were *KIR/HLA* gene concordant, namely (1) *KIR2DL3/KIR2DL3+C2/C2* (i.e. lacking a C1 ligand) associated with increased risk for IP transmission, and (2) *KIR2DL1+C1/C2* associated with decreased risk for IU transmission. Moreover both these associations were maintained post adjustment for mVL (**Table 4.3**) and, strikingly, when adjusting for mVL, there was almost a nine-fold increased risk of IP transmission when mother-infant were concordant for *KIR2DL3/KIR2DL3+C2/C2* (OR=8.81). We postulated that mother-infant *KIR/HLA* concordance influenced the risk of transmission in a similar fashion to that of the ‘arming’ and ‘disarming’ model of NK cell self-tolerance first proposed by Roulet and Vance (2006).

In this model (**Figure 4.4**), immature NK cells become functionally mature and 'armed' through the interactions with HLA-class-I-expressing target cells, whereas NK cells that fail to interact with HLA-class-I-expressing target cells remain unarmed and unresponsive, and therefore do not attack MHC-class-I-deficient target cells (Raulet and Vance, 2006). Therefore, in the case of *KIR2DL3/KIR2DL3+C2/C2* concordance, when both mother and infant lack their cognate ligand (C1) for KIR2DL3, NK cells which expressed KIR2DL3 in the absence of C1 were not licensed and are unresponsive to HIV-1-infected target cells which may contribute to increased risk of IP-transmission. Furthermore given that, *KIR2DL3* is a fixed locus of the group A haplotype (Gendzekhadze et al., 2009) and 9 (75%) out of 12 mother-infant pairs that were concordant for *KIR2DL3/KIR2DL3+C2/C2* were homozygous for the A haplotype, this suggested that having this inhibitory genotype in both mother and infant may increase the risk of MTCT of HIV-1. Alternatively, the increased risk for IP transmission may also be due to NK cell anergy. NK cells that fail to express inhibitory receptor specific for self-MHC during their development receive continuously stimulating signals because of normal activating receptor expression leading to a status of hyporesponsiveness or anergy (Hasenkamp et al., 2008). It may be that each of these factors contribute towards increased the risk of HIV-1 transmission.

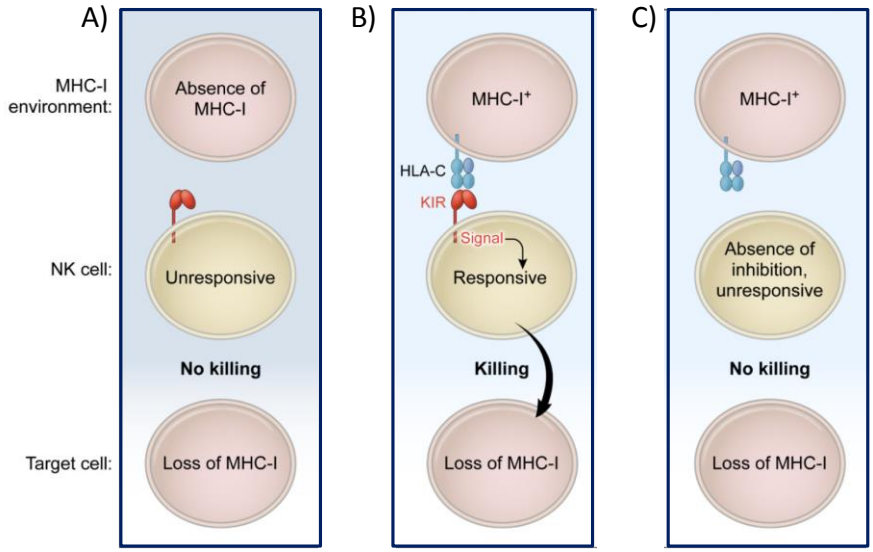


Figure 4.4 Comparison of arming and disarming mechanisms of NK cell self-tolerance. NK cells detect the loss but not the absence of MHC-I. (A) In the absence of self MHC-I, inhibitory receptors on NK cells are not engaged and NK cells remain unresponsive. (B) In the presence of MHC-I, inhibitory KIR deliver an inhibitory signal that prevents disarming and/or a signal that renders NK cells responsive (i.e., arming signal). The two options are not mutually exclusive and have the same outcome: NK cells become licensed by either maintaining or acquiring responsiveness to kill target cells. (C) NK cells that lack inhibitory receptors for self MHC-I are not licensed. Reproduced from (Long et al., 2013).

Conversely, using the ‘altered self” model, the protective association observed with *KIR2DL1+C1/C2* concordance (i.e. decreasing the risk for IU-transmission) may be explained in that engagement of KIR2DL1 NK cells (a gene that had a 99% frequency in MTCT1 mothers) with C2 ligands would license NK cells, thus enabling them to recognize and kill HIV-1 infected target cells that have been altered.

In addition, with regards to the role of infant NK cell alloreactivity in HIV-1 acquisition and/or the lack thereof, in MTCT1 we found that both ‘matched’ and ‘missing self’ *KIR/HLA* combinations were associated with IU acquisition of HIV-1. When infants were heterozygous for *KIR2DL2/KIR2DL3* and mothers possessed the cognate ligands, *C1/C1*, there was increased risk for IU acquisition of HIV-1 (IU: *P*=0.047, OR=4.02 and IU2: *P*=0.019, OR=3.45) which was maintained post adjustments for mVL and sdNVP, thereby suggesting that when HIV-1 infected maternal cells possessed C1/C1 inhibitory ligands possible engagement with infant NK cells bearing KIR2DL2/KIR2DL3 would not result in activation and thus allow IU HIV-1 infection to occur. Similarly, this hypothesis is in accordance with the principles of ‘self-recognition’ and NK cell education (Hoglund and Brodin, 2010), wherein recipient NK cells sensing self HLA on incoming index cells (matched) would result in NK cell inhibition and therefore fail to eliminate HIV-1 infected cells.

Likewise, Jennes et al. (2013) investigated whether allogeneic *KIR/HLA* combinations between HIV-1 concordant (HIV-1 transmission from one partner to the other) and HIV-1 discordant (one partner HIV-1 positive, the other HIV-1-negative) Senegalese couples would influence adult HIV-1 transmission and found that HIV-1 concordant couples showed an increased frequency of a specific "matched" *KIR/HLA* combination, while, HIV-1 discordant couples showed an increased frequency of a specific "missing self" *KIR/HLA* combinations. Thus, in agreement with the “self-recognition” model, NK cells sensing "missing self" as opposed to "matched" KIR/HLA combinations could potentially provoke alloreactive NK cell killing of HIV-1 infected partner cells preventing HIV-1 acquisition (Jennes et al., 2013). However, in MTCT1 “missing self” associated with increased risk for IU acquisition. IU infants had significantly greater representation of *KIR2DL2/KIR2DL2+C1/C2* in the presence of mothers that possessed *C2/C2* compared to EU infants (**Table 4.2**, 7% vs. 0%, *P*=0.011), but this association was not maintained post adjustments for mVL, maternal sdNVP or CD4+ T cell count. It is possible that this finding may be an artefact caused by the low representation of this *KIR/HLA* combination. Nonetheless, these allogeneic associations

found in MTCT1 suggest that alloreactive NK cells have an important role in MTCT transmission, particularly IU transmission of HIV-1, in a manner comparable to that of sexual transmission reported by Jennes et al. (2013).

In MTCT2, where mothers primarily received dual ART and the overall rate of HIV-1 transmission was significantly lower than that of MTCT1 (P<0.001, **Chapter 2, Figure 2.3**), maternal and infant *KIR/HLA* genotypes reported by Paximadis et al. (2011) that associated with decreased risk of transmission in MTCT1 were not evident in MTCT2. While NT mothers in MTCT1 had significantly higher total *KIR* gene number and greater number of inhibitory *KIR* genes than IU-TR mothers (Paximadis et al., 2011), no significant associations were seen in either mothers or infants when *KIR* gene numbers (total, inhibitory and activating) were compared in MTCT2. However, the total AA1 haplotype frequency was significantly lower in MTCT2 mothers compared to MTCT1 mothers (13.9% vs. 27.2%, P=0.029). This lower representation in MTCT2 was more evident in NT than IP-TR or IU-TR mothers (9.3% vs. 18.2% or 20%, respectively) but was not associated with decreased risk for HIV-1 transmission. Furthermore, given the similarity in AA1 representation between IP-TR and IU-TR, we compared the AA1 haplotype in NT to the total group of TR mothers (IP- + IU- TR mothers), but found no association with vertical transmission (P=0.328). In addition, comparison of the AA1 haplotype frequency in MTCT2 mothers to their infants showed a significant difference (13.9% vs. 33.7%, P=0.005, **Figure 4.1A**), suggesting that the majority of infants inherited an A Haplotype from their father. Nevertheless, in infants the higher prevalence of AA1 haplotype did not associate with increased/decreased risk for HIV-1 acquisition (**Figure 4.1C**).

Thus, given that the AA1 haplotype differed significantly across the two MTCT cohorts we compared our *KIR* data to another cohort of HIV-1 negative Black South Africans (Gentle et al, unpublished data). The *KIR* data represented a diverse cross-section of the Black ethnic groups of South Africa (n=167) sourced from the ESKOM (Electricity Supply Commission) cohort, termed as such because it involved collection of blood from individuals working at sites of this South African electricity public utility countywide. Overall, the total AA1 haplotype frequency in HIV-1-uninfected Black South Africans was 26.9% (45/167) which was most comparable to AA1 haplotype frequency in MTCT1. However, when stratifying these *KIR* data according to home language, namely: Xhosa (N=33), Northern Sotho (previously termed Pedi, N=24), Zulu (N=23), Venda (N=21), Tswana (N=20), South

Sotho (N=16), Swati (N=13), Ndebele (N=12) and Tsonga (N=5), we found the AA1 haplotype frequency was the highest in individuals that spoke Tswana or Tsonga (45% and 40%, respectively) and lowest in individuals who spoke Xhosa, South Sotho or Northern Sotho (12%, 13% and 17%, respectively), whereas the other dialects Zulu, Ndebele, Venda and Swati ranged from 30% to 38%. In agreement, the Xhosa AA1 haplotype frequency matched the Xhosa group reported by Middleton et al. (2007a). Thus, it is possible in our study that mothers from MTCT1 represented a more diverse ethnic group compared to mothers from MTCT2, which may have been skewed towards individuals having a Xhosa or Sotho background. Moreover, it could further be postulated that in MTCT2 paternal origins may have come from the South African dialects with the greater AA1 haplotype frequencies (Tswana or Tsonga), but since paternal data are not available this remains speculation. Thus, when comparisons are made between MTCT1 and MTCT2, this difference should be kept in mind.

Nevertheless, in both MTCT cohorts the Bx32 genotype associated with increased risk of transmission. In MTCT1, Paximadis et al. (2011) reported that IP-TR mothers had significantly higher frequency of Bx32 than NT mothers (P=0.014, OR=17.2), and in MTCT2 (**Figure 4.1**), both IP-TR and IU-TR mothers had increased representation of Bx32 compared to NT mothers (P=0.038 and P=0.005, respectively). Moreover, in MTCT1 Bx20 also associated with decreased risk of transmission (P=0.02, OR=0.12). Examination of *KIR* gene components revealed that the only difference between Bx32 (consisting of *KIRs 2DL1*, *2DL3*, *2DL4*, *2DL5*, *2DS4*, *2DS5*, *2DP1*, *3DL1*, *3DL2*, *3DL3* and *3DP1*) and Bx20 was the presence of *KIR2DL2* in Bx20. Likewise highlighting the fact that the Bx32 genotype is *KIR2DL3* homozygous and is one of the few homozygous *KIR3DL3* genotypes currently reported in South Africa; others in MTCT1 included AA1, Bx2, Bx10, Bx27, Bx35 and Bx48 (Paximadis et al., 2011).

While we observed no significant differences in distribution of *KIR2DL2/L3* genotypes among MTCT2 groups, in MTCT1 maternal *KIR2DL3* homozygosity significantly associated with increased risk for IP transmission (Paximadis et al., 2011). Similarly in another study, proportions of *KIR2DL3* homozygosity alone and in the presence of *C1/C2* heterozygosity were significantly higher in seropositive sex workers compared to exposed seronegative sex workers (Jennes et al., 2006b). Conversely, although not HIV related, *KIR2DL3* and *HLA*-C1 were also reported to play a role in the resolution of HCV in the context of low infectious

doses of HCV but not in individuals with high-dose exposure. (Khakoo et al., 2004). The authors suggest that distinct *KIR/HLA* genotypes are able to regulate NK cell activity sufficiently to alter the outcome when faced with low-dose HCV, but not in those with high-dose exposure during which the innate immune response is likely overwhelmed. However, in each of these studies as well as our own, the actual mechanisms that may be at play remain elusive.

We then evaluated the role of HLA-ligands in MTCT2, using the real-time PCR method described in Chapter 2 (Section 2.5). We found that both maternal and infant possession of the *HLA-A Bw4:80I* allele significantly increased the risk for IU transmission and/or acquisition of HIV-1 (**Figure 4.2 and 4.3**). Since our real-time PCR assay broadly grouped the *HLA-A* alleles into two main groups: those with an isoleucine at position 80 as *Bw4:80I* (*A*23*, *A*24*, *A*25* and *A*32*) and those without as *non-Bw4:80I*, we were unable to identify which specific *Bw4:80I* allele was responsible for the increased risk of IU transmission in mothers and infants. However, using the *HLA-A*, *-B* and *–C* frequency data for the Black ESKOM cohort (Paximadis et al., 2012) as a reference, we could extrapolate that the most likely *HLA-A Bw4:80I* alleles contributing towards increased IU-transmission in MTCT2 were *A*23:01* (8.04%), *A*24:02* (2.01%) or *A*32:01* (1.26%). In agreement with our finding, in perinatal cohort from Nairobi, Kenya maternal possession of *A*23:01* was found to associated with increased risk of HIV-1 transmission (Mackelprang et al., 2010). In addition, the same study reported that maternal *A*23:01* associated with higher plasma HIV-1 RNA VL and the *A*23:01/B*15:03/C*02:02* haplotype, but not the *B*15:03/C*02:02* haplotype associated with increased transmission. Of note, since *A*23:01* associated with increased risk for HIV-1 transmission both before and after adjusting for maternal VL the Mackelprang et al. (2010) suggested that *A*23:01* may alter infectivity through mechanisms other than influencing HIV-1 VL. This further supports our findings, given our NT and TR mothers MTCT2 cohort were matched on the basis of maternal VL, CD4+ T cell count and ART regimens.

Similarly other studies have also associated *A*23:01* with HIV-1 transmission, in a group of Kenyan commercial sex workers, women carrying *A*23:01* were found to have a 3.6-fold increased risk of HIV-1 seroconversion (MacDonald et al., 2000), and in Zambian HIV-1 serodiscordant couples, possession of the *A*23/B*07/Cw*07* haplotype was enriched in the transmitter group (Tang et al., 2008). Thus, these *A*23:01* associations as well as our own

finding of *HLA-A Bw4:80I* increasing the risk of IU HIV-1 transmission, might suggest that *HLA-A Bw4:80I* alleles in MTCT2 might exert immune pressure that selects for viral isolates that are more infectious in infants. Alternatively, since KIR3DL1 is able to bind to HLA-A antigens with the Bw4 epitope, recognition of these inhibitory ligands would allow HIV-1infected cells to escape lysis by KIR3DL1-expressing NK cells (Stern et al., 2008).

With regards to the other *HLA-ligands* (*HLA-B* and *HLA-C*), in the HLA-B group, although IU-TR mothers had significantly lower representation of *Bw4:80T* compared to NT mothers (*P*=0.030, OR=0.20), frequencies of HLA-B ligand genotypes (*Bw4/Bw4*, *Bw4/Bw6* and *Bw6/Bw6*) did not significantly differ amongst TR and NT mothers. Additionally, maternal HLA-C ligands showed no differences amongst MTCT2 mothers. Thus, we analysed paired *KIR/HLA* combinations in MTCT2 and found two *KIR/HLA* combinations that significantly associated with transmission of HIV-1 (**Table 4.7**). *KIR3DL1* in combination with *HLA-A/B Bw4:80I* was significantly higher in both IP-TR and IU-TR mothers compared to NT mothers, whilst *KIR3DL1+Bw4:80T* was over-represented in NT mothers compared to IU-TR mothers, suggesting that the *Bw4* allotype in combination with *KIR3DL1* influenced the risk of HIV-1 transmission. This differs from the other reports that implicated the *KIR3DL1* and *Bw4* (*HLA-B*57*) with protection against HIV-1 infection as well as slower time to AIDS and viral control (Martin et al., 2007, Boulet et al., 2008a, Boulet et al., 2010).

Given that *KIR3DL1* alleles are highly polymorphic and differ in their cell surface expression levels ranging from high (*h), intermediate (*i), low (*l) and null (*KIR3DL1*004*), Boulet et al. (2008a, 2008b and 2010) sought to determine which *KIR3DL1/S1* genotypes associated with decreased susceptibility to HIV-1 infection observed in EUs. They reported that co-carriage of the *KIR3DL1* genotypes that possess high expressing alleles in the absence of any low expressing alleles together with *HLA-B*57* (a *Bw4:80I*) conferred elevated NK cell functional potential and reduced risk of HIV-1 infection (Boulet et al., 2008a, Boulet et al., 2010). They proposed that the stronger inhibitory KIR3DL1 partnership with its Bw4:80I ligand enhanced 'licensing' of KIR3DL1+ NK cells that when HIV-1-infected cells downregulate cell surface expression of HLA-B, NK cells 'licensed' through KIR3DL1 would be NK cells activated to eliminate target. Therefore, one could speculate that in our MTCT2 cohort, the *KIR3DL1+Bw4:80I* associations with increased risk for both IP and IU HIV-1 transmission may be due to *KIR3DL1* alleles that represent the low to intermediate or null expressing alleles, which potentially may result in reduced NK cell functionality and thus

the inability to effectively target HIV-1-infected cells. However, this needs to be determined. Notably, NT mothers that possessed the weaker *Bw4:80T* ligand (Cella et al., 1994) in combination with *KIR3DL1* associated with protection from IU transmission, but individually neither *KIR3DL1* nor *Bw4:80T* showed a protective association, suggesting that the combined *KIR/HLA-ligand* interaction was necessary for a protective effect.

Considering that mother-infant pairs in MTCT1 showed significant associations with respect to concordance for *KIR/HLA* combinations, as well as infant allorecognition of maternal HLA, we also evaluated whether *KIR/HLA* concordance and allorecognition altered risk of HIV-1 transmission in mother-infant pairs from MTCT2, but found no significant associations. Overall, the study of these MTCT cohorts revealed various *KIR/HLA-ligand* combinations that associated with vertical transmission, some shared (e.g. the maternal Bx32 genotype associating with IP transmission in both MTCT1 and MTCT2), some different (e.g. both findings of mother-infant concordance for *KIR2DL3/KIR2DL3+C2/C2* associating with IP transmission, and infant allorecognition *iKIR2DL2/KIR2DL3+mC1/C1* associating with IU transmission in MTCT1 but neither not observed in MTCT2), and the presence of *HLA-A Bw4* associated with IU transmission in MTCT2 but was not evaluated in MTCT1. It is possible that the ability to show common and/or different associations within these MTCT cohorts are limited by the small sample numbers in MTCT2 and/or the overall under-representation of the maternal *KIR* AA1 haplotype. Moreover, these findings may also reflect the influence of different drug therapies, as the ever-changing drug environment may be selecting for infants with a higher susceptibility risk. Nevertheless, collectively, our results suggest that at the *KIR* gene-level as well as the *KIR/HLA* genotype level, irrespective of ART regimen, *KIR* and *HLA-ligands* play a significant role in MTCT of HIV-1.

CHAPTER 5

Differential effects of *KIR2DS4* on MTCT of HIV-1

The findings from MTCT1 presented in this Chapter have been in published: Hong et al. (2013) *Clinical Immunology* 149, 498 - 508.

5.1 Introduction

As previously mentioned, the ability of NK cells to recognize and kill virus-infected or tumour-transformed cells is determined by the action of different surface receptors that either activate or inhibit NK cell cytolytic activity. Among the 14 functional *KIR* genes, *KIR2DS4* is the only activating *KIR* gene in the Group A haplotype (**Figure 5.1**), and of the 13 *KIR2DS4* alleles described to date, only four (*KIR2DS4*001*, **011*, **014* and **015*) encode full-length (KIR2DS4-f) receptors while the remaining nine alleles (*KIR2DS4*003*, **004*, **006*, **007*, **008*, **009*, **010*, **012* and **013*) encode truncated/deleted (KIR2DS4-v) protein products (Robinson et al., 2013a). Alleles encoding KIR2DS4-v have a 22-bp deletion in exon 5 which causes a frame shift mutation yielding a truncated KIR2DS4 protein with loss of the transmembrane and cytoplasmic domains, thus, these variants are not anchored to the cell membrane but are potentially secreted (Hsu et al., 2002, Maxwell et al., 2002, Middleton et al., 2007a). Therefore, only *KIR2DS4-f* variants can serve as activating receptors upon binding to their specific HLA ligands (HLA-A: *A*11:01*, *A*11*:02 and HLA-C: *C*01:02*, *C*14:02*, *C*16:01*, *C*02:02*, *C*04:01* and *C*05:01*) (Mandelboim et al., 1997, Graef et al., 2009).

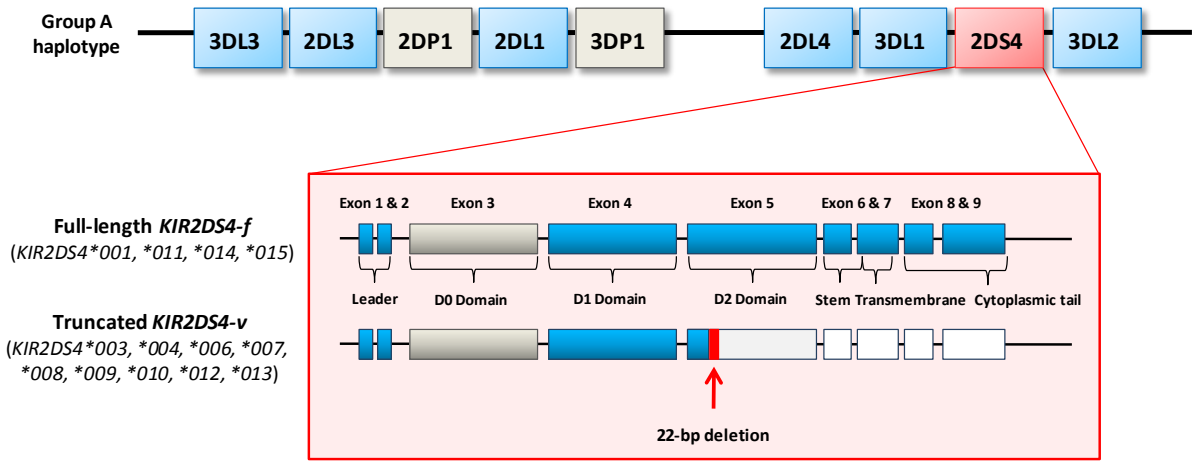


Figure 5.1 A schematic representation of the Group A haplotype and the allelic variants of *KIR2DS4* (*KIR2DS4-f* and *KIR2DS4-v*). Within the Group A haplotype inhibitory *KIR* genes are indicated in blue, the singular activating *KIR* gene (*KIR2DS4*) is indicated in pink, and the pseudogenes *KIR2DP1* and *KIR3DP1* are indicated in grey.

Several studies have described the importance of NK cells in the control of HIV-1 infection through recognition of virally infected cells by both activating and inhibitory KIRs (Alter and Altfeld, 2009, Berger and Alter, 2011, Tiemessen et al., 2011, Carrington and Alter, 2012, Jost and Altfeld, 2013) and the strategies evolved by HIV-1 to specifically evade NK cell-mediated immune responses (Jonjic et al., 2008, Alter et al., 2011, Jost and Altfeld, 2012). With regards to *KIR2DS4*, Merino et al. (2011) identified that *KIR2DS4-f* alleles in HIV-1-chronically infected Zambians independently associated with accelerated transmission of HIV-1 to cohabiting seronegative partners, as well as with higher VL (Merino et al., 2011). In addition, they also observed a positive association between *KIR2DS4-f* and the presence of genital ulcers in the seroprevalent index partners but not in the seroconverters. This suggested that in chronically HIV-1-infected individuals *KIR2DS4-f* might increase the likelihood of HIV-1 transmission through tissue inflammation and/or increased shedding of HIV-1. Furthermore, the *KIR2DS4-f* association with HIV-1 transmission was only weakly enhanced by the presence of its reported C2 ligand, HLA-C*04. More recently, the same group reported that *KIR2DS4-f* promoted HIV-1 pathogenesis during chronic infection. They observed that KIR2DS4^{+} and KIR2DS4^{-} NK cells (determined through flow cytometric staining) derived from subjects with chronic HIV-1 infection often differed in their functional properties after stimulation with HLA-deficient K562 cells, especially in terms of degranulation (production of CD107a) and/or induction of IFN-γ and MIP-1β. By all three measurements (individually or in various combinations), NK cells with the *KIR2DS4-f* genotype (g+) but negative for the membrane-bound KIR2DS4 staining (p−) universally behaved like those that were negative for both (g−/p−), while polyfunctional NK cells co-expressing CD107a, IFN-γ and MIP-1β were highly enriched in the g+/p+ NK cells when compared with the g+p− and g−p− NK cells. It was postulated that prolonged or excessive NK cell activities (as seen in chronic HIV-1 infection) may fuel inflammation and promote HIV-1 pathogenesis. Similarly, in line with this hypothesis elevated levels of pro-inflammatory cytokines and activated NK cells were found to be predictive of HIV-1 acquisition in African women (Naranbhai et al., 2012).

These observations have raised the question of how allelic variants of *KIR2DS4* might influence the likelihood of MTCT of HIV-1 in the context of single or dual ART. Thus, the aim of this chapter was to investigate the distribution of *KIR2DS4-f* and *KIR2DS4-v* in HIV-1 TR and NT mothers and their HIV-1 infected and uninfected infants, using the two MTCT cohorts (MTCT1 and MTCT2) receiving different PMTCT regimens.

5.2 Results

5.2.1 Distribution of *KIR2DS4* in mothers and infants from MTCT1

Upon genomic *KIR* typing using the commercial Olerup kit (Chapter 2, section 2.4.1), we found *KIR2DS4* was present in 98.6% (214/217) mothers from cohort 1 (MTCT1). In mothers the total allelic frequency for *KIR2DS4-f* was 87.8% (188/214) and 50.9% (109/214) for *KIR2DS4-v* (**Figure 5.2A**), of which 105 (49.1%) mothers were homozygous for *KIR2DS4-f* and 26 (12.1%) mothers were homozygous for *KIR2DS4-v*. Out of 59 (27.1%) mothers who were homozygous for Group-A haplotype (AA1), six were *KIR2DS4-v* carriers (10.2%), therefore in MTCT1, only 2.8% (6 of 214) of the mothers lacked a functional activating *KIR* gene. When comparing mVL and CD4+ T cell counts of mothers with *KIR2DS4-f* and those without (*KIR2DS4-v*), no significant associations were found (**Figure 5.2B** and **5.2C**). We then compared the frequency of the AA1 genotype and the presence of *KIR2DS4-v* to other population groups (**Figure 5.3**) and found MTCT1 was clearly separate from the Caucasian and Asian cluster and most comparable to the Mexican population.

5.2.2 Frequency of putative KIR2DS4 HLA-C ligands in MTCT1

Putative HLA-C ligands for functional KIR2DS4 are reported in **Table 5.1**. The ratio of total maternal C1 to C2 alleles was 1:2 (7.4 % vs. 16.4%) with *C*16:01* (6.5%) being the most common in the C1 group and *C*04:01* (14.5%) in the C2 group. Frequencies of HLA–C ligands between the NT and TR (IP, IU and IU2) mothers did not significantly differ. Infants had comparable C1 and C2 frequencies to the maternal group, however comparison of HLA-C ligands in the infants, showed *C*16:01* frequency was significantly higher in IP infants compared to EU infants (15.5% vs. 6.9%; *P*=0.038, OR=2.48) and in combination with *KIR2DS4-v*, showed a strong trend towards increased risk of IP HIV-1 acquisition (*P*=0.074, OR=10.52) but post adjustment for mVL, sdNVP, CD4+ T cell count and combined adjustments, both associations were not significant (data not shown). Since the majority of the KIR2DS4 HLA-C alleles had a frequency below 10%, the C1 and C2 alleles were grouped together (i.e. C1+C2) and were compared across the maternal and infant groups (**Table 5.1**). We found no evidence of combined C1+C2 ligands having an effect on maternal transmission of HIV-1 or infant acquisition of HIV-1.

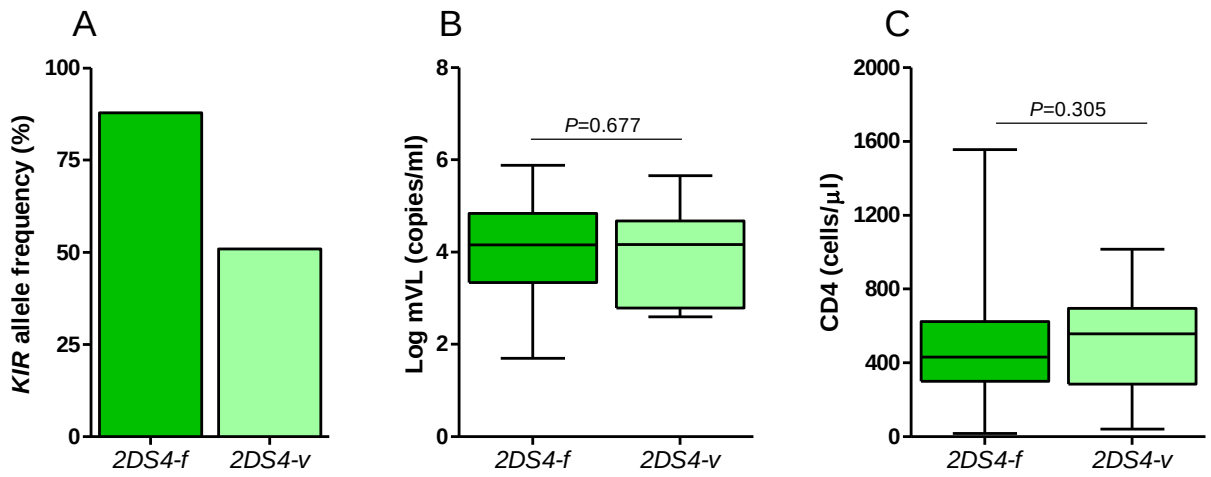


Figure 5.2 (A) Total *KIR2DS4-f* and *KIR2DS4-v* frequencies in mothers from MTCT1 (n=214); (B) comparison of maternal VL and (C) maternal CD4+ T cell count amongst mothers with at least one copy of *KIR2DS4-f* and those without (*KIR2DS4-v*).

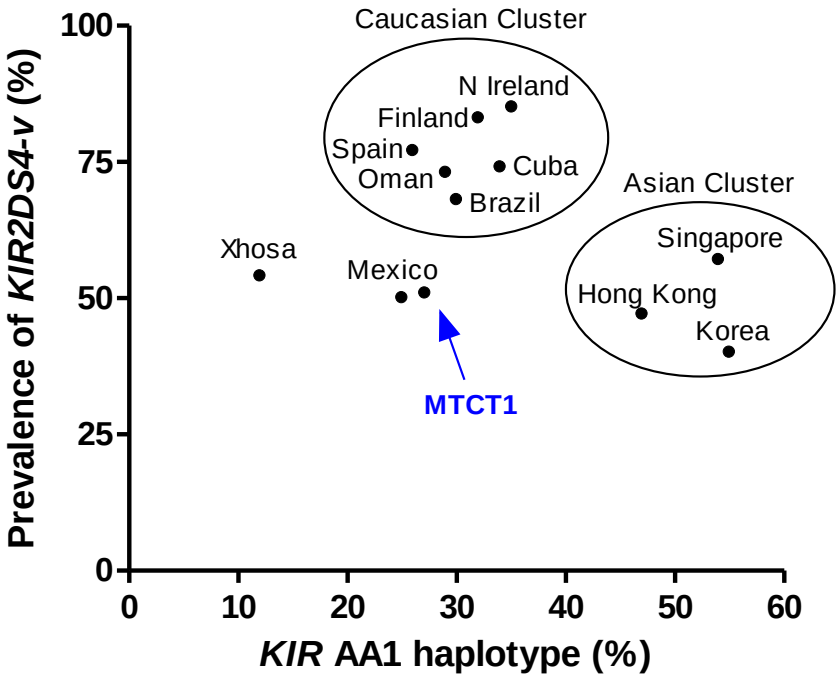


Figure 5.3 A scatter plot comparison of AA1 haplotype frequencies and the prevalence of *KIR2DS4-v* within different population groups, including MTCT1 (Blue). Reproduced and modified from (Whang et al., 2005, Middleton et al., 2007a).

Table 5.1 Carrier frequencies for KIR2DS4 HLA-C ligands in mothers and infants from MTCT1, N (%)

Maternal HLA-C ligands	Total (2N=434)	NT (2N=290)	IP-TR (2N=58)	IU-TR (2N=38)	IU2-TR (2N=86)	IP-TR vs. NT		IU-TR vs. NT		IU2-TR vs. NT	
						OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
C1	32 (7.37)	20 (6.90)	6 (10.34)	3 (7.89)	6 (6.98)	1.56 (0.59-4.06)	0.409	1.16 (0.32-4.09)	1.000	1.01 (0.39-2.61)	1.000
*C*01:02*	1 (0.23)	1 (0.34)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
*C*14:01*	3 (0.69)	3 (1.03)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	0.590
*C*16:01*	28 (6.45)	16 (5.52)	6 (10.34)	3 (7.89)	6 (6.98)	1.66 (0.62-4.41)	0.397	1.47 (0.41-5.29)	0.709	1.28 (0.49-3.39)	0.795
C2	71 (16.36)	45 (15.52)	8 (13.79)	8 (21.05)	18 (20.93)	0.87 (0.39-1.96)	0.843	1.45 (0.62-3.37)	0.481	1.44 (0.78-2.65)	0.251
*C*02:02*	3 (0.69)	1 (0.34)	1 (1.72)	1 (2.63)	1 (1.16)	5.07 (0.31-82.3)	0.306	7.81 (0.47-127.5)	0.219	3.40 (0.21-54.9)	0.406
*C*04:01*	63 (14.52)	41 (14.14)	7 (12.07)	6 (15.79)	15 (7.44)	0.83 (0.35-1.96)	0.835	1.14 (0.44-2.89)	0.806	1.28 (0.67-2.45)	0.491
*C*05:01*	5 (1.15)	3 (1.03)	0 (0.00)	1 (2.63)	2 (2.33)	-	1.000	2.59 (0.26-25.5)	0.390	2.28 (0.37-13.9)	0.593
C1+C2	103 (23.73)	65 (22.41)	14 (24.14)	11 (28.95)	24 (27.91)	1.10 (0.56-2.13)	0.864	1.41 (0.66-2.99)	0.413	1.34 (0.77-2.31)	0.313
Infant HLA-C ligands	**Total (2N=434)**	**EU (2N=290)**	**IP (2N=58)**	**IU (2N=38)**	**IU2 (2N=86)**	**IP vs. EU**		**IU vs. EU**		**IU2 vs. EU**	
						OR (95% CI)	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
C1	37 (8.53)	23 (7.93)	9 (15.52)	2 (5.26)	5 (5.81)	2.13 (0.93-4.88)	0.081	0.65 (0.14-2.85)	0.752	0.72 (0.26-1.94)	0.643
*C*01:02*	1 (0.23)	1 (0.34)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
*C*14:01*	2 (0.46)	2 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
*C*16:01*	34 (7.83)	20 (6.90)	9 (15.52)	2 (5.26)	5 (5.81)	2.48 (1.07-5.76)	**0.038**	0.75 (0.17-3.34)	1.000	0.83 (0.30-2.29)	0.811
C2	69 (15.90)	48 (16.55)	7 (12.07)	7 (18.42)	14 (16.28)	0.69 (0.29-1.61)	0.439	1.34 (0.47-2.74)	0.817	0.98 (0.51-1.88)	1.000
*C*02:02*	9 (2.07)	8 (2.76)	0 (0.00)	1 (2.63)	1 (1.16)	-	0.361	0.95 (0.11-7.83)	1.000	0.41 (0.05-3.36)	0.477
*C*04:01*	57 (13.13)	38 (13.10)	7 (12.07)	5 (13.16)	12 (13.95)	0.91 (0.38-2.15)	1.000	1.00 (0.37-2.73)	1.000	1.08 (0.53-2.16)	0.857
*C*05:01*	3 (0.69)	2 (0.69)	0 (0.00)	1 (2.63)	1 (1.16)	-	1.000	3.89 (0.34-43.97)	0.310	1.69 (0.15-18.9)	1.000
C1+C2	106 (24.42)	71 (4.48)	16 (27.59)	9 (23.68)	19 (22.09)	1.17 (0.62-2.21)	0.740	0.96 (0.43-2.12)	1.000	0.87 (0.49-1.55)	0.670

C1 constitutes one or more HLA-C alleles *C*01:02*, *C*14:02* and *C*16:01* and C2 constitutes one or more HLA-C alleles C*02:02*, *C*04:01* and *C*05:01*. *C1+C2* represent the combination of C1 and C2 alleles. Bold *P* values indicate significant differences ($P<0.05$).

5.2.3 *KIR2DS4* alleles in HIV-1 transmission in MTCT1

KIR2DS4-f, *KIR2DS4-fv* and *KIR2DS4-v* representation alone and in combination with putative HLA-C ligands (C1+C2) did not significantly differ between NT and any of the TR (IP, IU and IU2) maternal groups in MTCT1 (**Figure 5.4A** and **Table 5.2**). IU2 infants had significantly higher representation of *KIR2DS4-v* alone (**Figure 5.4B**, P=0.022, OR=2.88) and in combination with C1+C2 ligands (P=0.006; OR=5.52, **Table 5.2**) compared to EU infants. However after adjusting for maternal VL, sdNVP, CD4+ T cell count and the combined group, only *KIR2DS4-v* in combination with C1+C2 maintained significance throughout the adjustments (**Table 5.3**). Since individuals homozygous for the A haplotype carry *KIR2DS4* as the only activating *KIR* gene, we subdivided AA1 haplotypes into AA-*f* (*KIR2DS4-f*), AA-*fv* (*KIR2DS4-f* and *KIR2DS4-v*) and AA-*v* (*KIR2DS4-v*). We found no significant differences between the NT and TR mothers (**Figure 5.4C**). In the infant group comparisons however (**Figure 5.4D**), EU infants had a significantly lower representation of AA-*v* compared to both IU and IU2 infants (P=0.04; OR=15.33 and P=0.004; OR=18.40, respectively), however only the EU versus IU2 comparison remained significant post adjustments for mVL (P=0.013, OR=16.8), sdNVP (P=0.036, OR=8.70) and CD4+ T cell count (P=0.046, OR=14.47).

5.2.4 Mother-infant concordance and discordance in MTCT1

In order to distinguish how *KIR2DS4* might impact on viral transmission from the mother versus HIV-1 acquisition by the infant, we analysed mother-infant pairs for concordant and discordant possession of *KIR2DS4-f*, *KIR2DS4-fv* and *KIR2DS4-v*. Possession of the select *KIR* allele in both mother and matched infant, i.e. mother-infant concordance (M+I+), showed no significant differences among any of the groups compared (**Figure 5.5A**). Similarly when determining if possession of putative KIR2DS4 ligands (C1+C2) altered HIV-1 transmission, we found no significant difference when *KIR2DS4* alleles were paired with C1+C2 ligands (**Table 5.2**). However, M+I- mother-infant allele discordance (**Figure 5.5B**) revealed significantly higher representation of *KIR2DS4-f* in IP-TR mothers when compared to NT mothers (P=0.005, OR=3.84), which maintained significance post adjustments for mVL (P=0.007, OR=3.93), sdNVP (P=0.003, OR=4.44), CD4+ T cell count (P=0.014, OR=3.75) and the three combined (P=0.014, OR=4.35). However, this association was not evident when *KIR2DS4-f* was in combination with C1+C2 ligands (**Table 5.2**).

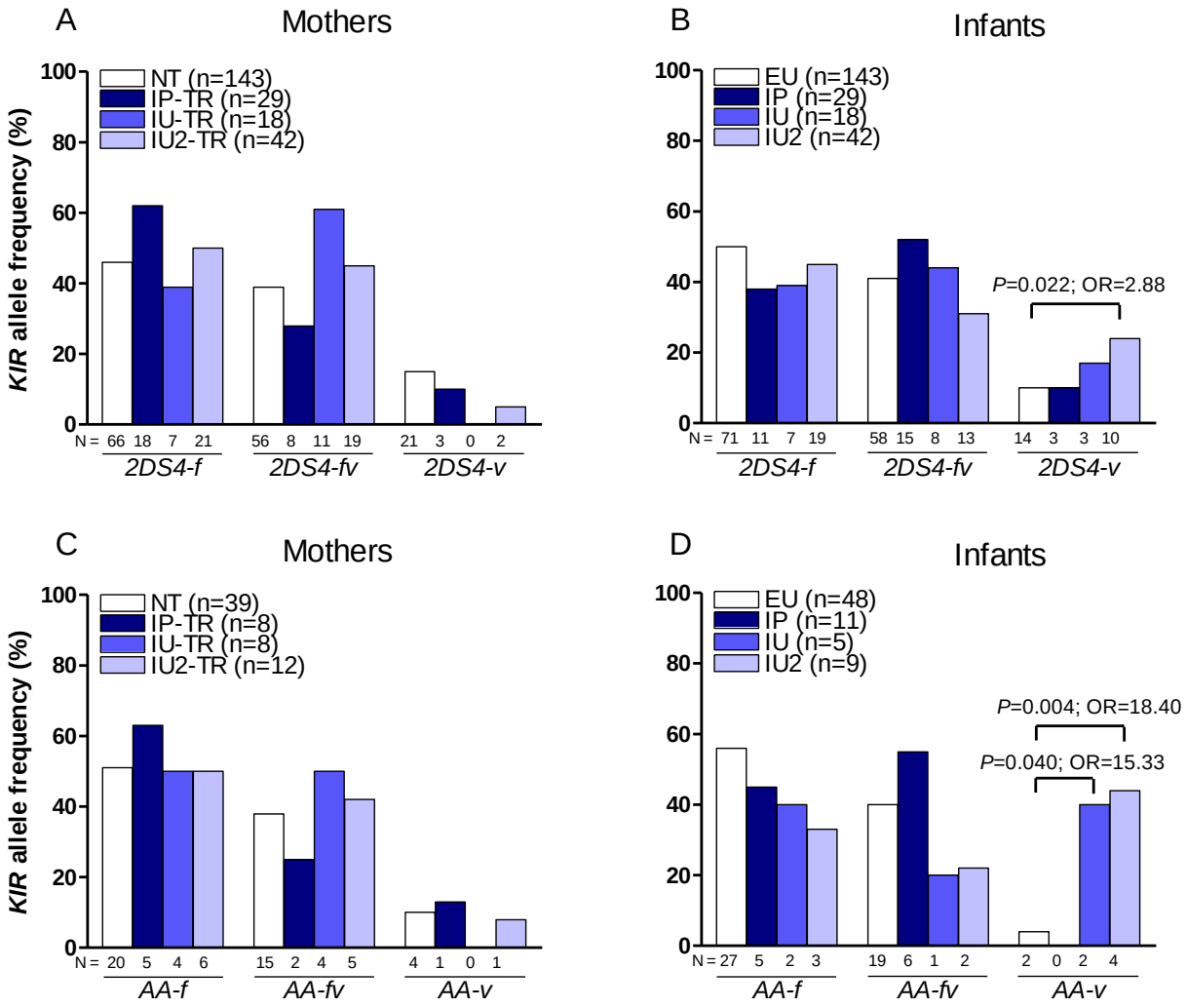


Figure 5.4 Frequencies of *KIR2DS4-f*, *KIR2DS4-fv* and *KIR2DS4-v* in the total group of mothers (A) and infants (B) in MTCT1; and the frequency of *KIR2DS4* variants amongst mothers (C) and infants (D) with AA haplotype. Non-transmitting (NT); Transmitting (TR); Intrapartum (IP); *In utero* (IU); Exposed uninfected (EU) infant. *KIR2DS4-f* indicates the presence of one or two copies of functional *KIR2DS4*; *KIR2DS4-fv* indicates the presence of both functional and non-functional *KIR2DS4*; and *KIR2DS4-v* indicates the presence of one or two copies of non-functional *KIR2DS4*.

Table 5.2 Carrier frequencies of *KIR2DS4* in combination with putative HLA-C1+C2 ligands in MTCT1

***KIR2DS4* and HLA-C ligands**		**Representation, N (%)**				**IP-TR/ IP vs. NT / EU**		**IU-TR / IU vs. NT / EU**		**IU2-TR / IU2 vs. NT / EU**	
		NT / EU	**IP-TR / IP**	**IU-TR / IU**	**IU2-TR / IU2**	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Mothers N=143	*2DS4-f C1+C2*	27 (18.88)	9 (31.03)	2 (11.11)	6 (14.29)	1.93 (0.79-4.71)	0.208	0.54 (0.12-2.47)	0.533	0.72 (0.27-1.87)	0.648
	2DS4-fv C1+C2	20 (13.99)	3 (10.34)	4 (22.22)	9 (21.43)	0.71 (0.19-2.56)	0.770	1.76 (0.52-5.88)	0.479	1.68 (0.69-4.03)	0.333
	2DS4-v C1+C2	6 (4.20)	1 (3.45)	0 (0.00)	2 (4.76)	0.82 (0.09-7.04)	1.000	-	0.620	1.14 (0.22-5.88)	1.000
Infants N=143	*2DS4-f C1+C2*	32 (22.38)	6 (20.69)	2 (11.11)	6 (14.29)	0.90 (0.33-2.41)	1.000	0.43 (0.09-1.98)	0.367	0.58 (0.22-1.49)	0.287
	2DS4-fv C1+C2	24 (16.78)	6 (20.69)	4 (22.22)	4 (9.52)	1.29 (0.47-3.51)	0.789	1.42 (0.43-4.68)	0.741	0.52 (0.17-1.60)	0.330
	2DS4-v C1+C2	5 (3.50)	2 (6.90)	1 (5.56)	7 (16.67)	2.04 (0.37-11.09)	0.603	1.62 (0.18-14.73)	1.000	5.52 (1.65-18.44)	**0.006**
M+I+ N=141	*2DS4-f C1+C2*	14 (9.93)	5 (17.24)	0 (0.00)	3 (7.14)	1.89 (0.63-5.74)	0.327	-	0.228	0.70 (0.19-2.55)	0.766
	2DS4-fv C1+C2	9 (6.38)	2 (6.90)	2 (11.11)	2 (4.76)	1.09 (0.22-5.31)	1.000	1.83 (0.37-9.24)	0.615	0.73 (0.15-3.53)	1.000
	2DS4-v C1+C2	2 (1.42)	1 (3.45	0 (0.00)	1 (2.38)	2.48 (0.22-28.32)	0.432	-	1.000	1.69 (0.14-19.17)	1.000
M+I- N=141	*2DS4-f C1+C2*	13 (9.22)	4 (13.79)	2 (11.11)	3 (7.14)	1.58 (0.47-5.23)	0.496	1.23 (0.25-5.96)	1.000	0.76 (0.20-2.79)	0.768
	2DS4-fv C1+C2	11 (7.80)	1 (3.45)	2 (11.11)	7 (16.67)	0.42 (0.05-3.40)	0.484	1.48 (0.30-7.27)	0.644	2.36 (0.85-6.54)	0.135
	2DS4-v C1+C2	4 (2.84)	0 (0.00)	0 (0.00)	1 (2.38)	-	0.605	-	1.000	0.84 (0.09-7.68)	1.000
M-I+ N=141	*2DS4-f C1+C2*	18 (12.77)	1 (3.45)	2 (11.11)	3 (7.14)	0.24 (0.03-1.91)	0.203	0.85 (0.18-4.02)	1.000	0.53 (0.15-1.88)	0.415
	2DS4-fv C1+C2	15 (10.64)	4 (3.79)	2 (11.11)	2 (4.76)	1.34 (0.41-4.39)	0.745	1.05 (0.21-5.02)	1.000	0.42 (0.09-1.91)	0.367
	2DS4-v C1+C2	3 (2.13)	1 (3.45)	1 (5.56)	6 (14.29)	1.64 (0.16-16.38)	1.000	2.71 (0.26-27.49)	0.385	7.67 (1.83-32.2)	**0.005**

C1+C2 constitutes one or more copies of HLA-C1 alleles (*C*01:02*, *C*14:02* and *C*16:01*) and HLA-C2 alleles (*C*02:02*, *C*04:01* and *C*05:01*). Non-transmitting mother (NT); Transmitting mother (TR); Intrapartum (IP); *In utero* (IU); Exposed Uninfected infant (EU). Matched mother and infant (M+I+); discordant mother-infant pair, where the mother possesses the KIR/HLA-C combination absent in the infant (M+I-) and when infant possesses the KIR/HLA-C combination absent in the mother (M-I+). Bold *P* values indicate significant differences ($P<0.05$).

Conversely in the M-I+ discordant group (**Figure 5.5C**), *KIR2DS4-v* was significantly increased in IU and IU2 infants compared to EU infants (P=0.048, OR=5.44 and P=0.002, OR=6.40, respectively). After adjusting for mVL, sdNVP and CD4+ T cell count alone, the frequency of *KIR2DS4-v* in IU2 infants remained significant (**Table 5.3**). Similarly, when *KIR2DS4-v* was combined with C1+C2 ligands, IU2 infants had significantly higher representation compared to EU infants (P=0.005, OR=7.67), and this association was maintained post adjustments for mVL, sdNVP and CD4+ T cell count.

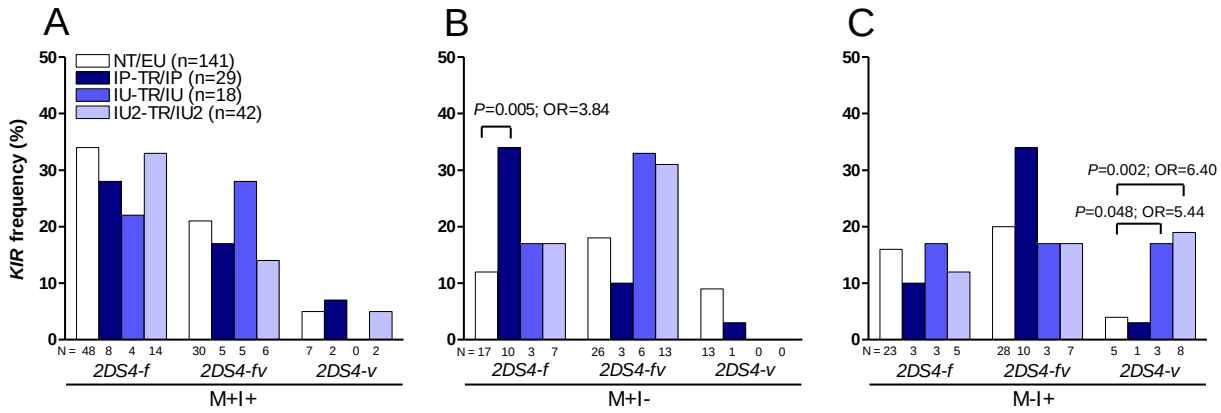


Figure 5.5 Comparison of *KIR2DS4* variants in MTCT1 (*KIR2DS4-f*, *KIR2DS4-fv* and *KIR2DS4-v*) amongst (A) Concordant mother-infant pairs (M+I+: both mother and infant are matched for *KIR2DS4* variant), (B) Discordant mother-infant pairs (M+I-: mothers possesses *KIR2DS4* variant that is absent in the infant), and (C) Discordant mother-infant pairs (M+I-: infants possesses *KIR2DS4* variant that is absent in the mother). Non-transmitting mother and exposed uninfected infant pair (NT/EU); intrapartum transmitting mother and intrapartum infected infant pair (IP-TR/IP); *in utero* transmitting mother and *in utero* infected infant pair (IU-TR/IU). The sum total of the frequencies in *KIR2DS4* variants between the groups of M+I+, M+I- and M-I+ will not equate 100%, as discordance will be represent twice, in context of M+I- and of M-I+.

Table 5.3 logistic regression analyses, multivariable adjustment made for maternal viral load (mVL), single dose nevirapine (sdNVP) and CD4+ T cell count

***KIR* or *KIR/HLA* combination**		**Association**		**Adjusted mVL**		**Adjusted sdNVP**		**Adjusted CD4+ T cell count**	
				OR (95% CI)	***P***	**OR (95% CI)**	***P***	**OR (95%CI)**	***P***
Infants	*2DS4-v*	EU	IU2	2.33 (0.84-6.43)	0.101	2.25 (0.84-5.98)	0.105	2.98 (1.09-8.15)	**0.033**
	2DS4-v C1+C2	EU	IU2	5.52 (1.43-21.3)	**0.013**	4.29 (1.21-15.3)	**0.024**	8.51 (1.97-36.7)	**0.004**
	AA-v	EU	IU	25.4 (0.66-966.0)	0.081	9.67 (0.97-95.7)	0.052	-	0.998
	AA-v	EU	IU2	16.8 (1.79-157.1)	**0.013**	8.70 (1.14-65.9)	**0.036**	14.47 (1.05-199.0)	**0.046**
	AA-v C1+C2	EU	IU2	7.02 (0.79-62.14)	0.080	7.25 (0.98-53.2)	0.052	14.47 (1.05-199.0)	**0.046**
M+I-	*2DS4-f*	NT	IP-TR	3.93 (1.44-10.7)	**0.007**	4.33 (1.63-11.5)	**0.003**	3.57 (1.29-9.89)	**0.014**
M-I+	*2DS4-v*	EU	IU	4.26 (0.55-32.5)	0.163	5.10 (1.09-23.8)	**0.038**	2.50 (0.25-24.8)	0.433
	2DS4-v	EU	IU2	5.68 (1.54-20.9)	**0.009**	5.02 (1.46-17.2)	**0.010**	5.73 (1.51-21.8)	**0.010**
	2DS4-v C1+C2	EU	IU2	7.40 (1.49-36.8)	**0.014**	4.93 (1.09-22.1)	**0.037**	9.66 (1.76-52.8)	**0.009**

EU, exposed uninfected infant; IU, *in utero* infected infant; IU2, enriched group of *in utero* infected infants; IP-TR, intrapartum transmitting mother. Bold *P* indicates significant differences ($P<0.05$).

5.2.5 Comparison of *KIR2DS4* variants in MTCT2

For MTCT2, mother and infant samples were *KIR2DS4* typed using the real-time PCR assay (Chapter 2, section 2.6). Out of the 79 mothers, *KIR2DS4* was absent in four mothers, thus the total *KIR2DS4* frequency was 94.9% (75/79) of which the frequencies for *KIR2DS4-f* and *KIR2DS4-v* were 86.7% (65/75) and 46.7% (35/75), respectively. In addition, out of the 11 mothers who were homozygous for Group-A haplotype, only one was homozygous for *KIR2DS4-v*, therefore, in MTCT2, only one mother (1.3%) lacked a functional activating *KIR* gene. Comparison of the AA1 haplotype frequency between the two maternal groups showed that the AA1 haplotype was significantly under-represented in mothers from MTCT2 compared to mothers from MTCT1 (13.9% vs. 27.2%, P=0.029). Thus, prevalence of *KIR2DS4-v* in mothers with the AA1 haplotype showed MTCT2 to cluster more closely to the South African Xhosa group (**Figure 5.6**). However, with regards to HIV-1 transmission, *KIR2DS4* variants (*KIR2DS4-f*, *KIR2DS4-fv* and *KIR2DS4-v*), as well as AA1 genotypes with *KIR2DS4* (*AA-f*, *AA-fv* and *AA-v*), did not significantly differ in the comparisons conducted within the maternal or the infant groups (**Figure 5.7**). Although, *KIR2DS4-f* showed a trend towards higher representation in IU-infected infants compared to EU infants (P=0.080, OR=2.61). With regards to paired mother-infant associations, neither *KIR2DS4* concordance nor discordance between mother-infant pairs showed any significant differences (**Table 5.4**).

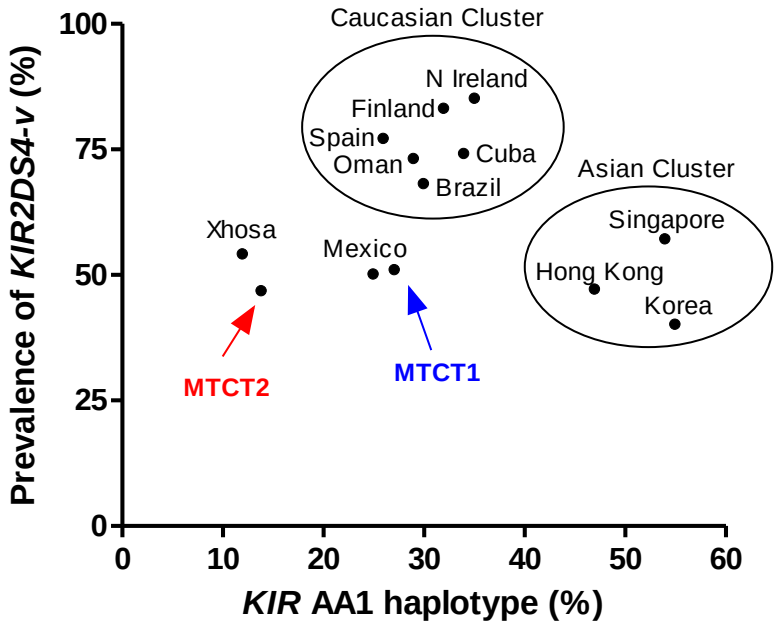


Figure 5.6 A scatter plot comparison of the *KIR2DS4-v* and AA1 haplotype prevalence amongst MTCT1 (Blue) and MTCT2 (Red) and other population groups. Reproduced and modified from (Whang et al., 2005, Middleton et al., 2007a).

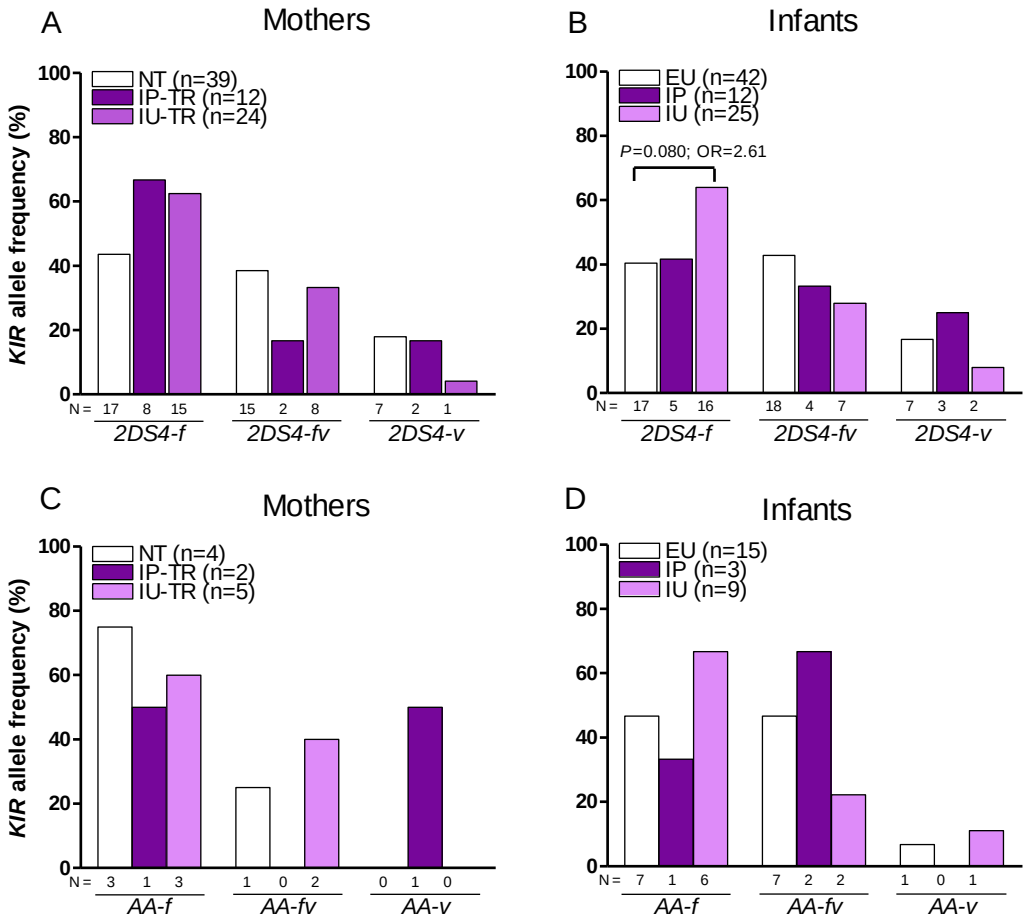


Figure 5.7 Frequencies of *KIR2DS4-f*, *KIR2DS4-fv* and *KIR2DS4-v* in the total group of mothers (A) and infants (B) in MTCT2; and the frequency of *KIR2DS4* variants amongst mothers (C) and infants (D) with AA haplotype. *KIR2DS4-f* indicates the presence of one or two copies of functional *KIR2DS4*; *KIR2DS4-fv* indicates the presence of both functional and non-functional *KIR2DS4*; and *KIR2DS4-v* indicates the presence of one or two copies of non-functional *KIR2DS4*.

Table 5.4 Carrier frequencies of maternal and infant *KIR2DS4* variants, as well as concordant and discordant mother-infant pairs in MTCT2

MTCT2*	*KIR2DS4*	Representation, N (%)			IP-TR / IP vs. NT / EU		IU-TR / IU vs. NT / EU	
		NT / EU	IP-TR / IP	IU-TR / IU	OR (95% CI)	*P*	OR (95% CI)	*P*
M+I+ (n=75)	*2DS4-f*	12 (30.77)	5 (41.67)	12 (50.00)	1.60 (0.42-6.10)	0.728	2.25 (0.78-6.43)	0.182
	2DS4-fv	8 (20.51)	2 (16.67)	4 (16.67)	0.78 (0.14-4.27)	1.000	0.78 (0.21-2.91)	0.755
	2DS4-v	2 (5.13)	2 (16.67)	1 (4.17)	3.70 (0.46-29.6)	0.232	0.80 (0.07-9.37)	1.000
M+I- (n=75)	*2DS4-f*	5 (12.82)	3 (25.00)	3 (12.50)	2.26 (0.45-11.3)	0.372	0.97 (0.21-4.49)	1.000
	2DS4-fv	7 (17.95)	0 (0.00)	4 (16.67)	-	0.177	0.91 (0.23-3.52)	1.000
	2DS4-v	5 (12.82)	0 (0.00)	0 (0.00)	-	0.323	-	0.147
M-I+ (n=75)	*2DS4-f*	5 (12.82)	0 (0.00)	3 (12.50)	-	0.323	0.97 (0.21-4.49)	1.000
	2DS4-fv	10 (25.64)	2 (16.67)	3 (12.50)	0.58 (0.11-3.11)	0.706	0.41 (0.10-1.69)	0.337
	2DS4-v	2 (5.13)	1 (8.33)	1 (4.17)	1.68 (0.14-20.3)	1.000	0.80 (0.07-9.37)	1.000

Concordant mother-infant pair (M+I+); discordant mother-infant pair, where the mother possesses the *KIR* combination absent in the infant (M+I-) and when infant possesses the KIR combination absent in the mother (M-I+). *Maternal NT and TR groups were matched according to maternal VL, CD4+ T cell counts as well as antiretroviral therapy, thus negating multivariate adjustments.

5.3 Discussion

Full-length functional variants of *KIR2DS4* (*KIR2DS4-f*) have been associated with increased risk for HIV-1 transmission as well as higher VL (Merino et al., 2011). In addition, Merino et al. (2011) also found a positive association between *KIR2DS4-f* and the presence of genital ulcers in seroprevalent index partners. More recently, the same group reported that the expression of *KIR2DS4-f* on the NK cell surface promoted HIV-1 pathogenesis during chronic infection (Merino et al., 2014). NK cells expressing *KIR2DS4-f* from individuals with untreated, chronic HIV-1 infection were polyfunctional and capable of degranulation (CD107a) and secretion of INF-γ and MIP-1β. The authors proposed that prolonged or excessive NK cell activities as seen in chronic HIV-1 infection may promote HIV-1 pathogenesis by means of creating a pro-inflammatory environment that promoted HIV-1 replication and/or mucosal shedding. These studies, therefore, highlighted the role for KIR2DS4 in NK cell activation and function in adult HIV-1 infection and its potential as a biomarker of HIV-1 pathogenesis. However, the role of *KIR2DS4* in MTCT of HIV-1 has not been determined. Thus, in this chapter we evaluated the role of allelic variants of *KIR2DS4* in MTCT of HIV-1 using two South African mother-infant cohorts recruited during two different PMTCT programs. Wherein half the mothers from MTCT1 received sdNVP at the onset of labour (monotherapy) and all mothers from MTCT2 received dual therapy consisting of daily AZT from 28 - 37 weeks gestation as well as sdNVP at the onset of labour (**Chapter 2, section 2.1**).

First we examined the allelic frequency of *KIR2DS4-f* and *KIR2DS4-v* across the two MTCT cohorts and then questioned the role of these variants in MTCT of HIV-1. Overall, maternal frequencies of *KIR2DS4-f* and *KIR2DS4-v* were similar in both MTCT cohorts and were comparable to the frequencies reported for Zambian (Merino et al., 2011) and Xhosa (Middleton et al., 2007a) individuals. Prevalence data for *KIR2DS4-v* and the AA1 haplotype across different population groups (Middleton et al. 2007a) showed that the two MTCT cohorts were clearly separate from the Asian and Caucasian population groups, however, MTCT1 clustered more closely to the Mexican group while MTCT2 clustered more closely to the Xhosa group. This deviation was the result of the significantly greater representation of the AA1 haplotype in MTCT1 compared to MTCT2 (27.2% vs. 13.9%, P=0.029). Given that the AA1 haplotype differed significantly across the two MTCT cohorts, we compared our *KIR* data to another cohort of HIV-1 negative Black South Africans (Gentle et al.,

unpublished data). As mention previously, the *KIR* data represented a diverse cross-section of the Black ethnic groups of South Africa sourced from the ESKOM (Electricity Supply Commission) cohort, wherein the overall AA1 haplotype frequency (26.9%) was similar to the MTCT1 cohort (27.1%) but significantly lower in the MTCT2 cohort (13.9%). Stratification of the *KIR* data according to home language indicated that among the different languages, the frequency of the AA1 haplotype also differed. Both Tswana and Tsonga speaking individuals had high frequencies of the AA1 haplotype (45% and 40%, respectively), whereas individuals who spoke Xhosa, South Sotho or Northern Sotho had the lowest frequencies of the AA1 haplotype (12%, 13% and 17%, respectively). Therefore, it could be postulated that although our two cohorts of Black South African mothers were recruited from the same hospital and were living in similar areas, mothers from MTCT1 and MTCT2 differed on the basis of home language. Mothers from MTCT1 (the larger cohort) may represent a more diverse black ethnic group, while mothers from MTCT2 (the smaller cohort) may have been skewed towards individuals having a Xhosa or Sotho background.

In MTCT1, of the mothers that carried the AA1 haplotype, six were homozygous for *KIR2DS4-v*, which meant that the overall percentage of mothers without any activating *KIR* gene was 2.8%, significantly lower than the 25.3% reported in the North Irish Caucasian population (Middleton et al., 2007b). However, *KIR2DS4-f* or *KIR2DS4-v* frequencies did not significantly differ amongst mothers who transmitted versus those that did not. Similarly, when maternal *KIR2DS4* variants were in combination with their putative HLA-C ligands we found no significant associations. Furthermore, neither *KIR2DS4-f* nor *KIR2DS4-v* showed an association with maternal CD4+ T cell count or VL. It was only in infants that *KIR2DS4-v* carriage alone, and in combination with C1+C2 ligands, was significantly associated with increased risk of IU acquisition of HIV-1, and this association was enhanced in those infants who were homozygous for the group A haplotype. Additionally, when mother and infant were paired, discordance in either direction (M+I- or M-I+) revealed two important associations. Firstly, in the M+I- discordant group, IP-transmitting mothers had significantly greater proportion of *KIR2DS4-f* compared NT mothers, and secondly, in the M-I+ discordant group, IU infants, as well as IU2 infants, had significantly greater representation of *KIR2DS4-v* compared to EU infants. These findings suggested that in MTCT1 maternal possession of *KIR2DS4-f* and its absence in infants increased the risk for IP transmission, whilst infant possession *KIR2DS4-v* increased the risk for IU acquisition of HIV-1, particularly when infants were homozygous for the A haplotype.

Given that IP and sexual transmission share similarities in their mode of transmission i.e. across the mucosal barrier, IP transmission can occur when there is direct contact of infant mucosa with HIV-1 infected maternal blood, amniotic fluid, or cervical/vaginal secretions (Van de Perre, 1999, Kwiek et al., 2006), and sexual transmission can occur when the genital or rectal mucosa comes into direct contact with cell-free or cell-associated virus (Shattock and Moore, 2003), it is plausible that the maternal *KIR2DS4-f* association with IP transmission is similar to that of *KIR2DS4-f* with sexual transmission (Merino et al., 2011, Merino et al., 2014). Thus, in the same manner by which Merino et al. (2011 and 2014) proposed that *KIR2DS4-f* in HIV-1 seropositive partners might increase the risk for sexual transmission through increased NK cell activation, mucosal inflammation and viral shedding. Given our findings, regardless of the recipient being a sexual partner or an infant, maternal possession of *KIR2DS4-f* NK cells might also produce a pro-inflammatory state that would increase viral replication and promote the likelihood of IP transmission of HIV-1.

Conversely, when infants carried the truncated *KIR2DS4* alleles (*KIR2DS4-v*) there was a greater likelihood of IU acquisition of HIV-1, especially when infants harboured the AA1 haplotype. Since the group A haplotype predominately consists of inhibitory *KIRs* (*KIR2DL1*, *KIR2DL3*, *KIR2DL4*, *KIR3DL1*, *KIR3DL2* and *KIR3DL3*), *KIR2DS4-v* homozygous individuals would possess NK cells completely in favour of strong inhibition. Consequently, these NK cells would be unable to eliminate HIV-infected maternal cells due to lack of NK cell activation resulting in IU infection. Similarly in another study, *KIR2DS4-v* homozygosity was significantly higher in patients with syphilis than in uninfected controls (Zhuang et al., 2012). In agreement with our findings, Zhuang et al. (2012) suggest that the lack of an activating *KIR* in individuals with AA1 haplotype would result in inhibition of NK cell cytotoxicity, thereby predisposing them to acquiring syphilis. Alternatively, since *KIR2DS4-v* alleles are able to encode soluble KIR2DS4 products (Maxwell et al., 2002), it has been speculated that soluble KIR2DS4 could possibly divert or obstruct NK cell function by acting as a ligand for another receptor or simply as a necessary decoy to mop up the available soluble HLA (Middleton et al., 2007a). However, these hypotheses are unlikely since the mRNA transcripts from *KIR2DS4-v* alleles were found to be unstable and associated with low expression levels (McErlean et al., 2010), and thus the true function of the molecules encoded by *KIR2DS4-v* alleles remains unknown.

Although full-length KIR2DS4 was previously shown to directly interact with HLA-C*04 (Katz et al., 2001), in our analyses neither the individual *HLA-C* alleles (*C*01:02*, *C*14:02, C*16:01, C*02:02, C*04:01* and C*05:01*) nor the combination of C1 and C2 alleles with *KIR2DS4-f* showed any significant differences between NT and TR (IP, IU and IU2) mothers, suggesting that in our MTCT1 cohort these HLA-C alleles were not associated with HIV-1 transmission.

Overall, the role of *KIR2DS4* in MTCT1 was striking and showed evidence that suggested that NK cell activation though *KIR2DS4-f* or the lack thereof with *KIR2DS4-v* influenced the outcome of HIV-1 transmission. In addition, the fact that these associations were maintained after correction for mVL, sdNVP and CD4+ T cell count lend credibility that *KIR2DS4* has a significant role in transmission of HIV-1 in MTCT1. Immune activation has been shown to strongly correlate with viraemia during untreated disease (Catalfamo et al., 2008, Catalfamo et al., 2011, Klatt et al., 2013). Correspondingly, some studies reported that a higher level of systemic immune activation was associated with increased risk of HIV-1 acquisition, thus suggesting that a suppressed or "quiescent" immune environment may be protective (Begaud et al., 2006, Jennes et al., 2006a, McLaren et al., 2010, Naranbhai et al., 2012). However, several studies reported that immune activation in both EU adults and infants did not necessarily favour HIV-1 transmission, but, rather may be contributing to their resistance (Romano et al., 2006, Tran et al., 2006, Suy et al., 2007, Ono et al., 2008). Likewise, using a selection of cord-blood samples from MTCT1, haematological and immunological studies conducted in our own laboratory showed that infants born to HIV-1 positive mothers, regardless of NVP exposure, had increased levels of immune activation markers when compared to HIV-1 unexposed infants, in addition, immune activation was increased by exposure to sdNVP, particular in IU-infants (Schramm et al., 2006). Moreover, EU infants from mothers with high CD4+ T cell counts (> 500 cells/μl) that had taken sdNVP had significantly elevated white blood cells, monocytes and basophils when compared to newborn infants of mothers with similar CD4+ T cell counts that had not taken sdNVP (Schramm et al., 2010). Thus, the authors suggested that not all immune activation is necessarily deleterious and, if caused *in vivo* by sdNVP, may well assist infants to preventing the establishment of infection, however, in infants already infected when exposed to sdNVP (i.e. those with IU infection), increased immune activation may well be deleterious.

However in MTCT2, where TR and NT mothers were matched on the basis of maternal VL, CD4+ T cell count and ART, neither maternal nor infant possession of *KIR2DS4-f* or *KIR2DS4-v* showed any significant associations with regards to HIV-1 transmission or acquisition. Moreover when mother and infant were paired, neither concordance nor discordance for *KIR2DS4* variants show any significant associations or tends. The absence of any significant *KIR2DS4* association in MTCT2 could be due to fact that the AA1 haplotype frequency was relatively low in mothers from this cohort (13.9%), which would imply that the majority of mothers would possess the at least one B haplotype and have more than one activating *KIR* gene present. It could, therefore, be postulated that having more than one activating *KIR* may be negating the *KIR2DS4-f* association with HIV-1 transmission seen in MTCT1.

Alternatively, dual ART consisting of AZT and sdNVP in mothers and infants could reduce the genetic influence of *KIR2DS4*. Unlike monotherapy consisting of sdNVP given at the onset of labour that has little time to reduce the maternal VL and primarily acts as an infant PrEP (Lehman and Farquhar, 2007), dual ART therapy with daily AZT given to the mothers from 28 weeks gestation may able to lower the maternal VL and improve maternal CD4+ T count cell prior to onset of labour. AZT given to pregnant women at least 2 days prior to anticipated delivery was shown to reduce VL (Jackson, 1995). Moreover, in another study that compared maternal CD4+ T cells counts before and after maternal AZT treatment starting at 36 weeks until start of labour showed that AZT treatment significantly improved the CD4+ T cell counts in mothers who did not transmit HIV-1 to their infants (Makokha et al., 2002). However, the increase in CD4+ T cells was not significant among mothers who infected their infants with HIV-1; and unfortunately, this study did not determine whether HIV-1 infected infants were IU- or IP- infected. Likewise, upon HAART, immune activation declines and there is a reduction in the amount of inflammatory cytokines and chemokines (Guihot et al., 2011, Klatt et al., 2013). However, this was not evident in MTCT2. Maternal VL was in fact found to be significantly higher in NT mothers from MTCT2 compared to MTCT1 and CD4+ T cell counts were significantly lower NT mothers from MTCT2 compared to MTCT1 (**Chapter 2, Figure 2.3**). This is intriguing and implies that in MTCT2, NT mothers from despite having high VL and low CD4 + T cell count did not transmit HIV-1 to their infants, and suggests there may be other factors involved in preventing HIV-1 transmission.

In summary, this chapter highlights the importance of analysing *KIR* allelic variation in MTCT of HIV-1, as *KIR2DS4-f* and *KIR2DS4-v* variants were shown to have differential effects on HIV-1 transmission and acquisition. The data from MTCT1 suggested that maternal NK cell activation through *KIR2DS4-f* or the lack thereof with *KIR2DS4-v* in infants associated with IP transmission or IU acquisition of HIV-1, respectively. Given that we only found *KIR2DS4* associations in MTCT1 and not in MTCT2, where mothers had significantly lower representation of the AA1 haplotype than MTCT1 mothers, suggests that future studies that investigate the role of NK cell function in HIV-1 transmission should match cases and controls on basis of their AA1 haplotype as this would control for the influence of other activating *KIR* genes. Taken together it is clear that aspects of immune activation (which cells, their numbers, timing relative to exposure, transmitter-recipient genotypes/phenotypes) need to be more clearly delineated to further our understanding as to which activated components are associated with protection as opposed to acquisition. Moreover, functional studies are necessary to understand the immune activation pathways in NK cells that express *KIR2DS4-f* and *KIR2DS4-v* alleles.

CHAPTER 6

The influence of *HLA-G* polymorphisms in MTCT of HIV-1

The findings from MTCT1 presented in this Chapter have been published: Hong et al. (2015) *Infection, Genetics and Evolution*, 30, 147 - 158.

6.1 Introduction

Recently there has been an increased interest in the role of *HLA-G* in MTCT, because of its preferential expression at the maternal-foetal interface on extravillous cytotrophoblasts, where it is plays an important tolerogenic role towards the semi-allogenic foetus. However, HLA-G expression is not limited to the maternal-foetal interface and has been found in the thymus, cornea, nail matrix, pancreas, and on erythroid and endothelial precursors as well as seminal plasma (Larsen et al., 2011, Dahl et al., 2014b, Lynge Nilsson et al., 2014). HLA-G can inhibit differentiation, proliferation, cytolysis, cytokine secretion and immunoglobulin production upon binding to their specific inhibitory receptors: immunoglobulin-like transcript (ILT)-2, ILT-4 and KIR2DL4 expressed by many immune cells, such as B and T lymphocytes as well as NK cells (Amiot et al., 2014).

Unlike the classical HLA Class I molecules (HLA-A, -B and -C), the non-classical HLA-G molecule has limited allelic polymorphism with only 50 alleles described to date (IMGT/HLA, version 3.16.0, 2014/04/14). These alleles encode 16 distinct transmembrane proteins (G*01:01, G*01:02, G*01:03, G*01:04, G*01:06, G*01:07, G*01:08, G*01:09, G*01:10, G*01:11, G*01:12, G*01:14, G*01:15, G*01:16, G*01:17 and G*01:18) and two truncated proteins (G*01:05N and G*01:13N) (Robinson et al., 2013b). Nevertheless, alternative splicing of the HLA-G primary transcript can generate seven mRNAs that encode membrane-bound (HLA-G1, -G2, -G3, and -G4) and soluble (HLA-G5, -G6, and -G7) protein isoforms (Donadi et al., 2011). In addition, HLA-G1 can also be proteolytically cleaved from the membrane and released as soluble HLA-G1 (sHLA-G) allowing it to have systemic immunoregulatory effects in the absence of localized tissue expression (Solier et al., 2002). Thus, the main isoforms present in plasma are shed HLA-G1 and secreted HLA-G5 proteins. Of note, in contrast to other *HLA* class I genes, exon 6 of the *HLA-G* encodes a premature stop codon that results in a truncated cytoplasmic tail which prolongs the half-life of HLA-G on the cell surface as endocytosis is reduced (Park et al., 2001).

Unlike the coding region, a high degree of variation exists within 5' and 3' untranslated regions (UTRs) and these polymorphisms, particularly within the 3'UTR, may affect the post-transcriptional regulation and biological functions of HLA-G (Castelli et al., 2010, Costa et al., 2012). Since the 3'UTR of *HLA-G* contains several regulatory elements (AU-rich motifs, a poly-A signal, as well as signals that regulate the spatial and temporal expression of an mRNA), polymorphisms within this region may alter mRNA stability, turnover, mobility, and splicing pattern (Lynge Nilsson et al., 2014). Indeed, several polymorphic sites within the 3'UTR have been associated with differential HLA-G expression profiles and at least eight of these polymorphic sites are frequently found in worldwide populations (Castelli et al., 2014). Well studied amongst these is the 14-bp insertion/deletion (rs66554220), wherein the presence of a 14-bp insertion (*Ins*) (5'-ATTTGTTCATGCCT-3') introduces an alternative splicing site that generates a 92-bp deletion in the 3'UTR that influences mRNA stability. Individuals with *Ins/Ins* genotype have been associated with lower mRNA production compared to *Ins/Del* and *Del/Del* genotypes (Hviid et al., 2003, Castelli et al., 2009, Castelli et al., 2010). Furthermore, other single nucleotide polymorphisms (SNPs) have also been implicated in the regulation of HLA-G expression. For example, the +3187 A/G SNP (located near an AU-rich motif in the HLA-G mRNA) has been associated with decreased *in vitro* mRNA stability, so that the presence of the +3187A allele may lead to decreased *HLA-G* expression due to the increased number of adenines in this AU-rich motif (Yie et al., 2008).

In the context of MTCT of HIV-1, several studies have associated the 14-bp indel with the risk of MTCT (Aikhionbare et al., 2006, Fabris et al., 2009, Segat et al., 2009, Segat and Crovella, 2012, Sanches et al., 2013, Segat et al., 2014) and two have associated *HLA-G* alleles with HIV-1 transmission (Luo et al., 2013, Turk et al., 2013). However, some of these reports have been contradictory. For example, in Zambian infants, the *Ins* (associated with lower with lower mRNA production) was linked with protection from IU and IP HIV-1 infection (Segat et al., 2014), yet in Brazilian children, the *Del* allele and *Del/Del* genotype (associated with more stable mRNA and increased HLA-G expression) was linked with protection from MTCT (Fabris et al., 2009). Whilst in other studies, *G*01:03*- alleles, which reportedly translate into lower plasma sHLA-G, were associated with a reduced risk of MTCT of HIV-1 (Luo et al., 2013). Whereas in the context of sexual transmission, *G*01:04:04*, an allele with reportedly higher levels of plasma sHLA-G, was associated with susceptibility to HIV-1 infection (Turk et al., 2013). Of note, amongst all these studies, few have assessed the role of *HLA-G* alleles and 3'UTR SNPs collectively.

Thus, the aim of this chapter was to investigate the role of *HLA-G* alleles (defined by the extracellular α1, α2 and α3 domains) as well as the 3'UTR in HIV-1 transmitting (TR) and non-transmitting (NT) mothers and their HIV-1 infected/uninfected infants in two unique MTCT cohorts recruited in Johannesburg, South Africa.

6.2. Results

6.2.1 *HLA-G* allele representation in MTCT1

A total of 216 mother-infant pairs were genotyped from MTCT1 (N=217) which included 144 NT/EU pairs and 72 TR/IP, IU or IU2 pairs (one NT/EU mother-infant pair was excluded due to insufficient DNA). Sixteen *HLA-G* alleles were identified in the study population (**Table 6.1**). These encoded six functional proteins (G*01:01, G*01:03, G*01:04, G*01:06, G*01:08 and G*01:11) and one truncated protein (G*01:05N). The three most common *HLA-G* alleles were *G*01:01:01*, *G*01:01:02* and *G*01:04:04* with frequencies greater than 15% in both mothers and infants, which corresponded with the most common genotypes *G*01:01:01/G*01:01:01*, *G*01:01:01/G*01:01:02* and *G*01:01:01/G*01:04:04* (**Table 6.2**). All mother and infant *HLA-G* allele frequencies were in HWE (P=0.120 and P=0.769, respectively).

6.2.2 *HLA-G* alleles and transmission of HIV-1 in MTCT1

To investigate the influence of *HLA-G* alleles and genotypes in vertical transmission of HIV-1 in MTCT1 we compared TR mothers to NT mothers as well as their respective infants. Maternal possession of the *G*01:01:02* allele was associated with increased risk for IU HIV-1 transmission (**Table 6.1**). Representation of *G*01:01:02* was higher in IU-TR and IU2-TR mothers compared to NT mothers (P=0.036, OR=2.26; P=0.078, OR=1.68, respectively). While *G*01:01:02/G*01:01:02* genotype showed a strong trend (P=0.072, OR=4.31) towards increased representation in IU-TR mothers compared to NT mothers (**Table 6.2**). In addition, the combination *G*01:01:02/G*01:03:01* was significantly higher in IU2-TR mothers compared to NT mothers (P=0.031, OR=4.61). All of these allele associations were significant post adjustments for mVL but only *G*01:01:02* homozygosity maintained significance post adjustment for maternal sdNVP and infant *KIR2DS4-v* (**Table 6.6**). Infant *HLA-G* alleles and *HLA-G* genotypes showed no significant associations with regards to HIV-1 acquisition (**Table 6.1** and **Table 6.3**, respectively).

6.2.3 *HLA-G* 3'UTR allele, genotype and haplotype frequencies in MTCT1

Sequencing analysis of *HLA-G* 3'UTR in mother-infant pairs from MTCT1 revealed the presence of 11 polymorphic sites. Nine of these have been previously described, namely: +2961*Ins/Del* (rs66554220), +3003T/C (rs1707), +3010C/G (rs1710), +3027C/A (rs17179101), +3032G/C (rs146339774), +3035C/T (rs17179108), +3142G/C (rs1063320), +3187A/G (rs9380142), and +3196C/G (rs1610696). Two new SNPs, +3076C/T and +3114G/A, were only found in EU infants, but had frequencies below 1% and were not considered in further analyses. Observed genotype frequencies at all variation sites were in adherence with HWE. Using HAPLOVIEW bioinformatics software (Barrett et al., 2005) and in combination with published 3'UTR haplotypes (Sabbagh et al., 2014), we found eight distinct 3'UTR haplotypes (UTR1-UTR7 and UTR20) within MTCT1 (**Figure 6.1** and **Figure 6.2**, respectively). LD analysis amongst 3'UTR SNPS revealed positions +3010C/G and +3142G/C were in complete linkage, whilst the 14-bp indel as well as position +3196C/G were in strong linkage disequilibrium (**Figure 6.1**). UTR1, UTR2 and UTR3 were the most frequent haplotypes (**Figure 6.2**), which together accounted for over 65% of the haplotypes in our South African study population, which was most comparable to the Senegalese population (SER, n=239) reported by Sabbagh et al. (2014).

Table 6.1 Representation of *HLA-G* allele frequencies in HIV-1 positive mothers and their infants from MTCT1, N (%)

Maternal	**NT**	**IP-TR**	**IU-TR**	**IU2-TR**	**IP-TR vs. NT**		**IU-TR vs. NT**		**IU2-TR vs. NT**	
***HLA-G* Alleles**	**(2n=288)**	**(2n=58)**	**(2n=38)**	**(2n=86)**	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
*G*01:01:01*	85 (29.51)	19 (32.76)	15 (39.47)	25 (29.07)	1.16 (0.63-2.12)	0.639	1.55 (0.77-3.13)	0.261	0.97 (0.57-1.66)	1.000
*G*01:01:02*	59 (20.49)	11 (18.97)	14 (36.84)	26 (30.23)	0.91 (0.44-1.86)	0.860	2.26 (1.10-4.64)	**0.036**	1.68 (0.97-2.89)	**0.078**
*G*01:01:08*	8 (2.78)	3 (5.17)	1 (2.63)	2 (2.33)	1.91 (0.49-7.42)	0.403	0.94 (0.11-7.77)	1.000	0.83 (1.07-3.99)	1.000
*G*01:01:09*	11 (3.82)	2 (3.45)	1 (2.63)	1 (1.16)	0.90 (0.19-4.16)	1.000	0.68 (0.08-5.42)	1.000	0.29 (0.04-2.32)	0.310
*G*01:01:15*	1 (0.35)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
*G*01:01:17*	1 (0.35)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
*G*01:01:19*	8 (2.78)	2 (3.45)	0 (0.00)	4 (4.65)	1.25 (0.25-6.04)	1.000	-	0.603	1.70 (0.50-5.81)	0.483
*G*01:01:20*	1 (0.35)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
*G*01:03:01*	23 (7.99)	3 (5.17)	3 (7.89)	7 (8.14)	0.63 (0.18-2.16)	0.592	0.98 (0.28-3.45)	1.000	1.02 (0.42-2.46)	1.000
*G*01:04:01*	11 (3.82)	5 (8.62)	0 (0.00)	1 (1.16)	2.37 (0.79-7.11)	0.160	-	0.375	0.29 (0.04-2.32)	0.310
*G*01:04:04*	53 (18.40)	10 (17.24)	3 (7.89)	15 (17.44)	0.92 (0.44-1.94)	1.000	0.38 (0.11-1.28)	0.116	0.93 (0.50-1.76)	0.875
*G*01:05N*	27 (9.38)	2 (3.45)	0 (0.00)	4 (4.65)	0.34 (0.08-1.49)	0.194	-	**0.056**	0.47 (0.16-1.38)	0.188
*G*01:06*	0 (0.00)	1 (1.72)	0 (0.00)	0 (0.00)	-	0.168	-	1.000	-	1.000
*G*01:11*	0 (0.00)	1 (1.72)	1 (2.63)	1 (1.16)	-	1.000	-	0.117	-	0.230
Infant	**EU**	**IP**	**IU**	**IU2**	**IP vs. EU**		**IU vs. EU**		**IU2 vs. EU**	
***HLA-G* Alleles**	**(2n=288)**	**(2n=58)**	**(2n=38)**	**(2n=86)**	**OR (95% CI)**	***P***	**OR (95%CI)**	***P***	**OR (95% CI)**	***P***
*G*01:01:01*	96 (33.33)	22 (37.93)	13 (34.21)	21 (24.42)	1.22 (0.68-2.19)	0.545	1.04 (0.51-2.12)	1.000	0.64 (0.37-1.12)	0.145
*G*01:01:02*	59 (20.49)	6 (10.34)	9 (23.68)	20 (23.26)	0.45 (0.18-1.09)	0.096	1.20 (0.54-2.68)	0.672	1.17 (0.66-2.09)	0.652
*G*01:01:08*	10 (3.47)	4 (6.90)	3 (7.89)	4 (4.65)	2.05 (0.62-6.81)	0.265	2.38 (0.62-9.07)	0.377	1.35 (0.41-4.43)	0.746
*G*01:01:09*	7 (2.43)	4 (6.90)	1 (2.63)	1 (1.16)	2.97 (0.84-10.5)	0.094	1.08 (0.12-9.06)	1.000	0.47 (0.05-3.89)	0.688
*G*01:01:19*	11 (3.82)	2 (3.45)	0 (0.00)	5 (5.81)	0.90 (0.19-4.17)	1.000	-	0.375	1.55 (0.52-4.60)	0.542
*G*01:03:01*	29 (10.07)	4 (6.90)	4 (10.53)	8 (9.30)	0.66 (0.22-1.95)	0.485	1.05 (0.35-3.17)	1.000	0.92 (0.40-2.08)	1.000
*G*01:04:01*	11 (3.82)	1 (1.72)	1 (2.63)	3 (3.49)	0.44 (0.06-3.49)	0.497	0.68 (0.08-5.42)	1.000	0.91 (0.25-3.33)	1.000
*G*01:04:04*	41 (14.24)	11 (18.97)	6 (15.79)	19 (22.09)	1.41 (0.67-2.94)	0.419	1.12 (0.44-2.87)	0.806	1.71 (0.93-3.13)	0.094
*G*01:04:05*	1 (0.35)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
*G*01:05N*	21 (7.29)	3 (5.17)	1 (2.63)	5 (5.81)	0.69 (0.20-2.41)	0.600	0.34 (0.05-2.63)	0.343	0.78 (0.29-2.15)	0.810
*G*01:06*	0 (0.00)	1 (1.72)	0 (0.00)	0 (0.00)	-	0.168	-	1.000	-	1.000
*G*01:08*	1 (0.35)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
*G*01:11*	1 (0.35)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000

NT: HIV-1 non-transmitting mother; IP-TR: intrapartum HIV-1 transmitting mother; IU-TR: *in utero* HIV-1 transmitting mother; IU2-TR: enriched group of *in utero* transmitting mothers; EU: HIV-1 exposed uninfected infant; INF; IP: intrapartum infected infant; IU: *in utero* infected infant; IU2: enriched group of *in utero* infected infants. Highlighted and bold *P* values indicate significant differences ($P<0.05$) and bold *P* values indicate trends ($P<0.090$).

Table 6.2 Representation of maternal *HLA-G* genotypes in MTCT1, N (%)

HLA-G* Genotypes	**NT (n=144)**	**IP-TR (n=29)**	**IU-TR (n=19)**	**IU-TR (n=43)**	**IP-TR vs. NT**		**IU-TR vs. NT**		**IU2-TR vs. NT**	
					OR (95% CI)	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
*G*01:01:01/G*01:01:01*	17 (11.81)	3 (10.34)	5 (26.32)	5 (11.63)	0.86 (0.23-3.16)	1.000	2.66 (0.85-8.34)	0.143	0.98 (0.34-2.84)	1.000
*G*01:01:01/G*01:01:02*	15 (11.81)	4 (13.79)	3 (15.79)	4 (9.30)	1.37 (0.42-4.49)	0.744	1.61 (0.42-6.18)	0.698	0.88 (0.27-2.81)	1.000
*G*01:01:01/G*01:01:09*	4 (2.78)	0 (0.00)	1 (5.26)	1 (2.33)	-	0.608	1.94 (0.21-18.3)	1.000	0.83 (0.09-7.65)	1.000
*G*01:01:01/G*01:03:01*	4 (2.78)	0 (0.00)	1 (5.26)	2 (4.65)	-	0.608	1.94 (0.21-18.3)	1.000	1.71 (0.30-9.66)	0.622
*G*01:01:01/G*01:04:01*	3 (2.08)	2 (6.90)	0 (0.00)	0 (0.00)	3.48 (0.56-21.8)	0.196	-	1.000	-	0.587
*G*01:01:01/G*01:04:04*	17 (11.81)	4 (13.79)	0 (0.00)	6 (13.95)	1.19 (0.37-3.85)	0.758	-	0.224	1.21 (0.44-3.29)	0.792
*G*01:01:01/G*01:05N*	7 (4.86)	1 (3.45)	0 (0.00)	0 (0.00)	0.70 (0.08-5.91)	1.000	-	0.602	-	0.205
*G*01:01:02/G*01:01:02*	6 (4.17)	2 (6.90)	3 (15.79)	4 (9.30)	1.70 (0.33-8.89)	0.623	4.31 (0.98-18.9)	**0.072**	2.35 (0.63-8.77)	0.242
*G*01:01:02/G*01:01:08*	3 (2.08)	1 (3.45)	0 (0.00)	1 (2.33)	1.67 (0.17-16.7)	1.000	-	1.000	1.11 (0.11-11.0)	1.000
*G*01:01:02/G*01:01:19*	4 (2.78)	0 (0.00)	0 (0.00)	0 (0.00)	-	0.608	-	1.000	-	0.575
*G*01:01:02/G*01:03:01*	4 (2.78)	0 (0.00)	2 (10.53)	5 (11.63)	-	0.608	4.11 (0.70-24.1)	0.145	4.61 (1.17-17.9)	**0.031**
*G*01:01:02/G*01:04:01*	3 (2.08)	0 (0.00)	0 (0.00)	1 (2.33)	-	1.000	-	1.000	1.11 (0.11-11.0)	1.000
*G*01:01:02/G*01:04:04*	10 (6.94)	2 (6.90)	2 (10.53)	4 (9.30)	1.00 (0.21-4.78)	1.000	1.57 (0.32-7.81)	0.634	1.37 (0.41-4.62)	0.741
*G*01:01:02/G*01:05N*	6 (4.17)	0 (0.00)	0 (0.00)	2 (4.65)	-	0.387	-	0.615	1.12 (0.22-5.77)	1.000
*G*01:01:08/G*01:04:04*	3 (2.08)	0 (0.00)	1 (5.26)	1 (2.33)	-	1.000	2.61 (0.26-26.5)	0.394	1.11 (0.11-11.0)	1.000
*G*01:01:09/G*01:05N*	3 (2.08)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	0.587
*G*01:03:01/G*01:04:04*	3 (2.08)	1 (3.45)	0 (0.00)	0 (0.00)	1.67 (0.17-16.7)	1.000	-	1.000	-	0.587
*G*01:03:01/G*01:05N*	4 (2.78)	0 (0.00)	0 (0.00)	0 (0.00)	-	0.608	-	1.000	-	0.575
*G*01:04:04/G*01:04:04*	6 (4.17)	0 (0.00)	0 (0.00)	0 (0.00)	-	0.387	-	0.615	-	0.339
*G*01:04:04/G*01:05N*	6 (4.17)	1 (3.45)	0 (0.00)	2 (4.65)	0.82 (0.09-7.09)	1.000	-	0.615	1.12 (0.22-5.77)	1.000

* indicates *HLA-G* genotype frequencies > 2% in the NT group. NT: HIV-1 non-transmitting mother; IP-TR: intrapartum HIV-1 transmitting mother; IU-TR: *in utero* HIV-1 transmitting mother; IU2-TR: enriched group of *in utero* transmitting mothers. Highlighted and bold *P* values indicate significant differences ($P<0.05$) and bold *P* values indicate trends ($P<0.090$).

Table 6.3 Representation of infant *HLA-G* genotypes in MTCT1, N (%)

HLA-G* Genotypes	**EU (n=144)**	**IP (n=29)**	**IU (n=19)**	**IU2 (n=43)**	**IP-TR vs. EU**		**IU-TR vs. EU**		**IU2-TR vs. EU**	
					OR (95% CI)	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
*G*01:01:01/G*01:01:01*	17 (11.81)	4 (13.79)	3 (15.79)	3 (6.98)	1.19 (0.37-3.85)	0.751	1.40 (0.36-5.31)	0.708	0.56 (0.16-2.01)	0.421
*G*01:01:01/G*01:01:02*	15 (10.42)	2 (6.90)	2 (10.53)	3 (6.98)	0.63 (0.14-2.94)	0.741	1.01 (0.21-4.81)	1.000	0.64 (0.18-2.34)	0.575
*G*01:01:01/G*01:01:09*	3 (2.08)	2 (6.90)	0 (0.00)	0 (0.00)	3.48 (0.56-21.8)	0.196	-	1.000	-	0.587
*G*01:01:01/G*01:01:19*	4 (2.78)	0 (0.00)	0 (0.00)	3 (6.98)	-	0.608	-	1.000	2.62 (0.56-12.2)	0.355
*G*01:01:01/G*01:03:01*	12 (8.33)	2 (6.90)	1 (5.26)	1 (2.33)	0.81 (0.17-3.85)	1.000	0.61 (0.07-4.98)	1.000	0.26 (0.03-2.07)	0.304
*G*01:01:01/G*01:04:01*	4 (2.78)	1 (3.45)	0 (0.00)	1 (2.33)	1.25 (0.13-11.6)	1.000	-	1.000	0.83 (0.09-7.66)	1.000
*G*01:01:01/G*01:04:04*	11 (7.64)	3 (10.34)	3 (15.79)	6 (13.95)	1.39 (0.36-5.34)	0.707	2.26 (0.57-8.99)	0.375	1.96 (0.67-5.65)	0.229
*G*01:01:01/G*01:05N*	10 (6.94)	2 (6.90)	0 (0.00)	0 (0.00)	0.99 (0.21-4.78)	1.000	-	0.374	-	0.120
*G*01:01:02/G*01:01:02*	9 (6.25)	0 (0.00)	0 (0.00)	2 (4.65)	-	0.228	-	0.390	0.73 (0.15-3.52)	1.000
*G*01:01:02/G*01:01:08*	3 (2.08)	2 (6.90)	2 (10.53)	3 (6.98)	3.48 (0.56-21.8)	0.196	5.52 (0.86-35.47)	0.104	3.52 (0.68-18.1)	0.136
*G*01:01:02/G*01:01:19*	3 (2.08)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	0.587
*G*01:01:02/G*01:03:01*	3 (2.08)	0 (0.00)	2 (10.53)	3 (6.98)	-	1.000	5.52 (0.86-35.47)	0.104	3.52 (0.68-18.1)	0.136
*G*01:01:02/G*01:04:01*	3 (2.08)	0 (0.00)	1 (5.26)	1 (2.33)	-	1.000	2.61 (0.25-26.45)	0.394	1.11 (0.11-11.1)	1.000
*G*01:01:02/G*01:04:04*	9 (6.25)	1 (3.45)	2 (10.53)	4 (9.30)	0.53 (0.06-4.39)	0.699	1.76 (0.35-8.85)	0.619	1.53 (0.45-5.26)	0.500
*G*01:01:02/G*01:05N*	3 (2.08)	0 (0.00)	0 (0.00)	2 (4.65)	-	1.000	-	1.000	2.29 (0.37-14.1)	0.591
*G*01:01:08/G*01:04:04*	4 (2.78)	0 (0.00)	0 (0.00)	0 (0.00)	-	0.608	-	1.000	-	0.575
*G*01:03:01/G*01:04:04*	3 (2.08)	2 (6.90)	0 (0.00)	3 (6.98)	3.48 (0.56-21.8)	0.196	-	1.000	3.52 (0.68-18.1)	0.136
*G*01:03:01/G*01:05N*	3 (2.08)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	0.587
*G*01:04:04/G*01:04:04*	3 (2.08)	2 (6.90)	0 (0.00)	1 (2.33)	3.48 (0.56-21.8)	0.196	-	1.000	1.11 (0.11-11.1)	1.000
*G*01:04:04/G*01:05N*	5 (3.47)	1 (3.45)	1 (5.26)	2 (4.65)	0.99 (0.11-8.83)	1.000	1.54 (0.17-13.9)	1.000	1.35 (0.25-7.25)	1.000

* indicates *HLA-G* genotype frequencies > 2% in the EU group. EU: HIV-1 exposed uninfected infant; IP: intrapartum infected; IU: *in utero* infected infant; IU2: enriched group of *in utero* infected infants.

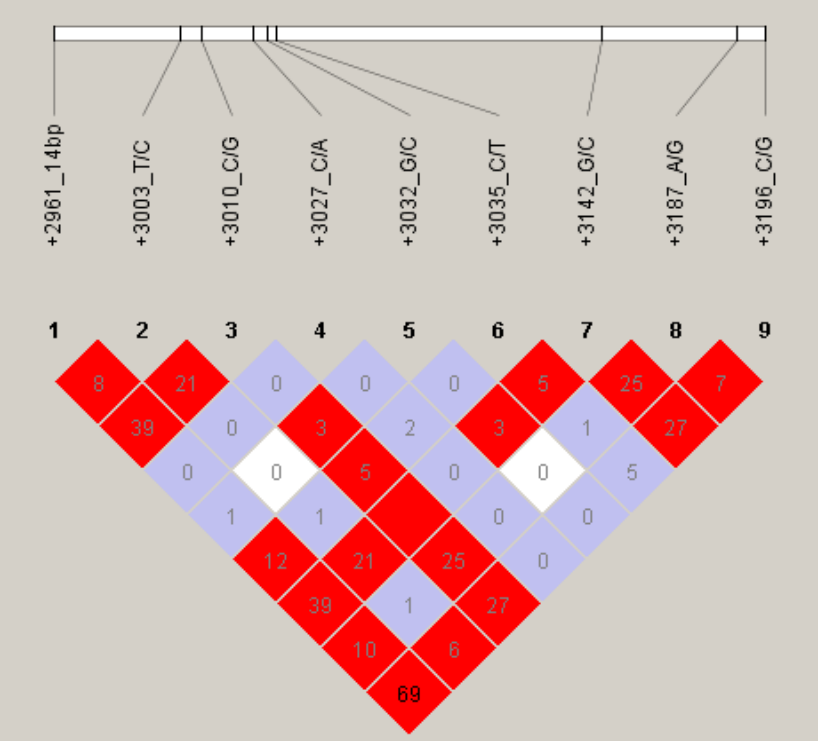
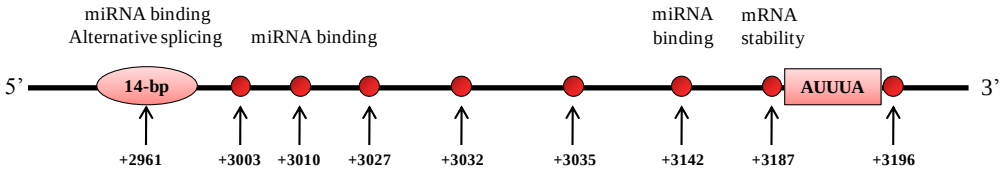


Figure 6.1 Linkage disequilibrium (LD) plot of *HLA-G* 3'UTR polymorphisms. At the top the SNPs are shown according to their succession in the 3'UTR of the *HLA-G*. Standard D'/LOD colour scheme was used, white and blue squares indicate a non-significant LD (LOD <2) and red squares indicate pairs in strong LD (LOD ≥ 2). Numbers indicate the R-square value expressed as a percentage and an empty box indicates an r^2 equal to 1.



Haplotype										% Representation				
										S.A[a]	ASW[b]	SER[b]	YAN[b]	LWK[b]
UTR1	Del	T	G	C	G	C	C	G	C	11.81	22.13	11.51	4.57	24.48
UTR2	Ins	T	C	C	G	C	G	A	G	34.49	31.15	34.31	18.00	32.29
UTR3	Del	T	C	C	G	C	G	A	C	22.45	16.39	27.20	28.00	11.98
UTR4	Del	C	G	C	G	C	C	A	C	10.19	6.56	5.44	10.57	8.85
UTR5	Ins	T	C	C	G	T	G	A	C	8.56	13.11	9.41	7.71	8.33
UTR6	Del	T	G	C	G	C	C	A	C	10.65	4.92	6.07	12.29	7.81
UTR7	Ins	T	C	A	G	T	G	A	C	0.23	0.82	0.00	0.29	0.00
UTR20	Del	T	G	C	C	C	C	A	C	1.62	1.64	0.00	1.43	2.08
Other UTRs[c]	-	-	-	-	-	-	-	-	-	0.00	3.28	6.07	7.14	4.17

Figure 6.2 Polymorphisms in the 3' untranslated region (UTR) of the *HLA-G* gene and the 3'UTR haplotypes. Polymorphic positions are relative to the first ATG codon at exon 1. UTR haplotypes were numbered according previously published reports (Castelli et al., 2014, Sabbagh et al., 2014). Del, Deletion; Ins, insertion.

[a] South Africa (SA) represents the total group of HIV-1 infected mothers from cohort 1 (n=216)

[b] Represents 3'UTR frequencies reported by (Sabbagh et al., 2014) which pooled some data from the 1000 genome project. ASW, people of African ancestry from the South Western United States (n=61); SER, Serer from Niakhar, Senegal (n=239); YAN, Yansi from Bandundu, Democratic Republic of the Congo (N=175); and LWK, Luhya from Webuye, Kenya (n=96).

[c] Represents other 3'UTR haplotypes not identified in SA

6.2.4 *HLA-G* 3'UTR polymorphisms and their association in MTCT1

When comparing these nine polymorphic sites within the 3'UTR with risk of MTCT, only one position (+3187A/G) showed an association with increased risk for IU MTCT (**Table 6.4**). Maternal frequencies of the *G* allele as well as *G/G* homozygosity at position +3187 were significantly over-represented in IU-TR mothers compared to NT mothers (*P*=0.011, OR=2.96 and *P*=0.011, OR=14.4, respectively). However, only *G/G* homozygosity maintained significance post adjustment for mVL (*P*=0.009, OR=29.6), but the confidence interval (CI) was considerably large (2.36-371.0), most likely due to the small number (**Table 6.6**). Comparison of haplotype associations in MTCT however confirmed the UTR1 haplotype (the only haplotype to have a *G* at position +3187), as well as the UTR1/UTR1 genotype (**Table 6.5**), were both significantly over-represented in IU-TR mothers compared to NT mothers (*P*=0.011, OR=2.96 and *P*=0.012, OR=13.3, respectively). The genotype UTR1/UTR2 also showed a strong trend towards increased representation in IU2-TR mothers compared to NT mothers (*P*=0.052, OR=3.66). Both UTR1/UTR1 and UTR1/UTR2 genotypes maintained significance after adjustment for mVL (*P*=0.009, OR=29.6 and *P*=0.019, OR=5.44, respectively) but not after adjustment for maternal sdNVP or *KIR2DS4-v* (**Table 6.6**).

Taken together, a Guanine at +3187 and/or the UTR1 haplotype in MTCT1, showed a significant association with increased risk for IU transmission. In contrast, IP-TR mothers had a significantly higher representation of the UTR3/UTR4 genotype compared to NT mothers (*P*=0.034, OR=8.19), but this association was not significant post adjustment for mVL or maternal sdNVP (**Table 6.6**). Comparison between genotypes UTR1/UTR1 (associated with IU transmission) and UTR3/UTR4 (associated with IP transmission) showed differences at four positions. The UTR3/UTR4 combination is heterozygous at positions +3003T/C, +3010C/G, +3142G/C, and has an Adenine at position +3187; while UTR1/UTR1 is homozygous at positions +3003T, +3010G, +3142C, and has a Guanine at position +3187.

Comparing the 3'UTR polymorphisms, as well as the haplotypes between the respective infant groups from MTCT1 revealed no significant associations or trends (data not shown), suggesting that HLA-G polymorphisms within the 3'UTR did not play a role in infant acquisition of HIV-1.

Table 6.4 Representation 3'UTR polymorphisms of *HLA-G* in HIV-1 positive mothers from MTCT1, N (%)

Polymorphism		NT (n=144)	IP-TR (n=29)	IU-TR (n=19)	IU2-TR (n=43)	IP-TR vs. NT OR (95% CI)	*P*	IU-TR vs. NT OR (95% CI)	*P*	IU2-TR vs. NT OR (95% CI)	*P*
	D	166 (57.64)	36 (62.07)	19 (50.00)	43 (50.00)	1.20 (0.67-2.14)	0.562	0.73 (0.37-1.45)	0.388	0.73 (0.45-1.19)	0.218
	I	122 (42.36)	22 (37.93)	19 (50.00)	43 (50.00)	1		1		1	
+2961 (Indel)	**DD**	53 (36.81)	11 (37.93)	6 (31.58)	12 (27.91)	1.61 (0.47-5.48)	0.564	0.58 (0.17-1.97)	0.527	0.58 (0.23-1.45)	0.344
	ID	60 (41.67)	14 (48.28)	7 (36.84)	19 (44.19)	1.81 (0.55-5.96)	0.414	0.52 (0.16-1.69)	0.348	0.81 (0.35-1.90)	0.668
	II	3 (21.53)	4 (13.79)	6 (31.58)	12 (27.91)	1		1		1	
	C	31 (10.76)	9 (15.52)	1 (2.63)	4 (4.65)	1.52 (0.68-3.40)	0.366	0.22 (0.03-1.69)	0.149	0.40 (0.14-1.18)	0.095
	T	257 (89.24)	49 (84.48)	37 (97.37)	82 (95.35)	1		1		1	
+3003.T/C	**CC**	4 (2.78)	1 (3.45)	0 (0.00)	0 (0.00)	1.39 (0.15-13.1)	1.000	-	1.000	-	0.573
	TC	23 (15.97)	7 (24.14)	1 (5.26)	4 (9.30)	1.69 (0.36-4.45)	0.418	0.28 (0.03-2.22)	0.311	0.52 (0.17-1.60)	0.329
	TT	117 (81.25)	21 (72.41)	18 (94.74)	39 (90.70)	1		1		1	
	G	100 (34.72)	22 (37.93)	15 (39.47)	26 (30.23)	1.14 (0.64-2.06)	0.653	1.23 (0.61-2.45)	0.590	0.81 (0.48-1.37)	0.516
	C	188 (65.28)	36 (62.07)	23 (60.53)	60 (69.77)	1		1		1	
+3010.C/G	**GG**	23 (15.97)	4 (13.79)	5 (26.32)	5 (11.63)	1.06 (0.31-3.66)	1.000	1.61 (0.49-5.32)	0.518	0.66 (0.22-1.95)	0.609
	CG	54 (37.50)	14 (48.28	5 (26.32)	16 (37.21)	1.58 (0.66-3.76)	0.379	0.69 (0.22-2.18)	0.582	0.90 (0.43-1.89)	0.853
	CC	67 (46.53)	11 (37.93)	9 (47.37)	22 (51.16)	1		1		1	
	A	1 (0.35)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
	C	287 (99.65)	58 (100.00)	38 (100.00)	86 (100.00)	1		1		1	
+3027.C/A	**AA**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
	CA	1 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
	CC	143 (99.31)	29 (100.00)	19 (100.00)	43 (100.00)	1		1		1	
	C	4 (1.39)	1 (1.72)	1 (2.63)	2 (2.33)	1.00 (0.11-8.66)	1.000	1.52 (0.17-13. 5)	1.000	1.34 (0.26-7.07)	1.000
	G	284 (98.61)	57 (98.28)	37 (97.37)	84 (97.67)	1		1		1	
+3032.G/C	**CC**	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
	GC	4 (2.78)	1 (3.45)	1 (5.26)	2 (4.65)	1.67 (0.16-16.6)	1.000	2.59 (0.26-26.4)	0.396	2.28 (0.37-14.1)	0.591
	GG	140 (97.22)	28 (96.55)	18 (94.74)	41 (95.35)	1		1		1	
	C	263 (91.32	53 (91.38)	34 (89.47)	78 (90.70	1.01 (0.37-2.75)	1.000	0.81 (0.26-2.46)	0.760	0.93 (0.40-2.14)	1.000
	T	25 (8.68)	5 (8.62)	4 (10.53)	8 (9.30)	1		1		1	
+3035.C/T	**CC**	121 (84.03)	24 (82.76)	15 (78.95	35 (81.40)	-	1.000	-	1.000	-	1.000
	CT	21 (14.58)	5 (17.24)	4 (21.05)	8 (18.60)	-	1.000	-	1.000	-	1.000
	TT	2 (1.39)	0 (0.00)	0 (0.00)	0 (0.00)	1		1		1	
	C	100 (34.72)	22 (37.93)	15 (39.47)	26 (30.23)	1.14 (0.64-2.06)	0.653	1.23 (0.61-2.45)	0.590	0.81 (0.48-1.37)	0.516
	G	188 (65.28)	36 (62.07)	23 (60.53)	60 (69.77)	1		1		1	
+3142.G/C	**CC**	23 (15.97)	4 (13.79)	5 (26.32)	5 (11.63)	1.06 (0.31-3.65)	1.000	1.61 (0.49-5.33)	0.518	0.66 (0.22-1.95)	0.609
	GC	54 (37.50)	14 (48.28)	5 (26.32)	16 (37.21)	1.58 (0.66-3.76)	0.379	0.68 (0.21-2.18)	0.582	0.90 (0.43-1.89)	0.853
	GG	67 (46.53)	11 (37.93)	9 (47.37)	22 (51.16)	1		1		1	
	G	31 (10.76)	6 (10.34)	10 (26.32)	14 (16.28)	0.96 (0.37-2.41)	1.000	2.96 (1.31-6.67)	**0.011**	1.61 (0.81-3.19)	0.187
	A	257 (89.24)	52 (89.66)	28 (73.68)	72 (83.72)	1		1		1	
+3187.A/G	**GG**	2 (1.39)	0 (0.00)	3 (15.79)	3 (6.98)	-	1.000	14.4 (2.18-94.7)	**0.011**	5.39 (0.86-33.7)	**0.080**
	AG	27 (18.75)	6 (20.69)	4 (21.05)	8 (18.60)	1.11 (0.41-2.99)	1.000	1.42 (0.42-4.74)	0.740	1.06 (0.44-2.57)	1.000
	AA	115 (79.86)	23 (79.31)	12 (63.16)	32 (74.42)	1		1		1	
	G	97 (33.68)	17 (29.31)	15 (39.47)	35 (40.70)	0.82 (0.44-1.51)	0.545	1.28 (0.64-2.57)	0.586	1.35 (0.82-2.21)	0.249
	C	191 (66.32)	41 (70.69)	23 (60.53)	51 (59.30)	1		1		1	
+3196.C/G	**GG**	18 (12.50)	2 (6.90)	4 (21.05)	7 (16.28)	0.52 (0.11-2.48)	0.514	1.81 (0.48-6.68)	0.464	1.68 (0.60-4.76)	0.399
	CG	61 (42.36)	13 (44.83)	7 (36.84)	21 (48.84)	0.98 (0.43-2.27)	1.000	0.93 (0.32-2.73)	1.000	1.49 (0.71-3.16)	0.347
	CC	65 (45.14)	14 (48.28)	8 (42.11)	15 (34.88)	1		1		1	

NT: HIV-1 non-transmitting mother; IP-TR: intrapartum HIV-1 transmitting mother; IU-TR: *in utero* HIV-1 transmitting mother; IU2-TR: enriched group of *in utero* transmitting mothers. Highlighted and bold *P* values indicate significant differences ($P<0.05$) and bold *P* values indicate trends ($P<0.090$).

Table 6.5 Representation of *HLA-G* 3'UTR haplotype and genotype frequencies in mothers from MTCT1, N (%)

3'UTR	NT (n=144)	IP-TR (n=29)	IU-TR (n=19)	IU2-TR (n=43)	IP-TR vs. NT OR (95% CI)	*P*	IU-TR vs. NT OR (95% CI)	*P*	IU2-TR vs. NT OR (95% CI)	*P*
UTR1	31 (10.76)	6 (10.34)	10 (26.32)	14 (16.28)	0.96 (0.38-2.41)	1.000	2.96 (1.31-6.67)	**0.011**	1.61 (0.81-3.19)	0.187
UTR2	97 (33.68)	17 (29.31)	15 (39.47)	35 (40.70)	0.82 (0.44-1.51)	0.545	1.28 (0.64-2.57)	0.586	1.44 (0.87-2.37)	0.155
UTR3	66 (22.92)	14 (24.14)	4 (10.53)	17 (19.77)	1.07 (0.55-2.07)	0.865	0.39 (0.13-1.16)	0.094	0.82 (0.46-1.51)	0.559
UTR4	31 (10.76)	9 (15.52)	1 (2.63)	4 (4.65)	1.52 (0.68-3.39)	0.366	0.22 (0.03-1.69)	0.149	0.40 (0.14-1.18)	0.095
UTR5	24 (8.33)	5 (8.62)	4 (10.53)	8 (9.30)	1.03 (0.39-2.84)	1.000	1.29 (0.42-3.96)	0.756	1.13 (0.49-2.61)	0.826
UTR6	34 (11.81)	6 (10.34)	3 (7.89)	6 (6.98)	0.86 (0.34-2.16)	0.827	0.64 (0.18-2.19)	0.596	0.56 (0.23-1.38)	0.238
UTR7	1 (0.35)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
UTR20	4 (1.39)	1 (1.72)	1 (2.63)	2 (2.33)	1.24 (0.14-11.3)	1.000	1.91 (0.21-17.6)	0.464	1.69 (0.30-9.39)	0.624
UTR1/UTR1	2 (1.39)	0 (0.00)	3 (15.79)	3 (6.98)	-	1.000	13.3 (2.07-85.7)	**0.012**	5.32 (0.86-32.9)	**0.081**
UTR1/UTR2	5 (3.47)	3 (10.34)	2 (10.53)	5 (11.63)	3.21 (0.72-14.3)	0.132	3.27 (0.59-18.2)	0.190	3.66 (1.01-13.3)	**0.052**
UTR1/UTR3	12 (8.33)	1 (3.45)	0 (0.00)	0 (0.00)	0.39 (0.05-3.14)	0.475	-	0.363	-	**0.071**
UTR1/UTR4	5 (3.47)	1 (3.45)	0 (0.00)	0 (0.00)	0.99 (0.11-8.83)	1.000	-	1.000	-	0.347
UTR1/UTR5	2 (1.39)	0 (0.00)	1 (5.26)	2 (4.65)	-	1.000	3.94 (0.34-45.7)	0.312	3.46 (0.47-25.4)	0.227
UTR1/UTR6	3 (2.08)	0 (0.00)	1 (5.26)	1 (2.33)	-	1.000	2.61 (0.26-26.5)	0.394	1.12 (0.11-11.0)	1.000
UTR1/UTR20	0 (0.00)	1 (3.45)	0 (0.00)	0 (0.00)	-	0.168	-	1.000	-	1.000
UTR2/UTR2	18 (12.50)	2 (6.90)	4 (21.05)	7 (16.28)	0.52 (0.11-2.37)	0.534	1.87 (0.56-6.25)	0.474	1.36 (0.53-3.51)	0.610
UTR2/UTR3	23 (15.97)	4 (13.79)	3 (15.79)	10 (23.26)	0.84 (0.27-2.64)	1.000	0.99 (0.26-3.66)	1.000	1.59 (0.69-3.68)	0.361
UTR2/UTR4	12 (8.33)	3 (10.34)	0 (0.00)	1 (2.33)	1.27 (0.33-4.81)	0.719	-	0.363	0.26 (0.03-2.07)	0.304
UTR2/UTR5	10 (6.94)	2 (6.90)	2 (10.53)	5 (11.63)	0.99 (0.21-4.78)	1.000	1.58 (0.32-7.81)	0.634	1.76 (0.57-5.47)	0.342
UTR2/UTR6	9 (6.25)	1 (3.45)	0 (0.00)	0 (0.00)	0.54 (0.06-4.40)	0.699	-	0.390	-	0.121
UTR2/UTR7	1 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
UTR2/UTR20	1 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
UTR3/UTR3	8 (5.56)	0 (0.00)	0 (0.00)	0 (0.00)	-	0.355	-	0.598	-	0.201
UTR3/UTR4	2 (1.39)	3 (10.34)	0 (0.00)	2 (4.65)	8.19 (1.30-51.5)	**0.034**	-	1.000	3.46 (0.47-25.4)	0.227
UTR3/UTR5	5 (3.47)	3 (10.34)	0 (0.00)	0 (0.00)	3.21 (0.72-14.3)	0.132	-	1.000	-	0.347
UTR3/UTR6	7 (4.86)	3 (10.34)	1 (5.26)	4 (9.30)	2.25 (0.55-9.31)	0.374	1.09 (0.12-9.35)	1.000	2.01 (0.56-7.21)	0.461
UTR3/UTR20	1 (0.69)	0 (0.00)	0 (0.00)	1 (2.33)	-	1.000	-	1.000	-	1.000
UTR4/UTR4	4 (2.78)	1 (3.45)	0 (0.00)	0 (0.00)	1.25 (0.13-11.6)	1.000	-	1.000	-	0.575
UTR4/UTR5	1 (0.69)	0 (0.00)	1 (5.26)	1 (2.33)	-	1.000	7.94 (0.48-132.6)	0.220	3.40 (0.21-55.6)	0.408
UTR4/UTR6	2 (1.39)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
UTR4/UTR20	1 (0.69)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	3.40 (0.21-55.6)	0.408
UTR5/UTR5	2 (1.39)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
UTR5/UTR6	2 (1.39)	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000	-	1.000
UTR6/UTR6	5 (3.47)	1 (3.45)	0 (0.00)	0 (0.00)	0.99 (0.11-8.83)	1.000	-	1.000	-	0.347
UTR6/UTR20	1 (0.69)	0 (0.00)	1 (5.26)	1 (2.33)	-	1.000	7.94 (0.48-132.6)	0.220	3.40 (0.21-55.6)	0.408

NT: HIV-1 non-transmitting mother; IP-TR: intrapartum HIV-1 transmitting mother; IU-TR: *in utero* HIV-1 transmitting mother; IU2-TR: enriched group of *in utero* transmitting mothers. Highlighted and bold *P* values indicate significant differences ($P<0.05$) and bold *P* values indicate trends ($P<0.090$).

Table 6.6 Multivariate analyses, adjustments made for maternal factors that influence HIV-1 transmission in MTCT1

Allele/genotype	**Association**	**Unadjusted**		**Adjusted mVL**		**Adjusted mNVP**		**Adjusted Infant *KIR2DS4-v***	
		OR (95% CI)	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
*G*01:01:02*	IU-TR vs. NT	2.26 (1.10-4.64)	**0.036**	3.22 (1.03-10.1)	**0.043**	2.02 (0.74-5.52)	0.168	2.13 (0.79-5.78)	0.134
*G*01:01:02*	IU2-TR vs. NT	1.68 (0.97-2.89)	**0.078**	2.39 (1.10-5.20)	**0.027**	1.54 (0.73-3.22)	0.252	1.79 (0.88-3.65)	0.105
*G*01:01:02/G*01:01:02*	IU-TR vs. NT	4.31 (0.98-18.9)	**0.072**	8.79 (1.46-52.8)	**0.017**	4.81 (1.04-22.3)	**0.044**	5.08 (1.12-22.9)	**0.035**
*G*01:01:02/G*01:03:01*	IU2-TR vs. NT	4.61 (1.17-17.9)	**0.031**	10.0 (2.15-46.8)	**0.003**	3.14 (0.73-13.5)	0.122	3.81 (0.89-16.3)	0.071
3187G	IU-TR vs. NT	2.96 (1.31-6.67)	**0.011**	2.17 (0.67-6.99)	0.192	2.56 (0.90-7.29)	0.077	2.42 (0.86-6.83)	0.094
3187GG	IU-TR vs. NT	14.4 (2.18-94.7)	**0.011**	29.6 (2.36-371.0)	**0.009**	-	-	-	-
3187GG	IU2-TR vs. NT	5.39 (0.86-33.7)	**0.080**	6.69 (0.91-49.4)	**0.062**	10.9 (1.06-113.6)	**0.044**	6.51 (1.04-40.7)	**0.045**
UTR1	IU-TR vs. NT	2.96 (1.31-6.67)	**0.011**	2.17 (0.67-6.99)	0.192	2.56 (0.90-7.29	0.077	2.42 (0.86-6.83)	0.094
UTR1/UTR1	IU-TR vs. NT	13.3 (2.07-85.7)	**0.012**	29.6 (2.36-371.0)	**0.009**	-	-	-	-
UTR1/UTR2	IU2-TR vs. NT	3.66 (1.01-13.3)	**0.052**	5.44 (1.32-22.4)	**0.019**	3.43 (0.91-12.8)	0.067	3.57 (0.95-13.3)	0.058
UTR3/UTR4	IP-TR vs. NT	8.19 (1.30-51.5)	**0.034**	6.17 (0.91-41.4)	0.061	4.24 (0.63-28.5)	0.137	N/A	N/A
Combined effect									
*G*01:01:02 & 3187G*	IU-TR vs. NT	4.12 (0.70-24.2)	0.145	6.62 (0.82-52.9)	0.074	3.81 (0.64-22.5)	0.140	4.19 (0.70-24.9)	0.116
*G*01:01:02* or *3187G*	IU-TR vs. NT	4.51 (1.25-16.1)	**0.014**	4.93 (1.24-19.5)	**0.023**	4.14 (1.14-15.1)	**0.031**	4.12 (1.13-14.9)	**0.031**

mVL: maternal viral load; mNVP: maternal single-dose Nevirapine; *KIR2DS4-v*: non-functional KIR2DS4; N/A: not an applicable adjustment, since infant possession of *KIR2DS4-v* has been association with IU HIV-1 transmission and not IP HIV-1 transmission. Highlighted and bold *P* values indicate significant differences ($P<0.05$).

6.2.5 Linkage disequilibrium (LD) between *HLA-G* alleles and the 3'UTR in MTCT1

To further investigate the relationship between *HLA-G* alleles and polymorphisms within the 3'UTR of MTCT1 mothers, pairwise LD between the *HLA-G* alleles, the 14-bp indel, as well as the other 3'UTR SNPs and haplotypes were calculated using the Lewontin method (Chapter 2, section 2.8.4.2). **Table 6.7** shows LD results for significant pairwise combinations only. Seven of the *HLA-G* alleles were in strong LD with the 14-bp indel: *G*01:01:01*, *G*01:04:01* and *G*01:04:04* were significantly linked with the *Del*; while, *G*01:01:02*, *G*01:03:01*, *G*01:05N* and *G*01:01:19* were significantly linked with the *Ins*. As such *G*01:01:01* was in strong LD with UTR1, UTR6 and UTR20, while *G*01:04:04* and *G*01:04:04* were in strong LD with UTR3, and *HLA-G* alleles associated with the *Ins* (*G*01:01:02*, *G*01:03:01*, *G*01:05N* and *G*01:01:19)* were found to be in significant LD with UTR2, UTR5 and UTR7. Thus, since the *G*01:01:02* allele and +3187G (UTR1) are not in LD (D'=-0.12; X^2=0.09, *P*>0.1), the effects of these two genotypic variations on IU transmission are independent of each other. Accordingly the comparison of IU-TR mothers vs. NT mothers having both *G*01:01:02* and +3187G (UTR1) did not show a significant additive association, likely due to the small number of individuals carrying both these variants (**Table 6.6**). However, comparison of IU-TR mothers with EU mothers and representation of *G*01:01:02* or +3187G (UTR1), was more significant (*P*=0.014; OR=4.51) than possession of *G*01:01:02* alone, but not more significant than having +3187G (UTR1) alone (**Table 6.6**). The significantly greater representation of having either *G*01:01:02* or +3187G (UTR1) was the only association maintained throughout all adjustments (**Table 6.6**).

6.2.6 *HLA-G* mother-infant concordance in MTCT1

Mother-infant concordance amongst the classical HLA molecules (-A, -B and -C) (MacDonald et al., 1998), as well as a synonymous SNP in exon 2 of *HLA-G* (Aikhionbare et al., 2001), have been described as risk factors for MTCT, thus we wanted to determine if mother-infant concordance for *HLA-G* alleles and 3'UTR had a similar effect. Within MTCT1 we found no significant associations with vertical transmission when mother-infant pairs were concordant for *HLA-G* alleles, the 14-bp indel, or 3'UTR haplotypes (**Table 6.8**). However, concordance at SNP +3187 was found to be significantly lower in IU-TR/IU mother-infant pairs compared to NT/EU pairs (*P*=0.014, OR=0.29) and remained significant post all adjustments (**Table 6.8**).

Table 6.7 Linkage disequilibrium (LD) between *HLA-G* alleles, 14-bp indel and 3'UTR in HIV-1 positive mothers from MTCT1 (n=216)

LD comparisons		N	(%)	D'	X^2	*P*-value
*G*01:01:01*	Del	126	29.17	0.95	45.19	<0.001
	UTR1	44	10.19	0.80	37.70	<0.001
	UTR4	33	7.64	0.64	19.97	<0.001
	UTR6	45	10.42	0.94	47.47	<0.001
	UTR20	6	1.39	1.00	5.93	<0.05
*G*01:01:02*	Ins	96	22.22	1.00	66.88	<0.001
	UTR2	96	22.22	1.00	98.49	<0.001
*G*01:01:19*	Ins	14	3.24	1.00	6.98	<0.01
	UTR2	14	3.24	1.00	10.27	<0.01
*G*01:03:01*	Ins	33	7.64	1.00	17.64	<0.001
	UTR5	33	7.64	1.00	189.72	<0.001
*G*01:04:01*	Del	17	3.94	1.00	4.16	<0.05
	UTR3	17	3.94	1.00	27.24	<0.001
*G*01:04:04*	Del	78	18.06	1.00	24.37	<0.001
	UTR3	78	18.06	1.00	159.40	<0.001
*G*01:05N*	Ins	33	7.64	1.00	17.64	<0.001
	UTR2	33	7.64	1.00	25.98	<0.001
	UTR7	1	0.23	1.00	5.83	<0.05
Del	+3003 C	44	10.19	1.00	11.94	<0.01
	+3010 G	148	34.26	1.00	65.44	<0.001
	+3035 C	245	56.71	1.00	7.30	<0.05
	+3142 C	148	34.26	1.00	65.44	<0.001
	+3187 G	51	11.81	1.00	14.23	<0.001
	+3196 C	245	56.71	1.00	126.48	<0.001
Ins	+3003 T	187	43.29	1.00	4.78	<0.05
	+3010 C	187	43.29	1.00	60.58	<0.001
	+3035 T	38	8.80	1.00	20.71	<0.001
	+3142 G	187	43.29	1.00	60.58	<0.001
	+3187 A	187	43.29	1.00	6.44	<0.01
	+3196 G	149	34.49	1.00	136.23	<0.001

N: the number of individuals; D': measure of linkage disequilibrium; X^2: Chi-Square value from which the *P*-value was determined significant at $P<0.05$.

Table 6.8 Representation of *HLA-G* concordance amongst HIV-1 transmitting and non-transmitting mother-infant pairs in MTCT1, N (%)

Concordance	NT/EU (n=144)	IP-TR/IP (n=29)	IU-TR/IU (n=19)	IU2-TR/IU2 (n=43)	IP-TR/IP vs. NT/EU		IU-TR/IU vs. NT/EU		IU2-TR/IU2 vs. NT/EU	
					OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
HLA-G alleles	51 (35.42)	12 (41.38)	9 (47.37)	18 (41.86)	1.28 (0.57-2.90)	0.673	1.64 (0.63-4.30)	0.322	1.31 (0.65-2.63)	0.474
14-bp	72 (50.00)	18 (62.07)	11 (57.89)	28 (65.12)	1.63 (0.72-3.71)	0.309	1.37 (0.52-3.62)	0.628	1.87 (0.92-3.78)	**0.085**
Del/Del	30 (20.83)	8 (27.59)	5 (26.32)	10 (23.26)	1.45 (0.58-3.59)	0.463	1.35 (0.45-4.07)	0.767	1.15 (0.51-2.59)	0.832
Del/Ins	31 (21.53)	9 (31.03)	5 (26.32)	13 (30.23)	1.64 (0.68-3.96)	0.333	1.30 (0.43-3.89)	0.768	1.58 (0.74-3.39)	0.305
Ins/Ins	11 (7.64)	1 (3.45)	1 (5.26)	5 (11.63)	0.43 (0.05-3.48)	0.489	0.67 (0.08-5.51)	1.000	1.59 (0.52-4.86)	0.533
UTR Haplotypes	62 (43.06)	10 (34.48)	8 (42.11)	16 (37.21)	0.69 (0.30-1.60)	0.418	0.96 (0.36-2.53)	1.000	0.78 (0.39-1.57)	0.598
+3003	123 (85.42)	21 (72.41)	15 (78.95)	36 (83.72)	0.45 (0.17-1.14)	0.103	0.64 (0.19-2.11)	0.498	0.87 (0.34-2.23)	0.809
+3010	84 (58.33)	18 (62.07)	10 (52.63)	26 (60.47)	1.16 (0.51-2.65)	0.837	0.79 (0.30-2.07)	0.806	1.09 (0.54-2.19)	0.861
+3027	143 (99.31)	29 (100.00)	19 (100.00)	43 (100.00)	-	1.000	-	1.000	-	1.000
+3032	141 (97.92)	28 (96.55)	17 (89.47)	39 (90.70)	0.59 (0.06-5.94)	1.000	0.18 (0.03-1.16)	0.104	0.21 (0.04-0.96)	**0.050**
+3035	115 (79.86)	26 (89.66)	14 (73.68)	36 (83.72)	2.19 (0.62-7.72)	0.297	0.71 (0.23-2.12)	0.552	1.29 (0.52-3.21)	0.664
+3142	84 (58.33)	18 (62.07)	10 (52.63)	26 (60.47)	1.16 (0.51-2.65)	0.837	0.79 (0.30-2.07)	0.806	1.09 (0.54-2.19)	0.861
+3187	109 (75.69)	21 (72.41)	9 (47.37)	26 (60.47)	0.84 (0.34-2.07)	0.814	0.29 (0.11-0.76)	**0.014**	0.49 (0.24-1.01)	**0.055**
+3196	80 (55.56)	17 (58.62)	13 (68.42)	28 (65.12)	1.13 (0.50-2.54)	0.839	1.73 (0.62-4.81)	0.332	1.49 (0.74-3.03)	0.295

NT/EU: paired HIV-1 non-transmitting mother and exposed infected infant; IP-TR/IP: paired intrapartum HIV-1 transmitting mother and intrapartum infected infant; IU-TR/IU: paired *in utero* HIV-1 transmitting mother and in utero infected infant; IU2-TR: enriched group of *in utero* transmitting mother and infant. Bold *P* values indicate trends ($P<0.090$). Multivariable logistic regression analysis on mother-infant concordance at SNP +3187, adjustments made for maternal viral load ($P=0.023$, OR=0.27, 95% CI=0.09-0.83), maternal sdNVP ($P=0.012$, OR=0.28, 95% CI=0.10-0.76) and infant possession of *KIR2DS4-v* ($P=0.029$, OR=0.33, 95% CI=0.12-0.89).

6.2.7 Comparison of maternal *HLA-G* alleles in MTCT2

Given that *HLA-G* analysis in mother and infant groups from MTCT1 highlighted the role of maternal *HLA-G* alleles and the 3'UTR in IU transmission of HIV-1 and showed no significant associations in the infant group, in MTCT2 due to infant sample availability, costs of *HLA-G* sequencing and time constraints we selected to first evaluate the role of *HLA-G* in mothers from MTCT2 and not their infants. Twelve *HLA-G* alleles were identified, which included four functional proteins (G*01:01, G*01:03, G*01:04 and G*01:10) as well as the null allele, G*01:05N (**Table 6.8**). As in MTCT1, *G*01:01:01* (30%) was the most common allele, followed by *G*01:01:02* (19%) and *G*01:04:04* (18%) in mothers from MTCT2. Correspondingly, *G*01:01:01* homozygosity was the most frequent genotype (**Table 6.9**).

6.2.8 *HLA-G* alleles and 3'UTR in transmission of HIV-1 in mothers from MTCT2

Unlike, in MTCT1 where representation of *G*01:01:02* was significantly higher in IU-TR mothers compared to NT mothers (**Table 6.1**: *P*=0.036, OR=2.26), in MTCT2 *G*01:01:01* and the genotype *G*01:01:01/G*01:01:04* were significantly over-represented in IP-TR mothers compared to NT mothers (*P*=0.028, OR=2.95 and *P*=0.001, OR=29.2, respectively). While representation of *G*01:01:02* showed a strong trend towards over-representation in NT mothers compared to IP-TR mothers (*P*=0.069, OR=0.15) but was not reflected in any *G*01:01:02* bearing genotypes (**Table 6.9**). In the 3'UTR of *HLA-G* (**Table 6.10**), both UTR1 haplotype and UTR1/UTR3 genotype were over-represented in IP-TR mothers compared to NT mothers (*P*=0.024, OR=3.27, *P*=0.018, OR=10.00, respectively); whereas the haplotype UTR3 and UTR3/UTR3 genotype were over-represented in IU-TR mothers compared to NT mothers (*P*=0.010, OR=3.07 and *P*=0.024, OR=10.25, respectively). SNP analysis showed three positions (+3010, +3142 and +3187) were associated with increased risk for IP transmission (**Table 6.11**). Heterozygosity at positions +3010 and +3142 (SNPs that are in complete LD, r^2=1) were significantly higher in IP-TR mothers compared to NT mothers (*P*=0.008) and at position +3187, a *G* allele as well as *A/G* heterozygosity were significantly over-represented in IP-TR mothers compared to NT mothers (*P*=0.024, OR=3.27 and *P*=0.002, OR=10.3, respectively).

Table 6.9 Representation of maternal *HLA-G* alleles and genotypes in MTCT2, N (%)

HLA-G **Alleles and Genotypes***	**NT (n=42)**	**IP-TR (n=12)**	**IU-TR (n=25)**	**IP-TR vs. NT**		**IU-TR vs. NT**	
				OR (95% CI)	***P***	**OR (95% CI)**	***P***
*G*01:01:01*	24 (28.57)	13 (54.17)	10 (20.00)	2.95 (1.16-7.50)	**0.028**	0.62 (0.27-1.45)	0.310
*G*01:01:02*	19 (22.62)	1 (4.17)	10 (20.00)	0.15 (0.02-1.17)	**0.069**	0.85 (0.36-2.02)	0.830
*G*01:01:08*	6 (7.14)	1 (4.17)	5 (10.00)	0.57 (0.06-4.93)	1.000	1.44 (0.42-5.00)	0.746
*G*01:01:09*	4 (4.76)	0 (0.00)	0 (0.00)	-	0.573	-	0.296
*G*01:01:19*	2 (2.38)	1 (4.17)	0 (0.00)	1.78 (0.15-20.5)	1.000	-	0.529
*G*01:01:20*	1 (1.19)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
*G*01:03:01*	7 (8.33)	1 (4.17)	5 (10.00)	0.47 (0.06-4.09)	0.681	1.22 (0.37-4.08)	0.762
*G*01:04:01*	1 (1.19)	0 (0.00)	2 (4.00)	-	1.000	3.45 (0.31-39.1)	0.555
*G*01:04:04*	11 (13.10)	5 (20.83)	12 (24.00)	1.74 (0.54-5.63)	0.515	2.09 (0.84-5.19)	0.154
*G*01:04:05*	0 (0.00)	0 (0.00)	1 (2.00)	-	1.000	-	0.373
*G*01:05N*	9 (10.71)	2 (8.33)	3 (6.00)	0.75 (0.15-3.77)	1.000	0.53 (0.14-2.06)	0.534
*G*01:10*	0 (0.00)	0 (0.00)	2 (4.00)	-	1.000	-	0.137
*G*01:01:01/G*01:01:01*	6 (14.29)	1 (8.33)	2 (8.00)	0.54 (0.06-5.03)	1.000	0.52 (0.09-2.81)	0.700
*G*01:01:01/G*01:01:02*	3 (7.14)	1 (8.33)	3 (12.00)	1.18 (0.11-12.5)	1.000	1.77 (0.33-9.54)	0.664
*G*01:01:01/G*01:01:08*	1 (2.38)	1 (8.33)	0 (0.00)	3.72 (0.21-64.5)	0.398	-	1.000
*G*01:01:01/G*01:01:19*	1 (2.38)	1 (8.33)	0 (0.00)	3.72 (0.21-64.5)	0.398	-	1.000
*G*01:01:01/G*01:03:01*	2 (4.76)	1 (8.33)	1 (4.00)	1.81 (0.15-21.9)	1.000	0.83 (0.07-9.69)	1.000
*G*01:01:01/G*01:04:04*	1 (2.38)	5 (41.67)	1 (4.00)	29.2 (2.96-289.7)	**0.001**	1.71 (0.10-28.6)	1.000
*G*01:01:01/G*01:05N*	2 (4.76)	2 (16.67)	0 (0.00)	4.00 (0.50-31.9)	0.210	-	0.525
*G*01:01:02/G*01:01:08*	2 (4.76)	0 (0.00)	2 (8.00)	-	1.000	1.73 (0.23-13.1)	0.626
*G*01:01:02/G*01:04:04*	5 (11.90)	0 (0.00)	2 (8.00)	-	0.336	0.64 (0.11-3.59)	0.704
*G*01:01:02/G*01:05N*	5 (11.90)	0 (0.00)	2 (8.00)	-	0.336	0.64 (0.11-3.59)	0.704
*G*01:03:01/G*01:03:01*	1 (2.38)	0 (0.00)	1 (4.00)	-	1.000	1.71 (0.10-28.6)	1.000
*G*01:03:01/G*01:04:04*	1 (2.38)	0 (0.00)	1 (4.00)	-	1.000	1.71 (0.10-28.6)	1.000
*G*01:04:04/G*01:04:04*	1 (2.38)	0 (0.00)	3 (12.00)	-	1.000	5.59 (0.54-56.9)	0.143

* indicates *HLA-G* genotype frequencies > 2% in the NT, IP-TR or IU-TR group. NT: HIV-1 non-transmitting mother; IP-TR: intrapartum HIV-1 transmitting mother; IU-TR: *in utero* HIV-1 transmitting mother. Highlighted and bold *P* values indicate significant differences ($P<0.05$) and bold *P* values indicate trends ($P<0.090$).

Table 6.10 Representation of *HLA-G* 3'UTR haplotype and genotype frequencies in mothers from MTCT2, N (%)

3'UTR	NT (n=42)	IP-TR (n=12)	IU-TR (n=25)	IP-TR vs. NT		IU-TR vs. NT	
				OR (95% CI)	*P*	OR (95% CI)	*P*
UTR1	13 (15.48)	9 (37.50)	5 (10.00)	3.27 (1.18-9.05)	**0.024**	0.61 (0.20-1.82)	0.440
UTR2	29 (34.52)	4(16.67)	13 (26.00)	0.38 (0.12-1.21)	0.132	0.67 (0.31-1.45)	0.340
UTR3	13 (15.48)	5 (20.83)	18 (36.00)	1.43 (0.45-4.53)	0.758	3.07 (1.34-7.02)	**0.010**
UTR4	12 (14.29)	0 (0.00)	3 (6.00)	-	**0.065**	0.38 (0.10-1.43)	0.167
UTR5	9 (10.71)	2 (8.33)	5 (10.00)	0.75 (0.15-3.77)	1.000	0.92 (0.29-2.93)	1.000
UTR6	6 (7.14)	2 (8.33)	6 (12.00)	1.18 (0.22-6.27)	1.000	1.77 (0.54-5.83)	0.363
UTR20	2 (2.38)	2 (8.33)	0 (0.00)	3.72 (0.49-27.9)	0.213	-	0.529
UTR1/UTR2	3 (7.14)	2 (16.67)	2 (8.00)	2.6 (0.38-17.7)	0.575	1.13 (0.17-7.27)	1.000
UTR1/UTR20	1 (2.38)	1 (8.33)	0 (0.00)	3.73 (0.21-64.4)	0.398	-	1.000
UTR1/UTR3	2 (4.76)	4 (33.33)	0 (0.00)	10.00 (1.56-64.2)	**0.018**	-	0.525
UTR1/UTR5	1 (2.38)	2 (16.67)	0 (0.00)	8.2 (0.67-99.7)	0.121	-	1.000
UTR2/UTR2	5 (11.90)	0 (0.00)	2 (8.00)	-	0.336	0.64 (0.12-3.59)	0.704
UTR2/UTR20	1 (2.38)	1 (8.33)	0 (0.00)	3.73 (0.21-64.4)	0.398	-	1.000
UTR2/UTR3	5 (11.90)	0 (0.00)	4 (16.00)	-	0.336	1.41 (0.34-5.82)	0.718
UTR2/UTR4	4 (9.52)	0 (0.00)	2 (8.00)	-	0.564	0.83 (0.14-4.87)	1.000
UTR2/UTR6	3 (7.14)	1 (8.33)	1 (4.00)	1.18 (0.11-12.5)	1.000	0.54 (0.05-5.51)	1.000
UTR3/UTR3	1 (2.38)	0 (0.00)	5 (20.00)	-	1.000	10.25 (1.12-93.7)	**0.024**
UTR3/UTR5	1 (2.38)	0 (0.00)	2 (8.00)	-	1.000	3.56 (0.31-41.5)	0.551
UTR3/UTR6	1 (2.38)	1 (8.33)	2 (8.00)	3.73 (0.21-64.4)	0.398	3.56 (0.31-41.5)	0.551

NT: HIV-1 non-transmitting mother; IP-TR: intrapartum HIV-1 transmitting mother; IU-TR: *in utero* HIV-1 transmitting mother. 3'UTR genotypes represented are those with frequencies greater than > 2% in the NT, IP-TR or IU-TR group. Highlighted and bold *P* values indicate significant differences ($P<0.05$).

Table 6.11 Frequencies of *HLA-G* 3'UTR polymorphisms in mothers from MTCT2, N (%)

Polymorphism		NT (n=42)	IP-TR (n=12)	IU-TR (n=25)	IP-TR vs. NT		IU-TR vs. NT	
					OR (95% CI)	*P*	OR (95% CI)	*P*
+2961 (Indel)	**D**	46 (54.76)	18 (75.00)	32 (64.00)	2.47 (0.89-6.86)	0.100	1.46 (0.71-3.02)	0.366
	I	38 (45.24)	6 (25.00)	18 (36.00)	1		1	
	DD	13 (30.95)	6 (50.00)	10 (40.00)	-	0.136	2.31 (0.49-10.8)	0.463
	ID	20 (47.62)	6 (50.00)	12 (48.00)	-	0.169	1.80 (0.41-7.98)	0.500
	II	9 (21.43)	0 (0.00)	3 (12.00)	1		1	
+3003.T/C	**C**	12 (14.29)	0 (0.00)	3 (6.00)	-	0.065	0.38 (0.10-1.43)	0.167
	T	72 (85.71)	24 (100.00)	47 (94.00)	1		1	
	CC	1 (2.38)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
	TC	10 (23.81)	0 (0.00)	3 (12.00)	-	0.093	0.42 (0.10-1.72)	0.340
	TT	31 (73.81)	12 (100.00)	22 (88.00)	1		1	
+3010.C/G	**G**	33 (39.29)	13 (54.17)	14 (28.00)	1.82 (0.73-4.56)	0.244	0.60 (0.28-1.28)	0.197
	C	51 (60.71)	11 (45.83)	36 (72.00)	1		1	
	GG	7 (16.67)	1 (8.33)	3 (12.00)	-	0.333	0.49 (0.11-2.26)	0.471
	CG	19 (45.24)	11 (91.67)	8 (32.00)	-	**0.008**	0.48 (0.16-1.43)	0.276
	CC	16 (38.10)	0 (0.00)	14 (56.00)	1		1	
+3027.C/A	**A**	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
	C	84 (100.00)	24 (100.00)	50 (100.00)	1		1	
	AA	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
	CA	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
	CC	42 (100.00)	12 (100.00)	25 (100.00)	1		1	
+3032.G/C	**C**	2 (2.38)	2 (8.33)	0 (0.00)	3.72 (0.49-27.9)	0.213	-	0.529
	G	82 (97.62)	22 (91.67)	50 (100.00)	1		1	
	CC	0 (0.00)	0 (0.00)	0 (0.00)	-	1.000	-	1.000
	GC	2 (4.76)	2 (16.67)	0 (0.00)	4.00 (0.50-31.9)	0.210	-	0.525
	GG	40 (95.24)	10 (83.33)	25 (100.00)	1		1	
+3035.C/T	**C**	75 (89.29)	22 (91.67)	45 (90.00)	1.32 (0.26-6.56)	1.000	1.08 (0.34-3.42)	1.000
	T	9 (10.71)	2 (8.33)	5 (10.00)	1		1	
	CC	34 (80.95)	10 (83.33)	21 (84.00)	-	1.000	0.61 (0.04-10.4)	1.000
	CT	7 (16.67)	2 (16.67)	3 (12.00)	-	0.421	0.43 (0.02-9.36)	1.000
	TT	1 (2.38)	0 (0.00)	1 (4.00)	1		1	
+3142.G/C	**C**	33 (39.29)	13 (54.17)	14 (28.00)	1.82 (0.73-4.55)	0.244	0.60 (0.28-1.28)	0.197
	G	51 (60.71)	11 (45.83)	36 (72.00)	1		1	
	CC	7 (16.67)	1 (8.33)	3 (12.00)	-	0.333	0.49 (0.10-2.26)	0.471
	GC	19 (45.24)	11 (91.67)	8 (32.00)	-	**0.008**	0.48 (0.16-1.43)	0.276
	GG	16 (38.10)	0 (0.00)	14 (56.00)	1		1	
+3187.A/G	**G**	13 (15.48)	9 (37.50)	5 (10.00)	3.27 (0.18-9.05)	**0.024**	0.61 (0.20-1.82)	0.440
	A	71 (84.52)	15 (62.50)	45 (90.00)	1		1	
	GG	2 (4.76)	0 (0.00)	1 (4.00)	-	1.000	0.74 (0.06-8.67)	1.000
	AG	9 (21.43)	9 (75.00)	3 (12.00)	10.3 (2.29-46.4)	**0.002**	0.49 (0.12-2.03)	0.510
	AA	31 (73.81)	3 (25.00)	21 (84.00)	1		1	
+3196.C/G	**G**	29 (34.52)	4 (16.67)	13 (26.00)	0.37 (0.12-1.21)	0.132	0.67 (0.31-1.45)	0.340
	C	55 (65.48)	20 (83.33)	37 (74.00)	1		1	
	GG	5 (11.90)	0 (0.00)	2 (8.00)	-	0.291	0.51 (0.09-3.05)	0.678
	CG	19 (45.24)	4 (33.33)	9 (36.00)	0.47 (0.12-1.85)	0.333	0.61 (0.21-1.75)	0.430
	CC	18 (42.86)	8 (66.67)	14 (56.00)	1		1	

NT: HIV-1 non-transmitting mother; IP-TR: intrapartum HIV-1 transmitting mother; IU-TR: *in utero* HIV-1 transmitting mother. Highlighted and bold *P* values indicate significant differences ($P<0.05$).

6.3. Discussion

The role of HLA-G has become increasingly evident in many infectious diseases, primarily due to its inhibitory effect on the innate and adaptive immune system. By directly binding to the inhibitory receptors KIR2DL4, ILT-2 and ILT-4 that are present on NK and CD8+ T cells, HLA-G can also inhibit differentiation, proliferation, cytolysis, cytokine secretion, and immunoglobulin production (Amiot et al., 2014). Therefore, certain *HLA-G* alleles and/or 3'UTR polymorphisms that alter HLA-G expression may influence the function of NK and CD8+ T cells and ultimately influence the susceptibility to HIV-1 transmission. Several studies have found associations between certain *HLA-G* alleles, as well as a 14-bp indel, with an altered risk for MTCT of HIV-1 (Aikhionbare et al., 2001, Aikhionbare et al., 2006, Segat et al., 2009, Moodley and Bobat, 2011, Segat and Crovella, 2012, Luo et al., 2013, Segat et al., 2014). However, these studies have largely focused on each of these parameters individually and, where studies have been comparable, the results have often not shown consensus. In addition, other SNPs within the 3'UTR have not been extensively analyzed for their clinical relevance in MTCT. In this study, we report on the combined analysis of *HLA-G* alleles as well as the 3'UTR of *HLA-G* in two Black South African MTCT cohorts recruited over the last 15 years. Although all infants born to HIV-1 positive mothers received sdNVP as a PEP soon after birth, maternal PMTCT therapies differed between MTCT1 and MTCT2 (Chapter 2, Section 2.1.1 and 2.1.2). In MTCT1, approximately half the mothers received sdNVP at the onset of labour while all mothers from MTCT2 received daily AZT from 28 - 37 weeks gestation as well as sdNVP at the onset of labour.

In both MTCT cohorts *G*01:01:01*, *G*01:01:02* and *G*01:04:04* were the most common alleles which corresponded with the most frequent genotypes: *G*01:01:01/G*01:01:01*, *G*01:01:01/G*01:01:02*, and *G*01:01:01/G*01:01:04*. Likewise both cohorts had similar 3'UTR haplotype frequencies with UTR2 being the most frequent haplotype followed by UTR3 and UTR1, respectively. However with regards to vertical transmission, in MTCT1 maternal possession of *G*01:01:02*, the UTR1 haplotype and a guanine at position +3187 of the 3'UTR significantly associated with increased risk for IU transmission (**Tables 6.1**, **6.3** and **6.4**), whilst in MTCT2, maternal possession *G*01:01:01*, *G*01:01:01/G*01:04:04* genotype, UTR1 haplotype, UTR1/UTR3 genotype as well as heterozygosity at positions +3010, +3142 and +3187 within the 3'UTR significantly associated with increased risk for IP transmission (**Table 6.9**, **6.10** and **6.11**). LD analysis between *HLA-G* alleles and the 3'UTR polymorphisms indicated that two independent *HLA-G* factors were associated with

increased risk for IU transmission in MTCT1, namely: (1) *G*01:01:02* in strong LD with the 14-bp *Ins,* and (2) UTR1 haplotype in complete LD with the 14-bp *Del* as well as +3187G SNP. Interestingly, the 14-bp *Ins* was previously shown to generate a 92-bp deletion in the 3'UTR of the HLA-G mRNA that changed mRNA stability and splicing (Rousseau et al., 2003b), and associated with lower mRNA levels (Hviid et al., 2003). On the contrary, the 14-bp *Del* and +3187G SNP associated with more stable the mRNA and result in higher HLA-G expression (Martelli-Palomino et al., 2013, Alegre et al., 2014). Moreover our data highlighted that the effect of having either *G*01:01:02* or +3187G (UTR1) was significantly over-represented in IU-TR mothers compared to NT mothers and was maintained throughout all adjustments, however, this association was stronger than the effect of *G*01:01:02* alone, but weaker than that of UTR1 alone (Hong et al., 2014). Whereas in MTCT2, *HLA-G* alleles *G*01:01:01* and *G*01:04:04* (in strong LD with the 3'UTR haplotypes UTR1 and UTR3, respectively) associated with IP transmission. Comparison of the UTR1 and UTR3 haplotype showed that while identical in six positions (including the 14-bp *Del*), UTR1 and UTR3 differed at three positions, namely +3010, +3142 and +3187, of which the SNPs at +3010 and +3142 have both been implicated in affinity interactions with microRNAs (Alegre et al., 2014).

Recent studies have associated certain 3'UTR haplotypes with differential levels of soluble HLA-G. UTR1 was found to associated with higher levels of plasma sHLA-G, whereas UTR5 and UTR7 (haplotypes containing the 14-bp *Ins*) were associated lower levels compared to subjects with haplotypes UTR3, UTR4 and UTR6 (haplotypes containing the 14-bp *Del*) that exhibited intermediate levels (Martelli-Palomino et al., 2013). In fact, in their study Martelli-Palomino et al. (2013) stated that UTR-1 (14-bp *Del*, +3003T, +3010G, +3027C, +3032G, +3035C, +3142C, +3187G, +3196C) was the only haplotype to undoubtedly associate with higher levels of sHLA-G, given that the majority of the polymorphisms within UTR1 correlate with increased expression of HLA-G, namely: the 14-bp *Del* that is associated with high levels of soluble HLA-G expression, cytosine at +3142 that is less sensitive to specific miRNAs (miR-148a, miR-148b and miR-152) and guanine at +3187 that increases mRNA stability.

Moreover, recent studies have associated HLA-G with heterosexual transmission of HIV-1. In the Pumwani sex worker cohort from Nairobi, Kenya, *G*01:01:01* was significantly enriched in HIV-1 resistant sex workers, whereas *G*01:04:04* was significantly associated

with susceptibility to HIV-1 infection (Turk et al., 2013). Yet in Zimbabwean women, *G*01:01:08* was associated with an increased risk of HIV-1 infection, while *G*01:05N* offered protection from heterosexual HIV-1 infection (Matte et al., 2004). Rebmann et al. (2001) reported that individuals with *G*01:04*- had higher levels of sHLA-G than individuals with the more frequent *G*01:01:01*, whereas *G*01:01:01* individuals had higher sHLA-G levels compared to individuals with *G*01:01:03* and *G*01:05N*. Therefore, in both HIV-1 transmission studies *HLA-G* alleles that potentially had higher levels of HLA-G expression were associated with increased risk of HIV-1 transmission.

Therefore in our study, *G*01:01:01* in strong LD with UTR1 haplotype, may share a similar mechanism to that of heterosexual HIV-1 transmission, through increased expression of HLA-G being a risk factor for both IU and IP transmission in MTCT1 and MTCT2, respectively. It can be postulated that higher HLA-G expression would exert greater NK and CD8+ T cell inhibition, which could result in the decreased ability of these cells to target viral-infected cells and thus increasing the risk for HIV-1 transmission (Matte et al., 2004, Moodley and Bobat, 2011, Luo et al., 2013, da Silva et al., 2014). As such, other studies have identified HLA-G1 is the major ligand by which HLA-G inhibits NK cell-mediated lysis (Navarro et al., 1999, Ponte et al., 1999, Marchal-Bras-Goncalves et al., 2001). In agreement with this hypothesis, it was reported that in ART naive mother-infant pairs from KwaZulu-Natal, South Africa, placental expression of HLA-G1 was 3.95 times more up-regulated in HIV-1 transmitting mothers compared to HIV-1 non-transmitting mothers (Moodley and Bobat, 2011). Furthermore, there was a significant correlation between placental HLA-G1 expression and the viral loads of the infants, which indicated that infants were more likely to become infected if placental HLA-G1 was increased.

However, while certain *HLA-G* alleles and 3'UTR polymorphisms predict either increased/decreased expression of HLA-G, this is not absolute. It was reported that even the null allele, *G*0105N*, had HLA-G protein expression (Le Discorde et al., 2005). *G*01:05N* is characterized by a single cytosine deletion in exon 3 that presents a stop codon in exon 4 blocking the translation of HLA-G1 and HLA-G5 isoforms; it does however, encode both membrane-bound and soluble functional HLA-G proteins that are able to inhibit NK-cell cytolysis (Le Discorde et al., 2005). It is possible that even low expressing *HLA-G* alleles, such as *G*01:01:02* and its strong LD with the 14-bp *Ins*, might have other mechanisms influencing HLA-G expression. Indeed, a fraction of HLA-G mRNA transcripts presenting

the 14-bp *Ins* can be alternatively spliced by the removal of 92 bases from the mature HLA-G mRNA, which yields smaller HLA-G transcripts, reported to be more stable than the complete mRNA forms (Rousseau et al., 2003b, Donadi et al., 2011). Using an *in silico* based study Donadi et al. (2011) reported that the deletion of 92 bases lead to a loss of key regions including the +3003 C/T and +3010 C/G polymorphic sites, which may be targeted by different microRNAs.

Interestingly in another study, *G*01:01:02*, in the heterozygous and homozygous state, was associated with high levels of sHLA-G in the genital mucosa in ART naive HIV-1 infected sex workers compared with those in both the HIV-1 uninfected sex workers (P=0.051) and non-sex workers (P=0.002) groups (Thibodeau et al., 2011). Additionally in a Canadian Human Papillomavirus (HPV) study, one of the most common sexually transmitted infections, both *G*01:01:02* and *G*01:03*- alleles (also in high LD with the 14-bp *Ins*) were associated with increased risk for HPV-16 infection and persistent infections with HPV types from the alpha species (Ferguson et al., 2011). It is likely that polymorphisms within the HLA-G promoter region (5'URR, the upstream regulatory region) may also influence HLA-G expression. For example, in a study of recurrent pregnancy loss, the tri-allelic polymorphism -725C/G/T (rs1233334) within the 5'URR was evaluated in relation to plasma levels of sHLA-G, and the CC genotype was found to have significantly lower levels of sHLA-G than the CG and CT genotypes (Jassem et al., 2012). Additionally, it was suggested that the presence of Guanine in position −725 may alter the methylation profile of CpG dinucleotides modifying gene expression (Costa et al., 2012). Indeed, *G*01:01:02* alleles were reported in 5'URR haplotypes having the -725C allele (Costa et al., 2012). However in our MTCT1 data, *G*01:01:02* was in strong LD with the UTR2 haplotype, a haplotype different from low expressing haplotypes (UTR5 and UTR7). While UTR2 contains the 14-bp *Ins*, it also bears the +3035C SNP which was associated with high levels of sHLA-G (Martelli-Palomino et al., 2013). Thus, it is possible that there may be other polymorphisms within the 5'URR and 3'UTR associated with either high/low expression of HLA-G which may be counteracting each other.

It must be noted that while we corrected for administration of maternal sdNVP in MTCT1 and had matched all MTCT2 mothers on the basis of their VL, CD4+ T cell count and ART regimens, it has been documented that ART can induce surface expression of HLA-G on blood peripheral monocytes from HIV-1-infected patients (Cabello et al., 2003, Rivero et al.,

2007). Cabello et al. (2003) reported that NRTIs, such as sdNVP, given as part of HAART may be directly related to high levels of monocytes expressing HLA-G. It is possible that in our MTCT cohorts ART may have an additive effect on HLA-G production in conjunction with the *HLA-G* polymorphisms which we report. However, since sdNVP at the onset of labour primarily acts as an infant PEP (Lehman and Farquhar, 2007) and reduces the risk for IP transmission but has little effect on IU transmission; it is questionable to what extent maternal sdNVP given at onset of labour might have on increasing HLA-G expression given the timing of administration. Moreover, in MTCT1 we found *HLA-G* associations were directed towards IU-transmission. Thus, in MTCT1 it is unlikely that maternal ART with sdNVP might have increased HLA-G expression. However, in MTCT2 mothers received daily dose AZT from 28 - 37 weeks gestation as well as sdNVP at the onset of labour, therefore it may be possible that AZT increased HLA-G expression and increased the risk for HIV-1 transmission. Further studies are required to conclusively validate this theory.

With regards to mother-infant concordance in MTCT1, we and others (Rousseau et al., 2003b, Segat and Crovella, 2012, Luo et al., 2013) found no association for *HLA-G* alleles and the 14-bp indel with risk of vertical transmission. Only one study reported an association between *HLA-G* discordance at codon 57 in exon 2 in non-transmitting mother-infant pairs, but the sample size was small (n=34) and the study did not consider different routes of transmission (Aikhionbare et al., 2001).

In conclusion, we show that certain maternal *HLA-G* alleles and 3'UTR haplotypes associate with an increased risk for either IU or IP MTCT, and that this is likely through modulation of maternal HLA-G expression at the maternal-foetal interface. However, further investigations in larger cohorts are necessary to confirm these associations and future studies should collectively assess *HLA-G* alleles, the 5'URR and 3'UTR as well as plasma and cervical levels of sHLA-G as these polymorphisms and changes in HLA-G expression may influence susceptibility to MTCT as well as adult HIV-1 transmission.

CHAPTER 7

Concluding Remarks

This study, through establishing and comparing the representations of various *KIRs* and their *HLA-ligands* in HIV-1 infected mothers and their infants, has identified several genetic associations with specific modes of MTCT. A brief summation of the relevant findings from each of the chapters follows:

From Chapter 3: (Hong et al. 2011. *Tissue Antigens*; 78, 185 - 194)

1. We developed two real-time PCR assays for the detection of *KIR* and *HLA-ligands.*
2. The real-time PCR assays were validated against well-known conventional *KIR* and *HLA* genotyping kits using reference samples that had been previously genotyped.
3. We demonstrated that the two real-time PCR assays were 100% and 99% concordant with the conventional *KIR* and *HLA* genotyping methods, respectively.
4. These two real-time PCR assays allowed for the quick, easy and cost-effective detection of all 16 *KIR* genes as well as their cognate HLA-A, -B and -C ligands.

From Chapter 4:

1. The influence of *KIR* genes and their *HLA-ligands* was evaluated in two distinct mother-infant cohorts that differed in ART and overall HIV-1 transmission rates.
2. In MTCT1, we found that concordance for *KIR2DL3/KIR2DL3+C2/C2* between mother and infant significantly increased the risk for IP transmission. In addition, "matched" allorecognition of infant *KIR2DL2/KIR2DL3* and maternal *HLA-C1/C1* significantly associated with increased risk for IU acquisition of HIV-1.
3. In MTCT2, total representation of the AA1 haplotype was significantly lower in the maternal group compared to the infant group. Moreover, the maternal AA1 haplotype was significantly under-represented in comparison to the frequency reported for MTCT1 mothers. However, the MTCT2 AA1 haplotype frequency did not associate with risk of vertical transmission.
4. In MTCT2, both maternal and infant possession of *HLA-A Bw4:80I* significantly increased the risk for IU transmission and IU acquisition of HIV-1. In addition, maternal possession of *KIR3DL1+Bw4:80I* associated with increased risk for IU and IP transmission.
5. In both MTCT cohorts, the Bx32 genotype was significantly higher in IP-TR mothers compared to the NT mothers and associated with increased risk of IP transmission.

From Chapter 5: (Hong et al. 2013. *Clinical Immunology*; 149, 498 - 508)

1. The findings demonstrate that allelic variants of *KIR2DS4* (functional, *–f* and non-functional, *–v*) alter the risk of MTCT of HIV-1.
2. In MTCT1, maternal possession of *KIR2DS4-f* while absent in their infants (i.e. M+I- discordance) associated with increased risk for IP transmission. However, infant possession of *KIR2DS4-v* significantly increased the risk of IU acquisition of HIV-1; this association was enhanced when infants possessed the AA1 haplotype.
3. In MTCT2, while we observed no significant differences in frequencies of allelic variants of *KIR2DS4* in the maternal, infant and paired mother-infant groups, a weak trend towards higher representation of *KIR2DS4-f* was observed in IU infants.
4. Scatter plot comparisons of *KIR2DS4-v* and the AA1 haplotype frequency showed that the two mother-infant cohorts differed considerably. This was largely due to the significant difference in the AA1 haplotype frequency between the mothers.

From Chapter 6: (Hong et al. 2015. *Infection, Genetics and Evolution* 30, 147 - 158)

1. We characterised *HLA-G* and showed that specific *HLA-G* alleles and 3'UTR haplotypes associated with IU or IP transmission of HIV-1.
2. In MTCT1 and MTCT2, the most common *HLA-G* alleles and 3'UTR haplotypes were *G*01:01:01*, *G*01:01:02* and *G*01:04:04*, and UTR2, UTR3 and UTR1, respectively. Pairwise LD analysis between *HLA-G* alleles and the 3'UTR polymorphisms showed *G*01:01:01, G*01:04:01* and *G*01:04:04* to be significantly linked with the 14-bp *Del*. However, the *HLA-G* alleles: *G*01:01:02, G*01:03:01, G*01:05N* and *G*01:01:19* were in significant LD with the 14-bp *Ins*. Strong LD was also observed between *G*01:01:01* and the haplotypes UTR1, UTR6 and UTR20, whereas *G*01:01:02*, *G*01:03:01*, *G*01:05N* and *G*01:01:19* were found to be in significant LD with UTR2, UTR5 and UTR7.
3. In MTCT1, maternal possession of *G*01:01:02* and the UTR1 haplotype independently associated with increased risk for IU transmission. However, in the infant group neither *HLA-G* alleles nor 3'UTR polymorphisms showed any significant association with HIV-1 acquisition.
4. In MTCT2, maternal possession of *G*01:01:01/G*01:04:04* genotype and the UTR1/UTR3 haplotype significantly associated with increased risk for IP transmission.

Concluding Remarks

To date the correlates of protective immunity against HIV-1 remain elusive, as there are no documented cases of individuals with complete viral clearance having long lasting resistance in the presence or absence of ART intervention. However, there have been recognized cases of individuals who remain HIV-1 uninfected despite repeated exposure to the virus (Kulkarni et al., 2003, Shacklett, 2006, Piacentini et al., 2008, Horton et al., 2010). These exposed uninfected (EU) individuals have been identified around the world and well known cohorts include the Pumwani CSWs from Nairobi, Kenya (Fowke et al., 1996), HIV-1 serodiscordant couples which can be either heterosexual or homosexual (men who have sex with men) (Tang et al., 2002, Hladik et al., 2003, Jennes et al., 2013), and the non-sexually exposed groups such as Vietnamese injection drug users (Scott-Algara et al., 2003, Tran et al., 2006) and the EU infants born to HIV-infected mothers (Rowland-Jones et al., 1998, Tiemessen and Kuhn, 2007, Tiemessen et al., 2009, Paximadis et al., 2011). Therefore, by studying the host genetics and the immune response in the EU individuals may offer key insights into what might qualify as protective responses for future vaccine design.

We sought to determine which immunogenetic factors influenced resistance and/or susceptibility to HIV-1 infection using the mother-to-child transmission model. Unlike in adult EU cohorts where the accuracy of HIV-1 exposure is often a concern (i.e. most rely on the participant's self-reported sexual history), in mother-infant birth cohorts clinical information regarding the maternal VL, CD4+ T cell counts and ART regimens at the time of delivery is usually available. Thus, viral exposure can be quantified more accurately and corrected for when identifying correlates of resistance and/or susceptibility. Importantly, having clinical specimens from the mother (transmitter and non-transmitter) and infant (infected and uninfected) may provide insights into which immunological factors may contribute to maternal HIV-1 transmission as well as infant HIV-1 acquisition of HIV-1. Furthermore, given that the mechanism of infant infection likely differs in IU- and IP-infection, a major advantage of our MTCT study is the stratification of HIV-1 infected infants according to their mode of acquisition (IU or IP) which few MTCT studies report. Moreover, since the rates of vertical transmission of HIV-1 have dropped dramatically from 30% to less than 2% with effective administration of PMTCT programs and ART, we evaluated in two mother-infant cohorts (MTCT1 and MTCT2) recruited during two periods of time where implemented ART regimens differed (i.e. monotherapy including sdNVP vs. dual therapy including AZT and sdNVP, respectively). This allowed us to more accurately understand and

determine which immunogenetic factors were associated with increased/decreased risk of HIV-1 acquisition in the context of changing ART regimens. Indeed, while it is essential that PMTCT programs and maternal ART administration continues to improve and reduce the rates of MTCT, over time the numbers of HIV-1 infected infants will decline. Moreover, the expense of establishing and following MTCT cohorts from birth with meaningful sample numbers will becomes exorbitant. Nevertheless, even with reduced numbers the findings generated provide valuable information regarding susceptibility to HIV-1 infection despite both maternal (PrEP) and infant (PEP) ART administration. Therefore, the use of MTCT specifically as a model to understand protective immunity is increasingly complicated by increased ART usage for prevention, making existing stored specimens exceptionally precious for the continued identification of additional factors important in MTCT.

Collective evidence has shown that NK cells through their KIR and HLA-ligand interactions have a role to play in the transmission and control of HIV-1 infection (Martin et al., 2002, Scott-Algara et al., 2003, Fauci et al., 2005, Tran et al., 2006, Martin et al., 2007, Ravet et al., 2007, Carrington et al., 2008, Tang et al., 2008, Tiemessen et al., 2009, Tiemessen et al., 2010, Wong et al., 2010, Paximadis et al., 2011, Pelak et al., 2011, Tiemessen et al., 2011, Martin and Carrington, 2013). In particular, the original work by Paximadis et al. (2011) from the greater MTCT1 cohort suggested that certain inhibitory *KIR/HLA-ligand* gene combinations associated with increased risk of vertical transmission of HIV-1. Moreover, the authors emphasized the need to further explore the relationship of concordance and allorecognition of *KIR* and *HLA-ligands* amongst mother-infant pairs. It was also noted that *KIR* allelic variance should be considered when determining associations for MTCT of HIV-1 as *KIR* alleles are not equally expressed on the surface of NK cells. In addition, it was also suggested that HLA-G be evaluated given its role as the cognate ligand for the framework *KIR2DL4*. Thus, in order to better understand the role of *KIR* and *HLA-ligand* genes in risk of and/or protection from vertical transmission we used a selection mother and infant samples from MTCT1 and second mother-infant cohort (MTCT2) that differed with respect to overall HIV-1 transmission rate and ART regimens.

Firstly, it was clear that the two MTCT cohorts differed significantly on the basis of their clinical and immunogenetic data. Although the overall rate of transmission was significantly lower in the MTCT2 compared to MTCT1, surprisingly this reduction in transmission was not due to reduced maternal VL. In fact, despite having received daily dose AZT starting

from 28 - 37 weeks gestation, VL in TR mothers was comparable in MTCT1 and MTCT2. Similarly, CD4+ T cells counts were significantly lower in TR mothers from MTCT2 compared to TR mothers from MTCT1 suggesting that there were other factors contributing to the decreased rate of transmission. It is likely that in MTCT2, the administration of ART reduced HIV-1 transmission primarily by preventing the establishment of HIV-1 infection establishment most likely through the IP route, this being consistent with the proportion of IP versus IU infants relative to MTCT1.

Additionally, representation of the common *KIR* AA1 haplotype was significantly lower in MTCT2 mothers (13.9%) when compared to their infants (33.7%), and in comparison to MTCT1 mothers (27.2%). This difference in the AA1 haplotype was marked when comparing scatter plot of the *KIR2DS4-v* and AA1 haplotype prevalence in MTCT1 versus MTCT2. Thus, to understand this difference we used *KIR* data from the Black ESKOM cohort, which represented a diverse cross-section of nine Black South African dialects (Gentle et al., unpublished data). While the overall representation for AA1 haplotype in the ESKOM cohort was 26.9% (similar to that of the MTCT1 cohort), individual representation of the AA1 haplotype across the dialects showed Tswana and Tsonga speaking individuals had the greatest representation of the AA1 haplotype (40-45%), whereas as Xhosa and Sesotho speaking individuals had the lowest representation of the AA1 haplotype (12-13%). The Xhosa *KIR* data also compared well with other published Xhosa *KIR* data (Middleton et al., 2007a). Thus, we speculated that mothers in MTCT1 represented a more diverse cross section of Black South African dialects, whereas mothers in MTCT2 may have been biased toward a larger proportion of Xhosa or Sesotho speaking individuals. Moreover, given the high proportion of the AA1 haplotype in MTCT2 infants, this also suggested that the majority of infants received a paternal copy of the A haplotype.

Nevertheless, while the AA1 haplotype itself did not associate with risk of vertical transmission in either the MTCT1 or MTCT2, components of this inhibitory haplotype associated with risk of transmission. Paximadis et al. (2011) previously reported that both maternal *KIR2DL3* homozygosity and possession of the Bx32 genotype significantly associated with IP transmission, and correspondingly, in this thesis we identified that concordance for *KIR2DL3/KIR2DL3+C2/C2* between mother-infant significantly associated with increased risk for IP transmission. Moreover, when adjusting for maternal VL mother-infant concordance for *KIR2DL3/KIR2DL3+C2/C2* showed a nine-fold increased risk for IP

transmission. Importantly, in this regard given that (1) *KIR2DL2* and *KIR2DL3* segregate as alleles of a single genetic locus (Uhrberg et al., 2002), (2) the presence of *KIR2DL2* associates with the group B haplotype while presence of *KIR2DL3* associates with group A haplotype (Hollenbach et al., 2009), and (3) *KIR2DL3/KIR2DL3* in MTCT1 mothers included the following haplotypes: AA1 (83.1%), Bx32 (7.0%), Bx35 (2.8%), Bx10 (2.8%), Bx48 (1.4%), Bx27 (1.4%) and Bx2 (1.4%), respectively, then *KIR2DL3* homozygosity likely correlated with the inhibitory AA1 haplotype. Thus, based on the principles of NK cell 'arming' model (Raulet and Vance, 2006), we postulated that homozygous *KIR2DL3* NK cells in the absence of their cognate C1 ligand would be unarmed and unresponsive to HIV-1 infected cells allowing viral infected cells to escape targeted lysis by NK cells. Additionally, given that *KIR2DL3* and *KIR2DL1* are in strong LD whereby 90% of individuals who bear *KIR2DL3* also possess *KIR2DL1* (Hollenbach et al., 2009), we could further extrapolate that NK cells in both mother and infant through a *KIR2DL1+C2/C2* interaction would promote NK cell inhibition and thus further contribute to increased risk of IP-transmission.

Similarly, while *KIR2DS4* can be found in B haplotypes it generally associates with the A haplotype (Gourraud et al., 2010). In MTCT1, we found maternal possession of *KIR2DS4-f* while absence in the infant (i.e. M+I- discordance) associated with increased risk for IP transmission. It may be that having one functional activating *KIR* on a predominantly inhibitory genotype with six other inhibitory *KIRs* is not sufficient to induce NK cell cytotoxicity toward viral infected cells. Alternatively, Merino et al. (2014) suggested that in chronic HIV-1 infection *KIR2DS4-f* promoted HIV-1 pathogenesis through the maintenance of an excessive pro-inflammatory state. The same group also reported that *KIR2DS4-f* associated with higher VL in HIV-1 positive partners and accelerated transmission of HIV-1 (Merino et al., 2011). Therefore, it may be that *KIR2DS4-f* NK cells in mothers with chronic HIV-1 infection are in a state of activation releasing pro-inflammatory cytokines that might promote IP transmission.

Conversely, in MTCT1 infant possession of *KIR2DS4-v* significantly associated with increased risk for IU acquisition of HIV-1 and this association was more prominent when infants were homozygous for the A haplotype. Accordingly, lack of a functional membrane bound *KIR2DS4* in a *KIR* genotype would be in complete favour of NK cell inhibition, would suggest that infant NK cells are unable to eliminate viral infected cells. Interestingly, in the Chinese Han (a population with high proportions of the A haplotype) possession of

KIR2DS4-v associated with increased susceptibility to syphilis (Zhuang et al., 2012). Therefore, in MTCT2 where the AA1 haplotype was significantly underrepresented in mothers it is perhaps not surprising that the *KIRDL3* and *KIR2DS4-f* associations were not evident. A greater proportion of Bx haplotypes would equate with the presence of more than three activating *KIRs* (i.e. *KIR2DS1*, *KIR2DS2*, *KIR2DS3* and *KIR2DS5*) which may possibly negate the effect of *KIR2DS4*. Taken together our data indicate that *KIR2DS4* variants are able to modulate the risk of vertical transmission, and this justifies further analysis into the direct and indirect roles of KIR2DS4 in NK cell function, but future studies should ideally match cases and controls in the context of the A haplotype.

In keeping with the NK cell inhibitory theory, it was also interesting to note that a "matched" inhibitory *KIR/HLA-ligand* combination between infant *KIR* and maternal *HLA* also increased the risk for vertical transmission. Again, in the MTCT1, infant possession of *KIR2DL2/KIR2DL3* paired with maternal *C1/C1* ligands increased the risk for IU acquisition of HIV-1. This suggested that if inhibitory KIRs on infant NK cells engage with cognate HLA-ligands expressed by HIV-1 infected maternal cells, this "matched" inhibitory KIR/HLA combination would fail to activate the cytolytic activity of the infant NK cell thereby allowing escape of the viral-infected cell. Indeed, our data concurs with the principles of 'self-recognition' (Hoglund and Brodin, 2010), and further supports the findings of "matched and miss matched" *KIR/HLA* allorecognition amongst HIV-1 concordant and discordant Senegalese couples (Jennes et al., 2013). Moreover, Jennes et al. (2013) in their *in vitro* NK cell alloreactivity assays using healthy blood donor derived NK cells and HIV-1 patient derived CD4+ T cells showed functional evidence of NK cell-mediated CD4+ T cell killing, which correlated with the presence of matched or mismatched allogeneic KIR/HLA combinations.

Although the concordant and allogeneic *KIR/HLA* associations were not evident in the MTCT2 cohort, the presence of a *HLA-A Bw4:80I* allele in mothers and infants significantly increased the risk for IU transmission and IU acquisition, respectively. Moreover, the combination *KIR3DL1+HLA-A/B Bw4:80I* was also significantly over-represented in both IP-TR and IU-TR mothers compared to NT mothers, notably another inhibitory *KIR/HLA-ligand* combination. However, since our real-time PCR assay broadly grouped the *HLA-A* alleles into those with an isoleucine at position 80 (*A*23*, *A*24*, *A*25* and *A*32*) and those without, we extrapolated which *HLA-A* allele/s could be increasing the risk for MTCT

by using *HLA-A* background frequencies collected from Black South African population (Paximadis et al., 2012). Using their *HLA-A* data, it was likely that *HLA-A Bw4:80I* in the MTCT2 primarily consisted of *A*23:01*. Therefore our data was consistent with other studies which reported that *A*23:01* associated with higher VL as well as increased the risk of transmission (MacDonald et al., 2000, Tang et al., 2008, Mackelprang et al., 2010). Similarly, in a Ugandan cohort of chronically HIV-1 infected individuals HLA-B Bw4 was associated with elevated frequencies of KIR3DL1+ $CD56^{dim}$ NK cells, and the size of the KIR3DL1+ NK cell subset correlated directly with VL (Eller et al., 2011). The authors noted this effect occurred only in HLA-B Bw4+ patients, suggesting that these cells expand in response to viral replication but may have relatively poor antiviral capacity.

Thus, collectively, in MTCT2 given that *HLA-A Bw4:80I* was significantly over-represented in TR mothers and their HIV-1 infected infants, and maternal *HLA-A/B Bw4:80I* in combination with *KIR3DL1* showed an association with increased risk for IP and IU transmission of HIV-1, it is plausible that the engagement of this inhibitory *KIR* might prevent NK cell lysis of HIV-1 infected cells possibly promoting HIV-1 transmission/acquisition. As such in light of these findings, while Paximadis et al. (2011) assessed only the *HLA-B* ligand groups and found no significant differences with regards to *HLA-B Bw4:80I* alone or paired with *KIR3DL1*, for future work it would be important to perform *HLA-A Bw4* genotyping the MTCT1 cohort.

Next, given that HLA-G is (1) the cognate ligand for *KIR2DL4,* a conserved framework *KIR* gene virtually expressed by all NK cells (Rajagopalan and Long, 2012), (2) can directly inhibit NK cell differentiation, proliferation, cytolysis and cytokine secretion (Amiot et al., 2014), and (3) HLA-G is preferentially expressed at the maternal-foetal interface on extravillous cytotrophoblasts, where it is plays an important tolerogenic role towards the semi-allogeneic foetus (Castelli et al., 2014), we evaluated the effect of *HLA-G* polymorphisms on vertical transmission of HIV-1. *HLA-G* alleles and 3'UTR haplotypes associations were evident in both MTCT cohorts. Representation of the UTR1 haplotype significantly associated with increased risk for IU- and IP- transmission in MTCT1 and MTCT2, respectively. Importantly, this haplotype has been reported to contain the majority of the polymorphisms that associate with increased expression of HLA-G, for example the 14-bp *Del*, +3142C and +3187G (Castelli et al., 2014). Moreover, the UTR1 haplotype was in strong LD with *G*01:01:01,* a *HLA-G* alleles that associated with higher levels of plasma

HLA-G than other *HLA-G* alleles, such as *G*01:01:03* and *G*01:05N* (Rebmann et al., 2001). In agreement with our findings, placental expression of HLA-G1 was 3.95 times more up-regulated in HIV-1 transmitting mothers compared to HIV-1 non-transmitting mothers (Moodley and Bobat, 2011). Other studies also identified HLA-G1 was able to inhibit NK cell-mediated lysis (Navarro et al., 1999, Ponte et al., 1999, Marchal-Bras-Goncalves et al., 2001).Thus in accordance with the NK cell inhibitory theory, we and others (Matte et al., 2004, Moodley and Bobat, 2011, Luo et al., 2013, da Silva et al., 2014), postulated that higher HLA-G expression would exert greater NK cell inhibition, which could result in the decreased ability of these cells to target viral-infected cells thereby increasing the risk for HIV-1 transmission.

Concerning *HLA-G* in infants, in MTCT1 we found no significant differences amongst the EU or HIV-1 infected infants, which suggested that *HLA-G* polymorphisms and potentially HLA-G expression by infant cells had little effect on HIV-1 acquisition. Thus, considering these findings as well as sample availability and cost of *HLA-G* genotyping we selected not to conduct *HLA-G* genotyping on the infant samples from MTCT2. This said, we cannot assume that *HLA-G* in MTCT2 infants would show no immunogenetic associations as found for MTCT1.

Figure 7.1 shows a summary of the major immunogenetic factors that were found to associate with the risk of MTCT of HIV-1. Overall, the data represented in this thesis confirm that both maternal and infant NK cells through their *KIR/HLA-ligand* interactions are able to influence the risk of vertical transmission of HIV-1. Moreover, potential alloreactive *KIR/HLA-ligand* combinations between mother and her infant holds important relevance to predicting which infants may be more susceptible to infection. While MTCT1 and MTCT2 received different ART regimens and were recruited almost a decade apart, the common emerging feature that appeared to associate with risk of transmission was a predicted overall inhibition of NK cell cytolysis, as indicated by representations of inhibitory *KIR* and their *HLA-ligands*.

In conclusion, in the context of MTCT, it remains an open question as to whether the small subset of infants still becoming HIV-1 infected despite receiving improved ART regimens, are a smaller subset of the group that would have normally become infected, or whether they are a uniquely susceptible group based on the altered drug environment. Given our findings

showing some common and uncommon genetic factors evident between MTCT1 and MTCT2, it is reasonable to suggest that there may well be such a shift in infant susceptibility. The study of additional genetic factors over time may allow this question to be more convincingly answered.

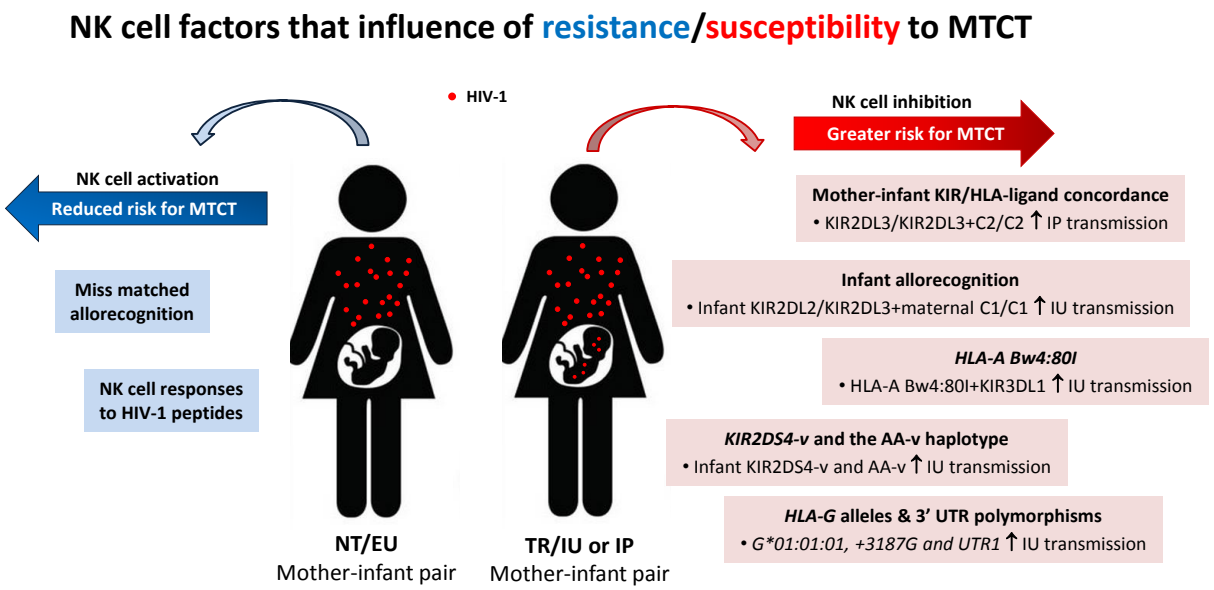


Figure 7.1 *KIR* and *HLA-ligand* factors that associate with risk of MTCT of HIV-1. NT/EU: HIV-1 non-transmitting mother and exposed infected infant; TR/IU or IP: HIV-1 transmitting mother and her *in utero* or intrapartum infected infant.

The findings in this thesis have important relevance to vaccine design as most HIV-1 vaccine have focused on the induction of nAbs to block infection by free virions and not virus-infected cells (Gooneratne et al., 2015). As both sexual and vertical transmission of HIV-1, transfer of HIV-1 infected lymphocytes takes place and evidence of this cell-to-cell transmission have been documented (Carr et al., 1999, Zhong et al., 2013), this implies vaccine design should incorporate the elicitation of immune responses capable of eliminating both cell-free and cell-associated virus. Given our *KIR/HLA* findings, as well as those reported in allogeneic stem cell transplantation, it is possible that allogeneic recognition of HIV-1 infected lymphocytes and their targeted lysis is achievable. Thus, future work should more thoroughly explore mechanisms to elicit these types of responses in the quest for a successful HIV-1 vaccine.

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Appendix A: Ethical clearance certificate

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130874

NAME: (Principal Investigator)	Ms Heather Hong
DEPARTMENT:	Centre for HIV and STIs/Virology National Institute for Communicable Diseases
PROJECT TITLE:	Killer Cell Immunoglobulin-Like Receptor (KIR) and Human Leucocyte Antigen (HLA) Genes in Mother-to-Child Transmission of HIV-1
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof C Tiemessen
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 04/09/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature — M130874 Date: 16 Oct 2013

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: Front covers of PhD publications

TISSUE ANTIGENS
IMMUNE RESPONSE GENETICS

Tissue Antigens ISSN 0001-2815

Killer-cell immunoglobulin-like receptor genotyping and HLA killer-cell immunoglobulin-like receptor-ligand identification by real-time polymerase chain reaction

H. A. Hong[1,2], A. S. Loubser[1,2], D. de Assis Rosa[1,3], V. Naranbhai[4], W. Carr[5,6], M. Paximadis[1], D. A. Lewis[7,8], C. T. Tiemessen[1,2] & C. M. Gray[1,9]

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Key words
genotyping; human leukocyte antigen killer-cell immunoglobulin-like receptor-ligand; killer-cell immunoglobulin-like receptor; real-time polymerase chain reaction

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Abstract

The effector function of natural killer (NK) cells is modulated by surface expression of a range of killer-cell immunoglobulin-like receptors (KIRs) that interact with human leukocyte antigen (HLA) class I ligands. We describe the use of real-time polymerase chain reaction (PCR) assays that allow easy and quick detection of 16 *KIR* genes and the presence/absence of KIR-ligands based on allelic discrimination at codon 80 in the *HLA-A/B Bw4* and *HLA-C C1/C2* genes. These methods overcome the tedious and expensive nature of conventional *KIR* genotyping and HLA class I typing using sequence-specific primer (SSP) PCR, sequence-specific oligonucleotide (SSO) hybridization or sequence-based typing (SBT). Using these two cost-effective assays, we measured the frequencies of KIRs, KIR-ligands and KIR/KIR-ligand pairs in a cohort of Black women recruited in South Africa.

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Clinical Immunology

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KIR2DS4 allelic variants: Differential effects on in utero and intrapartum HIV-1 mother-to-child transmission

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KEYWORDS:
Natural killer (NK) cells;
Killer cell immunoglobulin-like receptors (KIRs);
HLA-C ligands;
NK function;
Mother-to-child-transmission (MTCT)

Abstract *KIR2DS4* is the only activating gene within the A haplotype, and alleles of *KIR2DS4* can encode either functional (KIR2DS4-f) or non-functional (KIR2DS4-v) variants. To establish the role of *KIR2DS4* in the context of HIV-1 mother-to-child transmission, we *KIR* genotyped 145 HIV-1 non-transmitting mothers (NT) and their exposed uninfected infants (EU), and 72 HIV-1 transmitting mothers (TR) and their infected infants [intrapartum (IP), in utero (IU) or IU2 (an IU-enriched infected group)]. The frequency of *KIR2DS4-v* was significantly higher in IU2 infants compared to EU infants ($P = 0.022$, OR = 2.88); this association was more significant amongst AA haplotypes ($P = 0.004$, OR = 18.4). Possession of *KIR2DS4-f* in the mother with absence in the infant (M+I− discordance) was associated with a higher risk of IP transmission ($P = 0.005$, OR = 3.84); whilst in M−I+ discordance, infant possession of *KIR2DS4-v* was associated with increased risk of IU acquisition ($P = 0.002$; OR = 6.40). This study highlights the importance of *KIR2DS4* in HIV-1 transmission/acquisition.
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1. Introduction

Natural killer (NK) cells are cytotoxic lymphocytes crucial to the innate immune system in that they are able to provide rapid response to tumour and viral infected cells. NK cells are able to bridge the gap between the innate and adaptive immune systems through the release of cytotoxic granules and pro-inflammatory cytokines that signal to other immune cells such as dendritic cells and CD4+ T cells [1–4]. Several studies have described the importance of NK cells in HIV-1 infection [5–12] and strategies evolved by HIV-1 to specifically evade NK cell-mediated immune responses [1,3,13–15]. Furthermore, there is increasing evidence for a role of NK cells in

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Maternal human leukocyte antigen-G (HLA-G) genetic variants associate with *in utero* mother-to-child transmission of HIV-1 in Black South Africans

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ABSTRACT

A 14-bp insertion/deletion (indel) within the 3′ untranslated region (3′UTR) that affects *HLA-G* expression has been associated with HIV-1 mother-to-child transmission (MTCT). However, other 3′UTR single nucleotide polymorphisms (SNPs) that influence HLA-G mRNA stability have been described but not analysed in the context of MTCT, and little is known about the role of *HLA-G* alleles. We examined *HLA-G* alleles and 3′UTR SNPs, including the 14-bp indel, in 216 mother–infant pairs from Johannesburg, South Africa. Mother–infant pairs were classified as HIV-1 non-transmitting (NT, $n = 144$) or HIV-1 transmitting (TR, $n = 72$) with either intrapartum (IP, $n = 29$) or *in utero* (IU, $n = 19$) infected infants. We found *HLA-G* allele, *G*01:01:02* (in strong linkage disequilibrium with the 14-bp insertion) and +3187G SNP were significantly over-represented in IU-TR mothers compared to NT mothers ($P = 0.036$, OR = 2.26; $P = 0.011$, OR = 2.96, respectively). These findings suggest that maternal *HLA-G* alleles and/or SNPs that might alter expression of HLA-G potentially influence IU HIV-1 MTCT.

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1. Introduction

Prior to current prevention strategies, mother-to-child transmission (MTCT) or vertical transmission of HIV-1 occurred at an estimated rate of more than 30%, and still is the major cause of HIV/AIDS in children (Taha, 2011; da Silva et al., 2013). MTCT can occur during pregnancy (*in utero*, IU), at the time of delivery (intrapartum, IP), or postpartum (PP) through breast feeding (Duri et al., 2010; Kourtis et al., 2001). While the use of antiretroviral therapy (ART) during pregnancy has been shown to reduce the risk of MTCT, in the absence of ART, large proportions of infants remain HIV-1 uninfected and appear to have "natural protection". Thus, studying the mechanisms of natural protection in HIV-1 exposed but uninfected (EU) infants may help to determine correlates of protection in both infants and adults. Several studies have suggested that host genetic factors, such as human leukocyte antigen (HLA) class I and II alleles (Matt and Roger, 2001; Kuhn et al., 2004; MacDonald et al., 1998; Polycarpou et al., 2002) and killer immunoglobulin-like receptors (KIR) (Hong et al., 2013; Paximadis et al., 2011) contribute to MTCT. Recently, there has been increased interest in the role of *HLA-G* in MTCT, because of its preferential expression at the maternal–foetal interface and its immunosuppressive properties. HLA-G can inhibit differentiation, proliferation, cytolysis, cytokine secretion and immunoglobulin production upon binding to their specific inhibitory receptors: immunoglobulin-like transcript (ILT)-2, ILT-4 and KIR2DL4 expressed by many immune cells, such as B and T lymphocytes as well as natural killer (NK) cells (Amiot et al., 2014).

Unlike the classical class I HLA molecules, the non-classical HLA-G molecule has limited allelic polymorphism in the coding region, with only 50 *HLA-G* alleles having been described to date (IMGT/HLA, version 3.16.0, 2014/04/14) (Robinson et al., 2013). These alleles encode 16 distinct transmembrane proteins (G*01:01, G*01:02, G*01:03, G*01:04, G*01:06, G*01:07, G*01:08, G*01:09, G*01:10, G*01:11, G*01:12, G*01:14, G*01:15, G*01:16, G*01:17, G*01:18) and two truncated proteins (G*01:05N and G*01:13N) (Robinson et al., 2013). Alternative splicing of the HLA-G primary transcript can generate seven alternative mRNAs that encode membrane-bound (HLA-G1, -G2, -G3, and -G4) and soluble (HLA-G5, -G6, and -G7) protein isoforms (Donadi et al.,

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Appendix C: Cover page of Turnitin Report

PhDthesisKIRHLAgenesandmotherinfantHIV1transmissionTurnItIn.docx
by Heather Hong

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