CHAPTER ONE

LITERATURE REVIEW

1.1 HIV-1 prevalence and Africa

The United Nations Program on human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) estimates that more than 800 000 children continue to be infected with HIV-1 each year. Most of these infections occur in resource-poor countries and one-third to one-half of these infections are attributed to breastfeeding transmission (Bulterys *et al*., 2002; Nolan *et al*., 2002a; Mofenson and Munderi, 2002). Of the world's population of HIV-1 infected subjects, 4% are children (Chakraborty *et al*., 2003). Without highly active antiretroviral therapy (HAART), roughly 25% of these children will rapidly progress to AIDS within the first year of life, with the remaining 75% progressing to AIDS by the age of 7 years (Chakraborty *et al*., 2003). By the age of one year 35.2% of infected children will have died (Newel M-L *et* al., 2004) The figures indicate that sub-Saharan Africa has been the most severely affected by the HIV/AIDS epidemic. Of the 42 million people living in South Africa, over 5 million are HIV-1 infected with more than half of these infections occurring in women (Karim *et al*., 2002). For both biological (Langerhans cell physiology of the cervix, vulval and vaginal inflammation or ulceration, sexually and non-sexually transmitted diseases) and socio-cultural reasons (traditional customs and practices, male resistance to condom use, risk due to sexual behaviour of male partner, lack of education and employment opportunities and poverty forcing women into commercial sex work), women are particularly susceptible to HIV-1 (WHO/CHS/RHR/99.15 UNAIDS/99.35E). A study conducted in Kwa-Zulu Natal, South Africa has found the prevalence and incidence of HIV-1 to be highest in young women in their late teens and early twenties (Rollins *et al*., 2002). In industrialized nations, perinatal HIV-1 transmission rates in the absence of intervention ranges between 14 and 26%, whereas in developing nations the rates range from 21 to 43% (McGowan and Shah, 2000). This disparity is probably accounted for by the fact that in Africa prolonged breastfeeding (into the second year of life) is common, with the estimated risk of mother-to-infant HIV-1 transmission in the range from 25 to 48% (DeCock *et* al., 2000). However, it is important to recognize that there are other factors that affect perinatal acquisition of HIV-1. It has been projected that by 2010, if no AIDS

strategies are in place, AIDS infant mortality will increase by 25% and under-five mortality by over 100% in the regions most affected by the disease (WHO/CHS/RHR/99.15 UNAIDS/99.35E). Evidence suggests that the risk of transmission is greatest at delivery (50-80%), due to exposure of the infant to maternal blood and direct exposure to the virus during passage through the birth canal (Guay *et a*., 1999; McGowan and Shah, 2000). The fact that some infections do occur *in utero* suggests that the placental barrier is not impermeable to maternal pathogens (Clerici *et* al., 2000).

1.2 Maternal transmission of HIV-1

1.2.1 Factors influencing mode of perinatal transmission

Many factors have been implicated in maternal-foetal HIV-1 transmission, but to date there are no clearly defined factors associated with maternal transmission of HIV-1. Table 1.1 lists some of the factors (divided into maternal, viral, obstetrical, foetal and infant) that have been implicated in the maternal-foetal transmission of HIV-1 (WHO/RHT/98.24 UNAID/98.44; McGowan and Shah, 2000). Although the maternal viral load at delivery is very useful for determining the risk of transmission (Sperling *et al*., 1996; Cao *et al*., 1997; Mofenson 1997; Mofenson *et al*., 1999), there is no level above which transmission occurs nor a level below which transmission is never seen (Sperling *et al*., 1996; Cao *et al*., 1997; Mofenson 1997).

1.2.2 Factors that correlate with the uninfected status of an infant

Factors reported to exist in pregnant women, that inhibit HIV-1, and that could contribute to the lack of transmission of HIV-1 from mother-to-child include: (Bourinbaiar *et al*., 1992; Kondapi *et al*., 2002; Rossi *et al*., 1989; Goedert *et al*., 1989; Devash *et al*., 1990) (i) interferon-alpha (IFN-α) - elevated levels identified in maternal and umbilical cord blood and in placental trophoblasts being strongly associated with protection against vertical transmission of HIV-1 (Zachar *et al*., 1997), (ii) the action of female hormones produced by placental trophoblast cells that are associated with the detection of low HIV-1 virus titres in the foetus (Bourinbaiar *et al*., 1992; Bourinbaiar and Lee-Huang, 1995), (iii) the prevalence of maternal antibodies. Whilst studies report a correlation between the prevalence of maternal antibodies directed against the hypervariable region (V3 loop) of

Table 1.1 Factors associated with the risk of perinatal HIV-1 transmission

Factors	Description
Maternal	Clinical stage of the mother (HIV-1 status/AIDS status) [1; 2; 12; 30] Maternal immunological status (low CD4 count, p24 antigenaemia, neutralizing antibodies) [1; 2; 9; 12; 25; 26; 29] Maternal nutritional status (malnutrition / vitamin A deficiency) [1; 11; 14] Maternal behavioural factors (cigarette smoking, drug use, unprotected sexual intercourse during pregnancy) [1; 20] Background infections (sexually transmitted diseases) [12; 20] Antiretroviral treatment taken by the mother [1; 12; 18] Maternal cervicovaginal HIV-1 levels [7; 19] Maternal health (anaemia, fever) [29] Active genital ulcers, chorioamnioitis [12; 20; 29; 33]
Viral	Viral genotype and phenotype [1; 3; 4; 22; 26] Viral load [1; 5; 6; 8, 10; 12; 17; 18; 31; 35] Viral resistance [1] HIV-1 co-receptor usage [16] Viral characteristics including factors such as HIV-1 heterogeneity in the *env* region [36] changes in the V3 loop [34]
Obstetrical	Prolonged membrane rupture (>4 hours) [1; 15] Vaginal lacerations during labour or preterm delivery Mode of delivery [1] Intrapartum haemorrhage [1] Obstetrical procedures – placental abruption, chorionic villus sampling, amniocentesis, episiotomy [1; 30] Invasive foetal monitoring or use of foetal scalp electrodes [1; 30]
Foetal	Genetic factors [17; 27] Premature delivery [9; 13] Foetal cell susceptibility [22; 28]
Infant	Birth weight [20] Background infections [20] Breast-feeding – maternal breast milk viral load [23; 24] Gastrointestinal tract factors [21] Immaturity of the immune system [32]

[1] WHO/CHS/RHR/99.15 UNAIDS/99.35E; [2] Abrams *et al*., 2003; [3] Arroyo *et al* 2002; [4] Becker-Pergola *et al*., 2000; [5] Brandt *et al*., 1996; [6] Cao *et al*., 1997; [7] Chuachoowong *et al*., 2000; [8] Contopoulos-Ioannidis and Ioannidis, 1998; [9] The European Collaborative Study 1996; [10] The European Collaborative Study 1999; [11] Fawzi, *et al*., 2000; [12] Fawzi *et al*., 2001; [13] Goedert *et al*., 1989; [14] Greenberg *et al*., 1997; [15] The International Perinatal HIV Group 2001; [16] Kuiken *et al*., 1992; [17] MacDonald *et al*., 1998; [18] Mofenson *et al*., 1999; [19] Montano *et al*., 2003; [20] Nair *et al*., 1993; [21] Newell *et al*., 1997; [22] Ometta *et al*., 1995; [23] Richardson *et al*., 2003; [24] Rousseau *et al*., 2003; [25] Scarlatti *et al*., 1993b; [26] Scarlatti *et al*., 1993c; [27] Sei *et al*., 2001; [28] Sperduto *et al*., 1993; [29] St Louis *et al*., 1993; [30] Tess *et al*., 1998; [31] Thea *et al*., 1997; [32] Veazey *et al*., 2003; [33] Wabire-Mangen *et al*., 1999; [34] Wang *et al*., 1997a; [35]Weiser *et al*., 1994; [36] Wolinsky *et al*., 1992.

glycoprotein (gp) 120 and lack of transmission of HIV-1 from infected mothers to their infants (Rossi *et al*., 1989; Goedert *et al*., 1989; Devash *et al*., 1990) other studies do not confirm these findings (Halsey *et al*., 1992; Robertson *et al*., 1992). In addition, antibodies to specific regions of gp120 outside the V3 loop, including the CD4/gp120 binding site (Khouri *et al*., 1995) and epitopes within gp41 (Ugen *et al*., 1997) may be important in preventing vertical transmission of HIV-1, (iv) maternal immunoglobulin G (IgG) antibody-mediated neutralization has also been reported to correlate with reduced risk of mother-to-child transmission (MTCT) (Scarlatti *et al*., 1993a), (v) mother-child

class I human leukocyte antigen (HLA) discordance has been associated with reduced transmission of HIV-1 (MacDonald *et al*., 1998). Discordance in class II HLA determinants may be equally important in mediating protection however studies of the role of class II concordance are complex due to the diversity at the DR locus (Hader *et al*., 2002), (vii) glycoproteins isolated from tissues from pregnant women and reported to have anti-HIV-1 properties. These include a 50 kiloDalton (kDa) protein isolated from cultured placental stromal cell supernatants of chronic villus biopsy samples from HIV-1 seronegative patients and found to inhibit HIV-1_{MN}, and a 90 kDa glycoprotein detected in the placental tissue of HIV-1 seronegative pregnant women during the first trimester and shown to exhibit significant anti-HIV-1 activity by affecting HIV-1 infection through inhibition of CD4-gp120 binding (Kondapi *et al*., 2002).

1.2.3 Molecular mechanisms and viral determinants involved in vertical transmission

Molecular studies of the HIV-1 genome have defined the relative importance of various viral genes for infection and replication. Specific viral genetic regions have subsequently been identified and considered responsible for biological and serological properties such as cell tropism, kinetics of replication, cytopathogenicity, and sensitivity to antiviral antibodies. Molecular characterization of numerous HIV-1 genes including *gag* p17 (Hahn *et al*., 1999), *env* V3 (Ahmad *et al*., 1995), reverse transcriptase (Sundaravaradan *et al*., 2005), *gag* nucleocapsid (Wellensiek *et al*., 2006), *tat* (Husain *et al*., 2001), *rev* (Ramakrishnan *et al*., 2005), *vif* (Yedavalli *et al*., 1998a), *vpr* (Yedavalli *et al*., 1998b), *vpu* (Yedavalli *et al*., 2001a), *nef* (Hahn *et al*., 2003) and *env* gp41 (Ramakrishnan *et al*., 2006) from infected mother-infant pairs following perinatal transmission suggest a high conservation of functional domains of these genes and a close relationship between epidemiological linked mother-infant pairs. A limited heterogeneity of sequences and low conservation of functional domains from HIV-1 *env* (Matala *et al*., 2000), *vif* and *vpr* (Yedavalli and Ahmad, 2001b) and *gag* p17 (Hahn and Ahmad, 2001) regions has been reported from infected mothers who did not transmit HIV-1 to their infants. Essentially, the molecular and viral determinants associated with perinatal transmission are not clear making it difficult to develop strategies for the prevention and treatment of HIV-1 infection in children (Yedavalli *et al*., 1998a; Hahn *et al*., 1999).

1.2.4 Cellular mechanisms

Vertical transmission of HIV-1 has been reported to occur either *in utero*, intrapartum, or postpartum through breast milk (McGowan and Shah, 2000). Most transmission is thought to occur in late pregnancy or during labour (WHO/CHS/RHR/99.15 UNAIDS/99.35E). Potential mechanisms include mother-to-child transfer of HIV-1 through the placenta, exposure of the foetus or neonate to virus in the maternal blood or amniotic fluid at the time of parturition and exposure of the foetus or neonate to virus in cervicovaginal secretions throughout labour and delivery (Tuomala *et al*., 2003).

1.2.5 Cell-associated genital tract virus and vertical transmission of HIV-1

Despite the fact that HIV-1 tropism for placental cells in *in utero* transmission is less than 10% (Anderson *et al*., 2001), several receptors critical for HIV-1 infection have been reported on epithelial cells in the uterus including CD4, CXCR4, CCR5 and galactosylceramide (GalC) (Yeaman *et al*., 2003). While the lower reproductive tract tissues (vagina and cervix) are generally thought of as primary sites of infection in the human female reproductive tract, evidence suggests that the upper reproductive tract (cervix and uterus) may also serve as a site for initial infection by HIV-1 (Yeaman *et al*., 2003). Studies of HIV-1 infected women receiving no or minimal antiretroviral therapy (ART) during pregnancy have correlated increased risk of vertical transmission of HIV-1 with the presence of HIV-1 DNA (John *et al*., 2001) or HIV-1 RNA (Chuachoowong *et al*., 2000) in the genital tract.

1.3 Dynamics at the maternal-foetal interface

The placenta plays a critical role in providing an environment that supports optimal foetal growth, namely it provides the site of nutrient transfer, waste secretion (foetus to mother) and acts as an active endocrine organ capable of secreting hormones, growth factors, cytokines and bioactive products (Anthony *et al*., 1995). The placenta consists of two surfaces, the chorionic plate that faces the infant and to which the umbilical cord is attached and the basal plate which is adjacent to the maternal endometrium (Burton and Watson, 1997). The foetal component consists of villi that are bathed directly by maternal blood (Burton and Watson, 1997). Although the placental barrier (comprising the syncytiotrophoblastic layer (outer surface of villi), the stroma of the intravillous space

and the foetal capillaries) separates the blood in maternal and infant circulation, a wide range of substances are actively or passively transferred through the placenta to the foetus (Saji *et al*., 1999). Most low molecular weight compounds (<500 Da) diffuse through the placental tissue (Saji *et al*., 1999). Other low molecular weight substances such as amino acids (Cetin, 2001) and inorganic ions (Stulc, 1997) cross the placenta by active transport which is unidirectional (Saji *et al*., 1999). Water is presumed to cross the placenta passively and is possibly facilitated by the 28 kDa water channel-forming integral protein (Stulc, 1997). Bidirectional transfer of inflammatory cytokines (interleukin (IL)-6) has been reported (Zaretsky *et al*., 2004). High molecular weight compounds do not usually traverse the placenta although there are exceptions such as IgG (160 kDa). Immunoglobulins are transferred in an active, selective and affinity-restricted process, which is Fc-gamma receptor mediated and intracellular (Landor, 1995). The immunological relationship between the mother and the foetus is bidirectional, determined on the one hand by foetal antigen presentation and on the other hand by recognition of and reaction to these antigens by the maternal immune system. Evidence suggests that this immunological recognition contributes to implantation of the embryo, development of the placenta (Dealtry *et al*., 2000) and is important for the maintenance of gestation (Dealtry *et al*., 2000; Szekeres-Bartho, 2002). It is therefore not surprising that clinical and experimental evidence indicates that fluctuations occur in the balance of T-helper 1 or T-helper 2 (Th1/Th2)-type cytokines during pregnancy (Dealtry *et al*., 2000) and that the maternal immune response (particularly within the placenta (Dealtry *et al*., 2000)) is biased toward antibody production or Th2 cytokine expression (Lin *et al*., 1993; Wegmann *et al*., 1993).

1.3.1 The placenta and vertical transmission of HIV-1

The placenta is considered an important natural barrier to HIV-1 infection (Torres *et al*., 2001) however, HIV-1 infection has been reported in placental tissue (Maury *et al*., 1989; Sprecher *et al*., 1986; Zachar *et al*., 1994). Figure 1.1 depicts the anatomy of the maternal-foetal interface, where the foetal-derived placenta attaches to the mother's uterus. The finding that caesarian section reduces the risk of MTCT of HIV-1 by three- to four-fold suggests that the placenta functions to protect the infant during gestation (Behbahani *et al*., 2000). The barrier between maternal and foetal circulation is several layers thick and consists of syncytiotrophoblasts, placental macrophages (Hofbauer cells) and intervening stroma (Behbahani *et al*., 2000). Since placental macrophages and

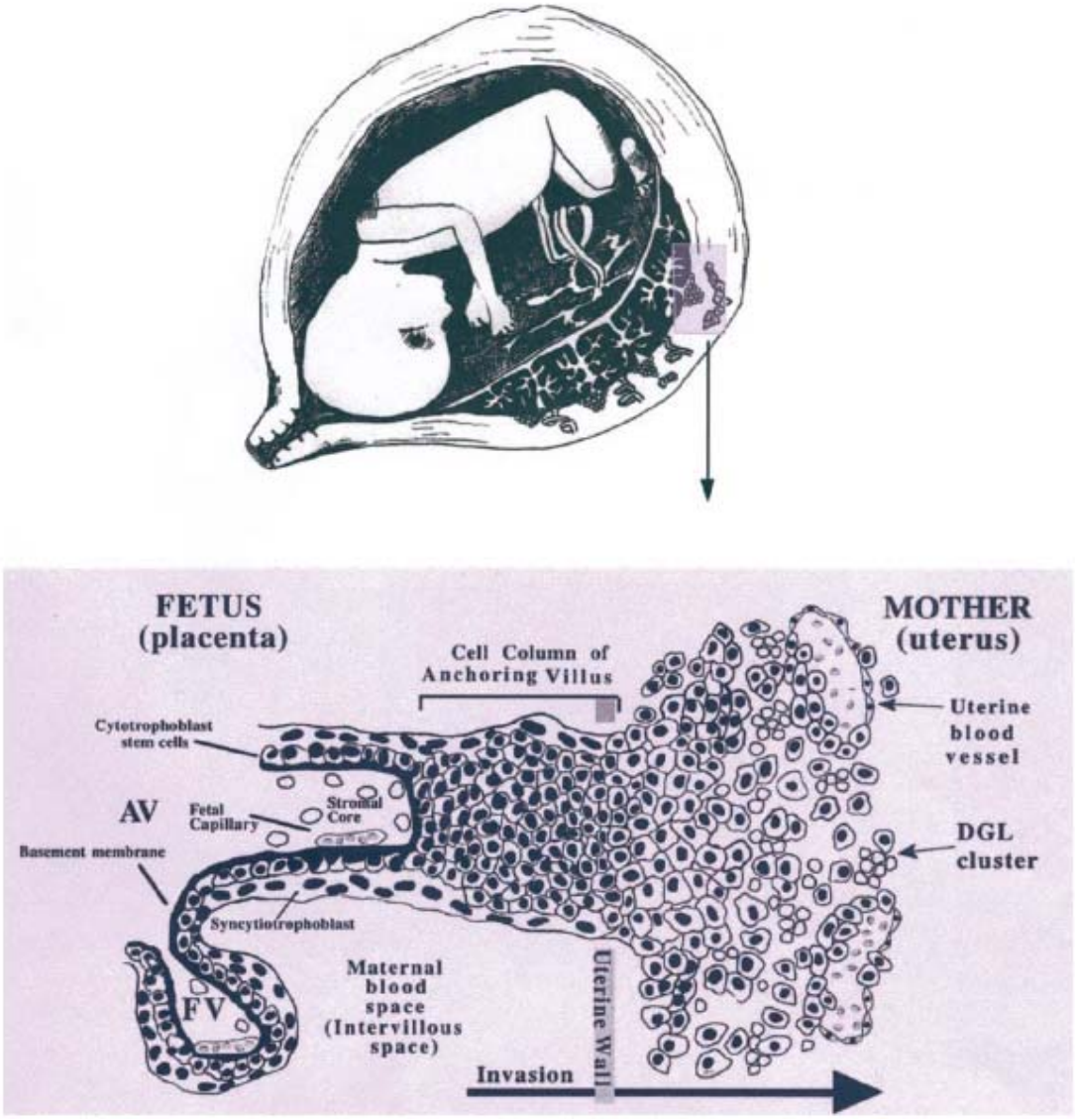


Figure 1.1 Maternal-foetal interface depicted on a gross level in the top diagram (lavender box) with the histology of this region shown in the bottom diagram. The basic structural unit of the placenta is the chorionic villus, composed of a stromal core with blood vessels, surrounded by a basement membrane, and overlain by cytotrophoblast stem cells. . These stem cells detach from the basement membrane and adopt one of two lineages – either fusing to form the syncytiotrophoblast, which covers floating villi (FV), or joining a column of extravillous cytotrophoblasts at the tips of anchoring villi (AV). The synciotrophoblast mediates nutrient, gas and waste exchange between foetal and maternal blood. The anchoring villi, through the attachment of cytotrophoblast columns, establish physical connections between the foetus and the mother. Invasive cytotrophoblasts penetrate the uterine wall up to the first third of the myometrium, encountering a population of maternal immune cells, termed decidual granulated leukocytes (DGLs), resident in the uterine stroma. A portion of the extavillous cytotrophoblasts home to uterine spiral arterioles and remodel these vessels by destroying the muscular wall and replacing the endothelial lining. Reproduced from Red-Horse *et al.,* 2001.

trophoblasts represent the main cellular components of the placenta, these cells may play an important role in transplacental HIV-1 transmission as HIV-1 has been reported to replicate in primary cultured placental cells (Mano and Chermann, 1991). MTCT of HIV-1 predominantly involves macrophage-tropic viral isolates (Ahmad *et al*., 1995). Differences have been reported in chemokine receptor expression between placentae from HIV-1 transmitting and non-transmitting women (Behbahani *et al*., 2000). These different expression patterns are attributed to distinctly different cytokine milieus in transmitting placentae compared to non-transmitting placentae with an up-regulation of type-2 placental cytokines (IL-4 and IL-10) in placentae from non-transmitting women compared to placentae from transmitting women that express type-1 cytokines (Behbahani *et al*., 2000). CCR5 and CXCR4 are expressed on placental macrophages and lymphocytes but not trophoblasts, although it has been suggested that the type-2 cytokine milieu drives the expression of CXCR4 selecting for HIV-1 isolates less likely to be transmitted whereas up-regulation of CCR5 expression by the type-1 cytokine milieu is associated with HIV-1 vertical transmission (Behbahani *et al*., 2000). This selection, based on chemokine receptor expression, is consistent with findings that a homogeneous subset of maternal R5 non-synctium-inducing HIV-1 isolates predominate in perinatally infected children (Behbahani *et al*., 2000).

1.3.2 Trophoblast involvement in *in utero* transmission of HIV-1

Trophoblasts are foetal-derived epithelial cells that are bathed in maternal blood. They express CD4, CD14, Fc receptors and receptors for many cytokines and constitutively secrete a variety of cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF), colony stimulating factor 1 (CSF-1), IL-1, IL-6, tumour necrosis factor (TNF)-α, transforming growth factors and platelet-alpha derived growth factor (PDGF) (Guilbert *et al*., 1993). There is little direct evidence that trophoblasts support HIV-1 infection, however recent data indicates that full-term trophoblasts express CXCR4 but not CCR5 and CD4 cell surface proteins and can be infected by CD4-independent, CXCR4-utilizing HIV-1 strains (Al-Harthi *et al*., 2002). Whilst trophoblasts express the host factors important for productive HIV-1 infection, the block to HIV-1 infection has been suggested to be at the level of entry (Al-Harthi *et al*., 2002).

A reduced risk of vertical transmission of HIV-1 has been reported among mother-child pairs that are discordant in HLA-G exon 2, a major histocompatibility complex (MHC)

class I gene which is uniquely expressed in extravillous cytotrophoblasts at the maternal-foetal interface and that functions to protect the trophoblast layer and the foetus from maternal T-cells, natural-killer (NK) cell-mediated lysis and peptide presentation (Aikhionbare *et al*., 2001).

1.3.3 Transplacental passage of pathogens - mechanism of selective transmission of HIV-1 through the placental barrier

Whilst the human placenta permits metabolic exchange between the mother and infant, several features thereof are considered possible obstacles to vertical transmission of pathogens (Burton and Watson, 1997). This includes the syncytial nature of the outer villous covering, namely the syncytiotrophoblast, and the ability of this tissue to secrete nitric oxide and interferons (Burton and Watson, 1997). However, other features such as the lack of expression of HLA class I antigens by the syncytiotrophoblast, its multiple vesicular and immunoglobulin transport pathways and the occurrence of defects in the trophoblast layers throughout gestation may facilitate transmission (Burton and Watson, 1997). Although damage to the trophoblast layers can occur throughout gestation, and while repair processes have been identified, transient exposure of the trophoblastic layer to pathogens or infected maternal cells is assumed (Burton and Watson, 1997). Macrophages or capillary endothelial cells and their junctions at the basement membrane or within the villous core have been implicated as defence mechanisms against pathogens (Burton and Watson, 1997). Placental inflammation e.g. chorioamnionitis (Wabmire-Mangen *et al*., 1999) and inflammatory cytokines (Raghupathy, 1997) can be responsible for placental damage. Trophoblastic cells of HIV-1 positive placentas have been reported to express significantly higher levels of IL-1β, IL-6 and TNF-α than control placentas and may contribute to a placental microenvironment that may facilitate vertical transmission of HIV-1 (Lee *et al*., 1997).

1.4 Antiretroviral prophylaxis of perinatal HIV-1 transmission in the developing world

There are currently twenty-two approved antiretroviral drugs or drug combinations available for the treatment of HIV-1 disease in adults (Bean, 2005) (Appendix B, Table 1). These target the two essential HIV-1 enzymes, reverse transcriptase (RT) and protease (Hammer, 2002). Several other agents such as entry inhibitors, integrase inhibitors (Buss

and Cammack, 2001; Hammer, 2002; De Clerq, 2001; Kilby and Eron, 2003) and new generation nucleoside reverse transcriptase inhibitors (NRTI), protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (McCarthy, 2002) are in clinical development (Appendix B, Table 2). Because of a reluctance to use new medication in children before efficacy and safety have been confirmed in adults, the need to develop liquid formulations and the need for age-specific pharmacokinetic data, there are fewer therapeutic options available for the treatment of HIV-1 infection in children (Appendix B, Table 3) (Cohen *et al*., 2002).

Despite the effectiveness of ART in reducing viral replication and plasma viraemia, incomplete adherence (due to drug toxicity/adverse side effects) or pharmacologic issues (limited potency of the drugs), the presence of replication competent HIV-1 in resting $CD4^+$ T-cells and concerns pertaining to drug resistance (from single and multiple drug regimens) have become critical issues that many researchers are addressing. Mathematical modeling of the HIV/AIDS epidemic suggests that depending on the country and manner in which ART therapy has been targeted, primary resistance may be observed in 25-100% of HIV-1 cases within 30 years (Nagelkerke *et al*., 2002), signifying there is a need for novel drug targets to combat the virus (Bean, 2005).

Perinatal HIV-1 transmission is reduced by administration of antiretroviral prophylaxis either during pregnancy and/or to the neonate (Mofenson and Munderi, 2002). The Paediatric AIDS Clinical Trials group Protocol 076 (PACTG 076) study, conducted in the United States of America (USA) and published in 1994, demonstrated the efficacy of a three-part regimen (antepartum (prenatal beginning at/after 14 weeks'gestation), intrapartum (intravenous infusion) and postpartum administration (6 weeks prophylaxis to exposed newborns)) of zidovudine (AZT) prophylaxis for prevention of perinatal transmission of HIV-1 (68% reduction) (Connor *et al*., 1994). In developed countries, triple antiretroviral regimens have dramatically reduced perinatal transmission to less than 2% among women avoiding breastfeeding (Mofenson and McIntyre, 2000). On the basis of the PACTG 076 findings, prenatal counselling and testing and AZT prophylaxis has been adopted as the standard of care in the USA and many parts of the world, where efficient resources exist, to reduce MTCT of HIV-1. Because of the logistics (cost, monitoring, introduction and continuation of treatment, drug reactions) this regimen is however not feasible in resource-poor countries with the consequence that numerous

trials have been undertaken to investigate shorter and less expensive antiretroviral regimens. These trials (Appendix C, Table 1) have investigated different antiretroviral drug combinations and varying administration periods and have proven to be effective in reducing perinatal HIV-1 transmission (Mofenson and Munderi, 2002).

1.4.1 Infant HIV-1 prophylaxis

Infants given antiretroviral prophylaxis who subsequently were found to be infected with HIV-1 tend to have higher virological set-points compared to adults and undetectable viral loads are attained at a lower rate with paediatric HIV-1 infection compared to adults (Mullen *et al*., 2002). While this is largely the result of the infants immature immune system, infant prophylactic regimens are faced with adherence difficulties due to complex dosing regimens, unpalatable liquid formulations and difficulty in swallowing large antiretroviral tablets, non-optimal drug dosing regimens and limited drug availability for children (only eight of the licenced drugs available for adults are registered for paediatric use) resulting in the development of drug resistance (Mullen *et al*., 2002). To date there is limited information on the prevalence and problems of drug resistance among paediatric populations.

1.5 Developing world prophylaxis using zidovudine (AZT) or nevirapine (NVP) to reduce perinatal HIV-1 transmission

In the developing world short-course prophylactic regimens have included the use of a NNRTI, nevirapine (NVP) and NRTI(s), AZT or a combination of zidovudine-lamivudine (AZT-3TC). Importantly, whilst NVP does not require intracellular modification, (therefore exhibiting similar antiviral properties in resting or activated cells), AZT must undergo intracellular modification to an active 5'-triphospate form, a process dependent on cellular kinases and phosphotransferases (Davis *et al*., 2001).

1.5.1 AZT prophylaxis

The PACTG Protocol 076 demonstrated a 67% reduction in the rate of perinatal transmission of HIV-1 to the newborn (Connor *et al*., 1994). Other trials confirm the efficacy of AZT prophylaxis and even demonstrate that reductions in the rates of perinatal

transmission of HIV-1 occur if abbreviated regimens of AZT prophylaxis are used (Wade *et al*., 1998; Shaffer *et al*., 1999a; Wiktor *et al*., 1999).

During pregnancy (17 to 21 weeks gestation) and at term, foetal peripheral blood mononuclear cells (PBMC) have been shown to take up AZT and generate the active AZT triphosphate metabolite, although as with adult PBMC the rate-limiting step is the phosphorylation of dideoxynucleoside monophosphate (AZT-MP) to dideoxynucleoside diphosphate (AZT-DP) (Agbaria *et al*., 2003).

1.5.1.1 Pregnancy and AZT therapy on maternal viral load

While AZT antepartum administration has been associated with a decrease in plasma HIV-1 RNA levels (Dickover *et al*., 1996), this association has not been found in asymptomatic pregnant women with low viral loads that initiate AZT therapy during pregnancy (Melvin *et al*., 1997). The exact mechanism by which AZT prevents MTCT of HIV-1 is unknown and while reduction in the maternal viral levels has been thought to play an important role (Sperling *et al*., 1996; Mofenson *et al*., 1999; Shaffer *et al*., 1999; Leroy *et al*., 2001), the effectiveness of short-course ART regimens in reducing MTCT suggest that the protective effect may not be due to the reduction in maternal viral levels but due to the prevention of HIV-1 reverse transcription in the newborn (Melvin *et al*., 1997).

1.5.2 NVP prophylaxis

Several characteristics of NVP including the quick absorption of NVP after oral dosing and its ability to cross the placenta, together with its long half-life and its activity against cell-free virions and cell-associated virions (Zhang *et al*., 1996), have favoured its use in single-dose prophylactic regimens. To date the most widely used NVP regimen is derived from the HIVNET 012 study which demonstrated that a single-dose of NVP to the mother in labour and a dose administered shortly after birth, to the infant, reduced transmission by 50% (Guay *et al*., 1999; Jackson *et al*., 2003). Theoretically, NVP given as an oral dose to HIV-1 infected pregnant women in labour would be effective in preventing MTCT of HIV-1 during the early intrapartum periods and the single-dose given to the infant within 72 hours of birth to early postpartum periods. Other important features of NVP include the fact that it can decrease plasma HIV-1 RNA levels by roughly 1.3 log

following a single-dose (Musoke *et al*., 1999) and does not need to be metabolized to an active form (Zhang *et al*., 1996). Short-course NVP regimens have proven to be effective (Eshleman *et al*., 2001a; Dorenbaum *et al*., 2002; Ayouba *et al*., 2003; Stringer *et al*., 2003) with perinatal transmission being reduced by 38-50% even amongst breastfeeding infants (Musoke *et al*., 1999).

1.6 Antiviral properties and characteristics of AZT and NVP

1.6.1 NRTIs

NRTIs are dideoxynucleoside analogues of endogenous nucleosides, that is, AZT is a thymidine analogue and 3TC a cytosine analogue (Li and Chan, 1999; Max and Sherer, 2000). NRTIs directly interfere with proviral DNA chain elongation due to the fact that they lack a 3'-hydroxyl group and are therefore incorporated into the elongating viral DNA chain preventing the 3'-5' phosphodiester bond from being formed (Mofenson and Munderi, 2002).

1.6.1.1 AZT

As mentioned earlier, AZT (3'azido-3'deoxythymidine), as with all NRTIs, must be phosphorylated intracellularly by specific host cell enzymes to form active triphosphates that function as inhibitors of HIV-1 RT (Li and Chan, 1999; Max and Sherer, 2000). The NRTI-5'triphoshate competes with cellular 2'-deoxynucleoside-5'-triphoshate for binding to the viral RT (Davis *et al*., 2001). The ratio NRTI-5'-triphosphate/2'deoxynucleoside-5'triphosphate determines the antiviral efficacy for the NRTI and its value reflects the activities of cellular enzymes responsible for its phosphorylation (Davis *et al*., 2001). The activities of these enzymes vary during the cell cycle (Gao *et al*., 1994).

1.6.1.2 AZT transport and metabolism

One of the most important factors thought to be involved in the anabolism of AZT is the cell type and the activation state of the target cell (Veal and Back, 1995; Cherry and Wesselingh, 2003). AZT is preferentially phosphorylated to dideoxynucleoside triphosphate (AZT-TP) in phytohaemagglutinin (PHA)-stimulated PBMC than resting

PBMC with the ratio of AZT-TP to deoxythymidine triphosphate (dTTP) being 10- to 17-fold greater in stimulated than resting cells (Gao *et al*., 1993). AZT permeates the cell membrane of human erythrocytes and lymphocytes by simple, non-facilitated diffusion (Zimmerman *et al*., 1987). The rapid diffusion of AZT has been attributed to the lipophilic nature of the molecule imparted to it by the replacement of a 3'-hydroxyl group of thymidine with an azido moiety (Zimmerman *et al*., 1987). AZT undergoes numerous phosphorylation steps to form the active triphosphate that exerts the antiviral activity (Veal and Back, 1995). These steps include phosphorylation by nucleoside kinase to form dideoxynucleoside-monophosphate (AZT-MP), phosphorylation by nucleoside monophosphate kinase (thymidylate kinase) to form dideoxynucleoside diphosphate (AZT-DP) and phosphorylation by nucleoside diphosphate (NDP) kinase to form AZT-TP (Veal and Back, 1995). The initial phosphorylation step of AZT by thymidine kinase is more efficient than the subsequent phosphorylation steps, with the conversion of AZT to AZT-MP being nearly as efficient as the conversion of thymidine to thymidine monophosphate (TMP) (Hazuda and Kuo, 1997). The conversion of AZT-MP to AZT-TP is less than 1% the efficiency of the conversion of TMP to thymidine triphospate (TTP) (Furman *et al.,* 1986). This results in the accumulation of high concentrations of AZT-MP relative to AZT-DP and AZT-TP (Hazuda and Kuo, 1997). Complex positive and negative feedback loops, which have not all been characterized, regulate these phosphorylation pathways (Stein and Moore, 2001). Inhibition of HIV-1 RT by AZT-TP occurs via a competitive mechanism (Li and Chan, 1999). The phosphorylation process is a minor pathway probably accounting for less than 1% of the overall metabolic profile, with the predominant pathway involving glucuronidation to 5'-glucuronyl zidovudine (GAZT) a metabolite renally excreted (Veal and Back, 1995). Reduction of the azido group on the ribose ring of AZT results in the formation of 3'-amino-3'-deoxythymidine (AMT) (Cretton and Sommadossi, 1991) a compound which in its own right has toxic effects and which was first reported from a clinical study carried out by Stagg *et al*. (1992).

Although cellular nucleoside triphosphate levels which compete with the active triphospate moiety are reduced in monocytes/macrophages compared to T-cells and while AZT phosphorylation is substantially reduced in monocytes/macrophages compared to T-cells, the ratio of the active triphosphate moiety (ddNTP) to cellular nucleoside

triphosphates (dNTP) may be the crucial factor determining the activity of dideoxynucleosides against HIV-1 (Perno *et al*., 1988).

1.6.1.3 Clinical pharmacokinetics of AZT

Maternal-foetal transfer of AZT occurs rapidly through simple diffusion (Mandelbrot *et al*., 2001) with levels of AZT in the foetal circulation being reported to be proportional to maternal concentrations (Liebes *et al*., 1990).

In adults, the half-life of AZT averages 1.1 hours since AZT is rapidly glucuronidated with both the parent compound and the glucuronide being excreted in the urine (Klecker Jr *et al*., 1987). AZT is well absorbed in newborn full-term infants (greater than 90%). This bioavailability decreases over the first week of life as hepatic glucuronidation and first-pass metabolism increase (Mirochnick *et al*., 1998a). AZT clearance increases and the half-life decreases with postnatal age (Mirochnick *et al*., 1998a), with the half-life of AZT in full-term infants older than 14 days being 1.87 hours (Boucher *et al*., 1993; Connor *et al*., 1994).

The efficacy of AZT is dependent on the dose and the rate of elimination from the blood as well as on the rate and extent of phosphorylation (Barry *et al*., 1996).

1.6.1.4 Safety and adverse effects of AZT

The common side effects of AZT include nausea, malaise, myalgias, insomnia and headache (Richman *et al*., 1987). Treatment with AZT is limited by the toxic effects of the drug on bone marrow cells as manifested by dose-dependent anaemia and neutropenia (Max and Sherer, 2000). At clinically achievable concentrations (1-2 μM), the mechanism of toxicity appears to be a direct dose-dependent inhibitory effect on human granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) (Sommadossi and Carlisle, 1987). AMT formation has been reported to be 5 to 7-fold more toxic to human haematopoietic progenitor cells than AZT, thus suggesting a role for AMT in the cytotoxicity of AZT observed in patients (Cretton and Sommadossi, 1991). With the plasma elimination half-life of AMT being two to three times longer than that of AZT, (Sommadossi, 1993) it has been speculated that the toxicity of AMT to the host combined with its antagonistic effect on the antiretroviral activity of AZT *in vitro* (Stagg

et al., 1992) may affect the drug's pharmacodynamic properties as they relate to HIV-1 replication and its cytotoxicity to host cells (Sommadossi, 1993; Veal and Back, 1995).

In addition to inhibiting HIV-1 DNA replication, nucleoside analogues have varying affinities for mitochondrial DNA polymerase gamma, and can inhibit the synthesis of mitochondrial DNA (mtDNA) (Mofenson and Munderi, 2002), resulting in mtDNA depletion and consequently mitochondrial dysfunction (Barret *et al*., 2003). Clinical reports of toxicities that relate to mitochondrial dysfunction include involvement of the central and peripheral nervous systems, myopathies (including cardiomyopathy), bone marrow disorders (anaemia), pancreatic dysfunction and metabolic abnormalities including hyperlactataemia, hepatic steatosis and lactic acidosis (Mofenson and Munderi, 2002; Cherry and Wesselingh, 2003). It has been speculated that incorporation of AZT into DNA may result in damage and the initiation of mutagenic effects that could lead to carcinogenesis (Mofenson and Munderi, 2002).

1.6.2 NNRTIs

NNRTIs include derivatives such as tetrahydro-benzodiazepine (TIBO) and dipyridodiazepinone, however more than 30 structurally different classes of compounds have been identified as NNRTIs (de Clercq, 1998; Spence *et al*., 1995; Podzamczer and Fumero, 2001). Three NNRTIs (nevirapine, efavirenz and delarvirdine) have been licensed for clinical use and several others e.g. thiocarboxanilide derivatives and quinoxaline derivates amongst others are in preclinical or clinical development (de Clercq, 1998; Spence *et al*., 1995). NNRTIs are non-competitive inhibitors with respect to primer-template and deoxynucleoside triphosphates (dNTPs) (Podzamczer and Fumero, 2001). Thus, these inhibitors are not incorporated into the growing DNA chain but bind directly to the HIV-1 RT in a hydrophobic pocket near the polymerization active site, thus slowing the rate of DNA synthesis (Podzamczer and Fumero, 2001; Mofenson and Munderi, 2002).

1.6.2.1 NVP

NVP (also known as BI-RG-587) (11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido-[3,2-b:2',3'-][1,4]-diazepin-6-one), a derivative of dipyridodiazepinone has an empirical

formula of $C_{15}H_{14}N_4O$ and a molecular weight of 266.3 (Podzamczer and Fumero, 2001). Antiviral activity is independent of cellular state (Podzamczer and Fumero, 2001) and exhibits a 50% inhibitory concentration (IC_{50}) of 84 nM in RT enzyme assays and an IC_{50} of 40 nM against HIV-1 replication in cell culture (Grob *et al*., 1992). The inhibition is highly selective and does not involve human α, β, γ or δ DNA polymerases (Merluzzi *et al*., 1990; Podzamczer and Fumero, 2001; Grob *et al*., 1992). NVP inhibits HIV-1 but is unable to inhibit RT from other retroviruses, including HIV-2, simian immunodeficiency virus (SIV) and feline leukaemia virus (Merluzzi *et al*., 1990; Cohen *et al*., 1991; Grob *et* al., 1992; Podzamczer and Fumero, 2001). The inhibitory activity of NVP is not competitive with respect to the template, primer and dNTP indicating that NVP does not act directly at the catalytic site of RT (Cohen *et al*., 1991; Kohlstaedt *et al*., 1992; Grob *et al*., 1992). Since NVP decreases the rate of the enzymatic catalysis (k_{cat}) but does not affect the binding of the substrate (k_s) this has indicated that NVP binds to the binary (RT:template primer) and ternary (RT:template primer:dGTP) complexes of the enzyme (Grob *et al*., 1992).

1.6.2.2 NVP binding pocket

NVP interacts with HIV-1 RT in a hydrophobic region of the side chain containing residues Tyr^{181} and Tyr^{188} of the RT p66 subunit, located near the catalytic site and the expected DNA primer terminus (Podzamczer and Fumero, 2001; Grob *et al*., 1992; Kohlstaedt *et al*., 1992). The NVP binding pocket lies between the β-sheets of the 'palm' and at the base of the 'thumb' subdomains (Kohlstaedt *et al*., 1992) where the drug interacts with thirty-eight protein atoms (Smerdon *et al*., 1994).

Two mechanisms of RT inhibition by NVP have been proposed (Kohlstaedt *et al*., 1992). Firstly, NVP may interfere with the movement of the 'thumb' domain thereby suppressing translocation of the primer template following nucleotide incorporation, or secondly, NVP may alter the conformation of β-strands 6, 9 and 10 and thus the orientation of conserved carboxylate side chains involved in catalysis (Kohlstaedt *et al*., 1992). It has also been proposed that NVP binds near the β-turn containing Asp^{185} and Asp^{186} (a highly conserved region for polymerases that has been proposed to participate in catalytic activity), altering the position of the carboxyl ligands and affecting the rate of the Mg^{2+}-dependent chemical reaction (Podzamczer and Fumero, 2001).

1.6.2.3 Clinical pharmacokinetics of NVP

NVP is highly lipophilic and distributes widely and evenly throughout the body (Havlir *et al*., 1995). Absorption of greater than 90% has been reported following oral administration (Podzamczer and Fumero, 2001). The median time to peak plasma concentration (C_{max}) is 4 hours, with this concentration increasing as the NVP dose increases (Podzamczer and Fumero, 2001). A secondary C_{max} is observed between 3 and 8 hours (24 and 28 hours for individuals receiving higher doses i.e. 200 mg or 400 mg) suggesting enterohepatic cycling (Cheeseman *et al*., 1993). The mean plasma elimination half-life ($t_{½}$) of NVP is 30 hours (Podzamczer and Fumero, 2001). Approximately 62% of NVP in plasma is bound to proteins (Cheeseman *et al*., 1993). NVP has been reported to enter HIV-1 reservoir sites such as the cerebrospinal fluid and the seminal fluid (Podzamczer and Fumero, 2001; Mirochnick *et al*., 1998b; Luzuriaga *et al*., 1996).

Pharmacokinetic parameters of NVP in pregnant women show more variation than seen in studies conducted on non-pregnant adults (Mirochnick *et al*., 1998b). Data has suggested that the physiologic processes associated with labour impact on NVP absorption, distribution and elimination (Mirochnick *et al*., 2001). NVP readily crosses the placenta and has also been detected in breast milk, reaching a concentration equal to 76% of that in maternal serum (Podzamczer and Fumero, 2001). Following maternal dosing with 200 mg NVP at the onset of labour, and a single 2 mg/kg oral dose to infants 48 to 72 hours after birth, concentrations exceeding 100 ng/ml (mean cord blood concentrations of 3.7 μM i.e. 10 times the *in vitro* IC_{50}) have been achieved in newborns (Mirochnick *et al*., 1998b; Bardsley-Elliot and Perry 2000). Similar results have been reported where mothers were given a 200 mg NVP dose on the day of delivery and the neonatal NVP plasma concentrations at 24 hours (1.39 mg/L; 5.2 μM) were determined to be 81% of maternal concentrations (1.71 mg/L) and 107% of cord blood concentrations (1.29 mg/L or 4.8 μM) (Taylor *et al*., 2000). Clearance is more rapid in chronic dosing studies than predicted by single-dose studies and is more rapid in younger children than in adolescent children (Luzuriaga *et al*., 1996). In infants, median $t_{½}$ ranges from 45 to 72 hours for elimination of the maternal NVP and from 37 to 46 hours for the elimination of a single 2 mg/kg neonatal dose (Bardsley-Elliot and Perry, 2000). NVP elimination in both mothers and infants is prolonged (median $t_{½}$ ranging from 36.8 to 65.7 hours) compared with that observed following single-doses in older children (mean 30.6 hours)

(Mirochnick *et al*., 1998b). Importantly, this implies that plasma concentrations of NVP (even though subtherapeutic) are detectable in mothers for several days after delivery. In fact, plasma NVP concentrations around the IC_{50} persist in some women for as long as 4 weeks after administration of single intrapartum NVP doses (Cressey *et* al., 2005).

1.6.2.4 NVP metabolism

NVP appears to be predominantly metabolized by two cytochrome P450 (CYP) enzymes, CYP3A4 and CYP2B6 (Riska *et al*., 1999; Erickson *et al*., 1999). Thus the drug is mainly metabolized in the liver and excreted by the urine in the form of glucuronide conjugates of 2-, 3-, 8-, and 12-hydroxyNVP (Riska *et al*., 1999; Podzamczer and Fumero, 2001; Erickson *et al*., 1999). The developmental pattern of the cytochrome enzymes is not clearly understood (Mirochnick *et al*., 1998b). CYP3A4 activity is reported to be low in infants, increasing to adult levels by 6 to 12 months, exceeding adult levels during years 1 to 4 and declining again to adult levels by the end of puberty (Mirochnick *et al*., 1998b). The pattern of CYP3A7 activity has not been elucidated but is high *in utero* and low during postnatal life (Mirochnick *et al*., 1998b). A possible problem to using perinatal NVP monotherapy is that on initiation of NVP therapy the hepatic cytochrome P450 enzyme pathway is induced resulting in a change of the plasma concentration of NVP until a steady state is reached. Autoinduction of NVP clearance with mean $t_{½}$ dropping from 45 hours after an initial dose to 20 to 30 hours after multiple dosing has been observed in adults (Mirochnick *et al*., 2001). Multiple maternal prenatal NVP dosing has been associated with increased neonatal NVP elimination suggesting *in utero* hepatic enzyme induction resulting in NVP plasma concentrations in newborns not being sustained (McGowan and Shah, 2000; Taylor *et al*., 2000; Mirochnick *et al* 2001).

1.6.2.5 Safety and adverse effects of NVP

The adverse effect most commonly associated with the long-term use of NVP is rash (Podzamczer and Fumero, 2001) which occasionally develops into severe rash or Stevens-Johnson syndrome (Bardsley-Elliot and Perry, 2000). The rate of rash attributable to NVP is 16% and approximately 7% of patients experience severe rash (Grade 3 or 4). The risk of experiencing rash is greatest during the first six weeks of NVP treatment. Hepatotoxicity is also associated with NVP (Podzamczer and Fumero, 2001).

The risk of hepatotoxicity is increased with hepatitis C coinfection (Martínez *et al*., 2001; González de Requena *et al*., 2002). Hypersensitivity reactions to NVP are characterized by rash, constitutional symptoms such as fever, arthralgia, myalgia and lymphadenopathy, and visceral involvement such as hepatitis, eosinophilia, granulocytopenia and renal dysfunction (Boehringer Ingelheim, 2000).

The short-term safety and tolerance of single-dose NVP has been demonstrated in clinical trials (Musoke *et al*., 1999). No rash or serious adverse effects, attributable to NVP were reported in the initial phase I/II trials PACTG 250 and HIVNET 006 (WHO/HIV_AIDS/2001.3). No significant differences in serious toxicity, occurrence of rash, anaemia, liver abnormalities or death between NVP and short-course regimens of AZT or AZT-3TC were reported in women or infants in the HIVNET 012 and SAINT studies (Guay *et al*., 1999; Moodley *et al*., 2003). Several studies, however, have shown different results regarding the real incidence of these adverse effects, the time of onset and the associated risk factors in patients treated with NVP.

NVP exhibits extremely low cytotoxicity in uninfected cells (Bardsley-Elliot and Perry, 2000). No cytotoxic effects have been observed on human bone marrow colonies at NVP concentrations up to 37.5 mmol/L (Merluzzi *et al*., 1990).

1.7 Drug resistance

The selection of resistance mutations during antiretroviral therapy is associated with a reduction in drug susceptibility and viral fitness (Nijhuis *et al.,* 2001). Primary (major) mutations are commonly the 'signature' mutations that occur *in vivo* or *in vitro* and are associated with large decreases in drug susceptibility (Deeks, 2003) Primary mutations are generally selected early and tend to be specific for each compound (Ammaranond *et al*., 2003). Secondary (minor) mutations are commonly selected during long-term virological failure and may act to restore replicative capacity (Deeks, 2003). Secondary mutations by themselves have little or no discernible effect on resistance but may be selected for because they compensate for reduced viral fitness (Ammaranond *et al*., 2003).

1.7.1 AZT drug resistance

Although nucleoside analogs have been the drugs of choice in attenuating the action of the virus, toxicity limits their use and long-term inhibition of RT is limited by the high frequency of virus mutations resulting in drug-resistant forms. A number of factors contribute to the development of AZT resistance. Many of these mechanisms relate to the intracellular metabolism of AZT and include: mutation of the HIV-1 RT (Larder and Kemp, 1989), AZT exclusion from HIV-1 infected cells via a mechanism similar to that observed in multidrug resistance (Antonelli *et al*., 1992), a decrease in the expression of thymidine kinase via hypermethylation of the DNA resulting in decreased AZT-MP formation *in vivo* (Di Vito *et al*., 1997) and mutation of HIV-1 integrase which is inhibited by phosphorylated metabolites of AZT (Mazumber *et al*., 1994). AZT-resistant HIV-1 strains appear within 1 year of treatment in many individuals using AZT monotherapy as the standard of care (Larder *et al*., 1989b). In many cases, HIV-1 strains develop gradual and stepwise resistance to AZT due to inadequate viral suppression, (Nolan *et al*., 2002b) by accumulating mutations at various codons in the RT (M41L, D67N, K70R, L210W, T215Y/T215F, K219Q/K219E and G333E, (Boucher *et al*., 1992; Clavel and Hance 2004; Deeks, 2003; O'Brien, 2000) and once present these mutations appear to persist long term (Nolan *et al*., 2002b). Of the mutations selected by AZT T215Y/F and K70R are generally considered primary, whereas D67N, L210W, or K219Q/E are considered secondary.

There have been case reports from numerous studies documenting transmission of AZT drug-resistant HIV-1 from mothers to their infants. Among some United States nested case studies the prevalence of any AZT mutation reported ranged from 4.2% (Eastman *et al.,* 1998) to 24% (Welles *et al.,* 2000) and the prevalence of high-level AZT resistance (with a codon 215 mutation) in these studies ranged from 0% to 6.3%. Studies have shown that genotypic sequences from infant viral isolates are less diverse than their mother's and reflect a minor subset of maternal variants (Wolinsky *et al.,*1992; Ahmad *et al.,* 1995; Colgrove *et al.,* 1998). Among a cohort of HIV-1 infected newborns who had perinatal antiretroviral exposure and genotypic evidence of drug resistance to HIV-1, the mutations detected correlated with at least one antiretroviral from the perinatal period (Parker *et al.,* 2003). Since some studies have suggested that drug resistance mutations are associated with increased risk of perinatal transmission (Colgrove *et al.,* 1998; Welles

et al., 2000) and the presence of drug resistant HIV-1 in pediatric infection has been shown to have an impact on clinical progression (Eshleman *et al.,* 2001b) the presence among pregnant women with phenotypic or genotypic AZT resistance is an emerging concern.

1.7.2 NVP drug resistance

NVP is associated with the rapid emergence of HIV-1 drug resistant mutants as a single amino acid in the hydrophobic pocket can reduce binding of the drug and confer a high level of drug resistance (Smerdon *et al*., 1994). Thus, the NVP-binding pocket and the 38 protein interactions that occur between NVP and the hydrophobic pocket from the p66 'palm' and 'thumb' sub-domains play an important role in determining NVP drug resistance (Smerdon *et al*., 1994). Most non-nucleoside drug resistance mutations map to residues in close contact with NVP. These mutations act by directly changing the shape of the pocket thus introducing steric changes (perturbations of van der Waals interactions) between the protein and the inhibitor and indirectly on the structure of the pocket. Single genetic mutations of the HIV-1 RT enzyme at codon positions K103N, Y181C/Y181I, Y188C, G190A/G190S confer primary mutations while those at codons A98G, L100I, V106A, V108I, P225H, P236L confer secondary mutations (Deeks, 2003; Clavel and Hance, 2004; O'Brien, 2000). High level NNRTI cross-resistance after a single mutation is common (Deeks, 2003). Other mutations may be selected for during long-term failure thus potentially limiting efficacy of second generation NNRTIs (Deeks, 2003).

Rapid emergence of resistance to NVP, if administered as monotherapy, has been reported in both adults and children. Standard sequencing-based methods detect minority species only when present at frequencies >20% of the viral population resulting in the under-estimation of resistant variants (Palmer *et al.,* 2005). More sensitive methods involving a sensitive point mutation assay (Flys *et al.,* 2005), real-time PCR (Johnson *et al.,* 2005) and allele-specific PCR analysis (Palmer *et al.,* 2006) have found that while NVP-resistant mutation decrease over time, they do persist above pretherapy levels for many more months (4 to 9 months (Johnson *et al.,* 2005) and even up to > 1 year (Flys *et al.,*2005; Palmer *et al.,* 2006) after single-dose NVP. The most frequently detected mutations conferring high-level resistance are Y181C, the predominant mutation, and K103N (Nolan *et al*., 2002b). Results from the HIVNET 006 Ugandan trial revealed that

the K103N mutation predominated (89%) in maternal samples whilst the Y181C mutation predominated (91%) over the K103N mutation (18%) in infant samples (Musoke *et al*., 1999; Eshleman *et al*., 2001c). HIV-1 variants with the K103N mutation can persist at frequencies greater than those found before exposure to NVP in some women and infants ≥ 1 year after administration of single-dose NVP (Flys *et al.,* 2005). Furthermore, quantitative analyses have determined that after single-dose NVP the K103N-containing variants are detected more frequently and at higher levels in women with subtypes C and D than A (Flys *et al.,* 2006)

1.8 Pathogenesis of HIV-1 infection in infants and children

The pathogenesis of HIV-1 infection in children differs in several respects from that of adults (Table 1.2). HIV-1 infected children have higher viral loads and a more rapid progression to AIDS than adults (Melvin *et al*., 1999). Rapid increases in viral loads (10^5 to 10^7 RNA copies/ml plasma) have been documented in vertically infected infants during the first weeks of life (Palumbo *et al*., 1998). These high levels of HIV-1 replication are sustained for a prolonged period following perinatal infection (up to the first 2 years of life), gradually declining through to 5 to 6 years of age (Mofenson *et al*., 1997b; Shearer *et al*., 1997; Biggar *et al*., 2001; Mullen *et al*., 2002). Similar to human infants in neonatal macaques, the sustained viral loads and enhanced progression to disease are generally attributed to the immaturity of the immune system (Veazey *et al*., 2003). However a study has suggested that both cellular and humoral immune responses develop early in life in most children infected with HIV-1 (Borkowsky *et* al., 1992). HIV-1 exposure occurs during delivery and HIV-1 DNA has been detected in gastric and oropharyngeal aspirates indicative of ingestion of HIV-1 containing fluids during birth (Gaillard *et al*., 2000). The $CD4^+$ T-cells in the intestinal lamina propria of newborns mostly express an activated, memory phenotype suggesting that intestinal lymphocytes are primed *in utero* rendering these cells more susceptible to SIV or HIV-1 infection and viral replication (Veazey *et al*., 2003). During foetal and neonatal life the increased T-cell production is associated with a more active thymus compared with that of the adult (Haynes *et al*., 1988), however, the spleen and intestinal lamina propria appear to be major sites of early viral replication and amplification in the neonatal host (Veazey *et al*., 2003). Thus, evidence suggests increased production, turnover and/or survival of activated $CD4^+$ T-cells may partially explain the high viral loads and rapid disease progression in infected human

(Kourtis *et al*., 2000) or macaque infants (Veazey *et al*., 2003). A further explanation for the higher and more persistent viral loads in HIV-1 infected infants is the progressively expanding lymphoid mass and increased numbers of circulating $CD4^+$ T-cells promoting replication of HIV-1 (Krogstad *et al.*,1999).

Table 1.2 Natural history of HIV-1 infection in children

Distinguishing features of HIV-1 infection in children
Perinatal transmission = primary infection
Immature immune system thus high viral load (declines slowly over 1 to 2 years)
$CD4^+$ counts in children higher than adults thus assessment must be in relation to the appropriate count for age of child
Symptoms and presentation of HIV-1 disease varies[1]
Disease progression rate[2] higher than in adults • Rapid progressors (20%) – develop serious signs and symptoms and progress rapidly to AIDS/death during first 12 to 24 months • Intermediate progressors (60%) – variable disease patterns , evidence of severe immunosuppression by 7 to 8 years of age, gradual loss of $CD4^+$ cells • Slow progressors (long term non-progressors; 20%) – minimal to no signs of HIV-1; normal to minimal decrease in $CD4^+$ cells to 9 years of age
Median survival time in perinatally infected children is 8 to 9 years

[1] The Centre for Disease Control has developed a classification based on clinical symptoms and immunological status

[2] Rates of progression associated with a variety of risk factors: timing of transmission, prematurity, mode of delivery, genetics, viral load, maternal health, viral strain, poor immune response or lack of maternal antibodies.

1.8.1 Molecular and biological properties of HIV-1 transmitted from mother-to-child

Elucidation of the molecular and biological properties of viruses transmitted perinatally to infants might provide important information for the development of effective strategies for the prevention and treatment of HIV-1 infection. It has not yet been established whether cell-free or cell-associated virus is transmitted or at what time transmission occurs (Briant *et al*., 1995). Reports suggest that M-tropic R5 viruses are more commonly transmitted from mother-to-child even when the predominant virus found in the mother uses the CXCR4 receptor (Wolinsky *et al*., 1992; Scarlatti *et al*., 1993). HIV-1 isolates recovered from infected infants are reported to replicate rapidly, infect macrophages and preferentially use the CCR5 co-receptor (Arroyo *et al*., 2002). A number of studies have reported the selective transmission of either minor or major maternal variants (Wolinsky

et al., 1992; Scarlatti *et al*., 1993d; Ahmad *et al*., 1995; Dickover *et al*., 2001; Matala *et al*., 2001). The transmission of multiple HIV-1 genotypes from mother-to-child has however also been reported (Briant *et al*., 1995; Dickover *et al*., 2001; Pasquier *et al*., 1998; Wade *et al*., 1998). Data suggests that maternal HIV-1 genotypes or subtypes that have rapid replication kinetics (Scarlatti *et al*., 1993c; Arroyo *et al*., 2002) are selected and transmitted to the infant, initially as an homogeneous population (Scarlatti *et al*., 1993d; Ahmad *et al*., 1995; Arroyo *et al*., 2002; Matala *et al*., 2001) then becoming diverse as the infant grows (Ahmad *et al*., 1995; Casper *et al*., 2002). Viral heterogeneity may play a role in vertical transmission since HIV-1 sequences found in mothers who failed to transmit HIV-1 to their infants (non-transmitting mothers) in the absence of ART have been shown to be less heterogeneous than those from transmitting mothers. Furthermore, a high conservation of intact and functional *gap* p17, *vif*, *vpr*, *vpu*, *tat*, and *nef* open reading frames has been demonstrated following MTCT of HIV-1 (Ahmad 2005). Also, the accessory genes *vif* and *vpr* have been found to be less functionally conserved in isolates from non-transmitting than transmitting mothers and their infants (Ahmad 2005). Vertically transmitted variants are reportedly also more resistant to neutralization by maternal plasma and have fewer glycosylation sites in the envelope (Wu *et al.*, 2006).

A study has shown that HIV-1 *env* sequences are detected in the placentas derived from HIV-1 seropositive mothers but that a negative selection of maternal HIV-1 quasispecies occurs within the placental villi (Menu *et al*., 1999). Whilst evidence has also suggested that mothers infected with subtype B viruses are more likely to transmit virus to their infants than mothers with subtype A virus (Yang *et al*., 2003), research is suggesting that subtype C viruses may display characteristics that make them distinct from subtype B and other subtypes, and that such differences may affect transmission and pathogenesis. Diagnosis of HIV-1 infection in newborns (within 48 hours) has been interpreted to reflect early *in utero* infection whilst lack of infection at delivery yet a positive outcome (from day 7 to day 90) to reflect late *in utero* infection (intrapartum) provided the infant has not been breast-fed (Bryson *et al*., 1992). Early *in utero* transmission has been suggested to account for a small proportion of MTCT of HIV-1, with transmission occurring mostly late in pregnancy or at delivery (Brossard *et al*., 1995). Whilst some researchers suggest that the first-born twin of a vaginal delivery is at a significantly higher risk of being infected than the second born (Goedert *et al*., 1991), suggesting that

transmission is more likely to occur during delivery, other researchers have not found birth order to be an important risk factor for infection of twins and suggest that birth-canal exposure is not a major contributor to perinatal HIV-1 transmission (Biggar *et al*., 2003). There is no consistent correlation between the maternal virus genotype found at different periods during pregnancy and infant virus genotypes that would indicate timing of transmission (Contag *et al*., 1997). In fact, studies have indicated that mucosal membranes, including the cervix, harbour HIV-1 envelope variants distinct from those found in PBMC and plasma (Panther *et al*., 2000; Poss *et al*., 1998). Depending on timing of transmission different selective pressures may be involved in determining the pattern of maternal HIV-1 variant transmission (Dickover *et al*., 2001).

1.8.2 Cellular responses in perinatally HIV-1 infected or exposed uninfected infants

HIV-1 specific T-helper and cytotoxic T-cells (CTL) have been detected in HIV-1 uninfected newborns of HIV-1 seropositive mothers (Borkowsky *et al*., 1990; Clerici *et al*., 1993a; Rowland-Jones *et al*., 1993; De Maria *et al*., 1994; Kuhn *et al*., 2002) indicating that the neonatal immune system is capable of responding to intrauterine exposure to HIV-1 or its soluble products. Low dose exposure to HIV-1 has been suggested to selectively activate the cellular arm of the immune system resulting in the presence of strong HIV-1 specific T-cell immunity (Shearer and Clerici, 1996). A decrease in this T-cell function is reported to occur in subsequent months following termination of the exposure to HIV-1 from the mother (Shearer and Clerici, 1996). The detection of HIV-1 specific responses in HIV-1 exposed but uninfected infants has been associated with protection from infection (Clerici *et al*., 1993a; Wasik *et* al., 1999). T-helper cell responses have also been reported to protect against intrapartum and breast-feeding transmission (Kuhn *et al*., 2001a).

1.8.3 Cytokine profiles in vertically HIV-1 infected children

Compared to vertically exposed but uninfected age-matched children, HIV-1 infected children born to HIV-1 seropositive mothers have shown a significant defect in Th1 cytokine production, without a concomitant increase in Th2 cytokines (Resino *et al*., 2001). A deficiency in mitogen stimulated type-1 cytokine production and excess type-2 cytokine transcription have been correlated with disease progression in vertically HIV-1

infected children (Lee *et al*., 1996). Interestingly, the stronger relative production of type-1 cytokines e.g IFN-γ to type-2 cytokines such as IL-10 in cord blood cells from infants of HIV-1 infected mothers may be associated with protection against perinatal HIV-1 infection (Kuhn *et al*., 2001b). The presence of HIV-1 specific T-helper responses at birth in uninfected newborns of HIV-1 positive mothers, but the absence of these responses in HIV-1 infected infants, has suggested that the development of HIV-1 specific T-helper responses associated with the β-chemokines CCL3, CCL4 and CCL5 play a protective role in vertical transmission of HIV-1 (Wasik *et al*., 1999).

1.8.4 Host genetic factors and vertical transmission

Much attention has focused on identifying genetic determinants of susceptibility and resistance to HIV-1 and AIDS. The HLA system plays a central role in the recognition and presentation of antigens to the immune system and represents the most polymorphic gene cluster in the human genome (Prugnolle *et al*., 2005). Extensive HLA class I and class II allelic variation occurs within and between different ethnic groups (Stephens, 2005). Polymorphism, which generates structural changes in a series of pockets in HLA class I extracellular protein domains, which accommodate certain amino acid residues of antigenic peptides, affects the biological function of class I molecules to bind and present microbial peptides to the antigen-specific receptors of $CD8^+$ CTL (Stephens, 2005). The inheritance therefore, of certain class I alleles has been associated with significantly more or less effective immunological control of diseases such as HIV-1 (Tang and Kaslow, 2003).

The disease-modifying effect of the 32 base pair deletion, within the coding region of the CCR5 receptor gene (CCR5Δ32), represents the prototypic association between a host genetic factor and altered HIV-1 disease progression (Nolan *et al*., 2004). Chemokine receptor polymorphism studies in infants have suggested that (i) CCR5Δ32 heterozygosity does not protect against perinatal infection, however it is unclear whether CCR5Δ32 affects transmission through breastfeeding (Contopoulos-Ioannidis *et al*., 2003), (ii) a maternal heterozygous stromal cell-derived factor (previously SDF-1: CXCL12) genotype (CXCL12 3'A/wt) is associated with perinatal HIV-1 transmission (John *et al*., 2000), (iii) the protective effect of the CCR5 wt/Δ32 genotype appears to be abrogated in children by the CXCL12-3'A genotype (Sei *et al*., 2001), (iv) children

(African and non-African) homozygous or heterozygous for CCR2B-64I (a variant of the chemokine receptor CCR2B reported to function as minor HIV-1 entry coreceptors (Zhang and Moore, 1999)), are not protected against HIV-1 infection through vertical transmission, nor does the CCR2B-64I allele have a beneficial effect on disease progression (Teglas *et al*., 1999).

The overall impact of chemokine receptor polymorphisms and chemokine receptor ligand polymorphisms may be considered in a manner similar to the MHC system (Nolan *et al*., 2004) since functional polymorphisms have been identified in chemokine receptor genes and chemokine receptor ligands such as macrophage inflammatory protein-1α: MIP-1α (CCL3), macrophage inflammatory protein-1β: MIP-1β (CCL4) and regulated upon activation, normal T-cell expressed and secreted: RANTES (CCL5), which affect chemokine receptor function (Mummidi *et al*., 1998; Gonzalez *et al*., 2001). A very recent study has demonstrated that genetic variation, specifically CCL3L1 copy number less than the population specific average is strongly associated with HIV/AIDS susceptibility (Gonzalez *et al*., 2005). Furthermore, CCL3L1 copy number may be a further factor that underlies the genetic basis of susceptibility to HIV-1 in infants born to HIV-1 seropositive mothers (Meddows-Taylor *et al*., 2006).

1.8.5 Disease progression among infected infants despite antiretroviral prophylaxis

Despite AZT, AZT-3TC and NVP prophylaxis given antepartum, intrapartum or postpartum to the mother and/or infant, many infants become infected. The data on disease progression among infants infected despite AZT-3TC is limited. Although some studies have suggested that the disease progression amongst infants infected with HIV-1 despite antiretroviral prophylaxis, compared with antiretroviral-unexposed infants, is more rapid (Sutthent *et al*., 2002), the evidence for this is not conclusive. The AZT placebo-controlled clinical trials conducted in the United States (PACTG 185), France (PACTG 076), Thailand (Bangkok Trial) and in two West African countries (Côte d'Ivoir Trial and DITRAME ANRS 049 Trial) have not indicated that the disease progression in HIV-1 infected children exposed to AZT was higher than a placebo control group of children (Mofenson and Munderi, 2002). Other observational perinatal cohort studies however have suggested that infants who become infected despite AZT prophylaxis may have a more rapid course of the HIV-1 disease and higher mortality compared with

infants who become infected without AZT exposure (Italian Registry for HIV Infection in Children, 1999).

The development of antitretroviral resistance or the adverse effect on disease course are long-term concerns for the child that becomes infected despite prophylaxis (Mofenson and Munderi, 2002). The clinical studies to date indicate that genotypic antiretroviral mutations will occur more rapidly following NVP and 3TC prophylaxis where single-point mutations can confer high-level resistance, in contrast to AZT prophylaxis where sequential multiple genetic mutations will confer resistance.

The theoretical reasons given for higher disease progression among infants infected with HIV-1 despite ART prophylaxis include selection for *in utero* transmission, more severe disease, more virulent viral phenotypes or reduced response to ART due to early induction or transmission of resistant virus (Nolan *et al*., 2002b; Mofenson and Munderi, 2002).

It is anticipated that in the absence of ongoing drug pressure single-point NVP or 3TC mutations will in asymptomatic HIV-1 infected women be outgrown by wild-type (dominant) virus. This has implications for postpartum transmission particularly where breastfeeding occurs. Also, the efficacy of antiretroviral prophylaxis for subsequent pregnancies comes into question (Nolan *et al*., 2002b). This is particularly relevant in the case of NVP where significant concentrations (around the IC_{50}) may persist in some women for as long as 4 weeks after administration of single intrapartum NVP doses (Cressey *et al.,* 2005) since single-dose NVP can select for resistant viruses within 1 week (Richman *et al.,* 1994), and persistence (up to 1 year) of NVP resistant variants has been reported (Palmer *et al.,* 2006).

The research questions regarding HIV-1 perinatal transmission and ART resistance differ between resource-rich and resource-limited settings, with concerns for the former setting relating to the potential negative consequences of treatment-related ART drug resistance on the rates of MTCT of HIV-1 while in the latter setting the concerns have focused on whether the widespread use of NVP or AZT-3TC will lead to increased treatment failure among women and those infants that become infected despite prophylaxis (Fowler *et al*., 2003).

1.9 Immunology of the newborn – immune competence in the neonate

Cellular immunity in the neonate has generally been considered 'incompetent' especially with respect to characteristics such as phenotype (Lewis 1998; Rich *et al.,* 1997), signal transduction pathways (Matsuzaki *et al*., 1989) and antigen presentation (Lewis 1998) however, other features such as the ability to respond to T-cell receptor (TCR)-mediated stimulation and to produce both type-1 and type-2 cytokines (Chipeta *et al*., 2000) suggests that the neonatal immune system may be more competent (Chipeta *et al*., 2000; Hermann *et al*., 2002) than previously appreciated, signifying infants are antigenically naïve. The ability of the neonate to respond to antigenic stimulation is dependent on the necessary immunocompetent cells and signalling mechanisms being acquired during foetal development (Table 1.3).

Table 1.3 Ontogeny of foetal and neonatal immunity (Billington, 1992)

Gestational age (weeks)	Developmental event
5	Yolk sac cells (pluripotent)
6	Thymus rudiment; early macrophages: HLA on foetal cells
7	Lymphopoiesis in liver
8	Pre-B cells
9	T-cell progeniors; NK cells; lysozyme
10	Further T-cell surface markers
11	C3 and C4 synthesis; dendritic cells
12	B cells in bone marrow; transmission of maternal IgG
13	Macrophages; reactive T-cells
14	Lymphocytes in peripheral blood
15	IgM and IgG synthesis; TCR expression on thymocytes
18	All complement components, except C9
19	$CD3^+$, $CD4^+$ and $CD8^+$ subpopulations increase
21	Increase in dendritic cell population; cytotoxic T-cells
22	Increased rate of maternal IgG transfer
Birth	All immune cells, lymphokines, complement components and non-specific factors present (immune system normally unprimed)
Breastfeeding	IgA and lysozyme (and immune cells) taken up from breast milk for protection of neonatal gut (and respiratory tract)

The extent to which the HIV-1 status of mothers influences immune responsiveness of their newborns is incompletely understood, with the result that changes in the ontogeny of the immune system of infants exposed/infected with HIV-1 remains poorly described. Due to the increased numbers of paediatric HIV-1 infections, primarily as a result of

transplacental or perinatal MTCT, it has become increasingly important to determine developmental time frames of the neonatal immune system that could permit one to distinguish between lack of immune function associated with developmental factors versus HIV-1 induced defects (Clerici *et al*., 1993b).

1.9.1 Thymic development and early T-cell or thymocyte development

The thymus is the primary site of T-cell maturation and development. The thymus begins to form around the 6^{th} to 7^{th} week of embryonic life. By 8 weeks of gestational age lymphoid cells with the prothymocyte phenotype migrate from the yolk sac and foetal liver and later from the bone marrow to colonize the foetal thymus. Prothymocytes interact with the stroma of the thymus and are triggered to undergo intense proliferation, differentiation and expression of the first T-cell-specific surface markers (CD2). Prothymocytes (do not express CD3, the T-cell receptor, CD4 or CD8), migrate into the subcapsular region where they undergo vigorous proliferation and give rise to progeny that continue to divide and rearrange their TCR genes (Lewis, 1998). These progeny express CD4 and CD8, migrate to the deeper layers of the thymus cortex where positive selection for self-major MHC restriction occurs, a process governed mainly by thymic cortical epithelial cells. More than 95% of thymocytes die with approximately 5% undergoing further differentiation. Negative selection which follows is mediated by the bone-marrow derived dendritic cells and macrophages and eliminates autoreactive cells either by clonal deletion or clonal anergy. Thymocytes that arrive in the medulla are considered mature and express either CD4 or CD8. These single-positive T-cells migrate from the thymus to the peripheral lymphoid organs. Mature $CD4^+$ and $CD8^+$ T-cells are first detected in the foetal liver and spleen at 14 weeks gestation (Arkachaisri and Ballow, 1999).

During foetal life the thymus is the largest lymphoid organ relative to body size. At birth the thymus is two thirds of its mature weight reaching its peak mass around 10 years of age. The thymus then begins to involute being replaced by adipose tissue (Arkachaisri and Ballow, 1999).

1.9.1.1 Generation of T-cell receptor diversity

The TCR is assembled from germ-line encoded gene segments and a complex sequence of DNA recombinational events generates the diversity in the αβ and γδ TCR and the immunoglobulin receptor (Arkachaisri and Ballow, 1999). DNA products formed during TCR rearrangement that specifically correlate with *de novo* T-cells can be measured using the technique of T-cell receptor excision circles (TRECs) (Douek *et al*., 1998). TRECS are generated during variable (V), diversity (D) and junctional (J) (V(D)J) gene recombination, a process responsible for the diversity of the αβ and γδ TCR repertoire. During this process, V(D)J gene segments are rearranged in order to generate a functional β and δ TCR chain (Al-Harthi *et al*., 2000a). Human thymocytes express a complete set of TCR by 15 weeks gestation (George and Schroeder, 1992) which is restricted due to diminished terminal deoxynucleotidyl transferase (TdT) levels resulting in shorter CDR3 regions (Schelonka *et al*., 1998a). By the third trimester the T-cell Vβ repertoire is similar to that seen in adults (Schelonka *et al*., 1998a). There is no evidence that the genetic composition of neonatal TCRs is responsible for diminished T-cell function and relative immunodeficiency (Schelonka and Infante, 1998b).

1.9.1.2 Foetal thymic precursors

Evidence suggests that differences exist between foetal and adult thymopoiesis (Adkins, 2003). This includes γδTCR-bearing progeny that arise from foetal thymic precursors that are not produced at detectable levels in the adult (Ito *et al*., 1989) and the relatively large percentage of T-lineage cells that undergo D-J recombination in Ig gene rearrangement in the adult but not the foetal thymus (Born *et al*., 1988). Indirect evidence for the differences has come from studies showing that the responses of neonatal T-cells are biased to Th2 function (Garcia *et al*., 2000). It has been argued that the Th2 bias is caused by an intrinsic inability of T-cells to interact and regulate the functions of antigen presenting cells (APC) (Bona and Bot, 1997). Whilst evidence indicates that the differentiation processes in the foetal and adult thymus are dissimilar, it is not clear whether these developmental differences influence the properties of mature cells that exit the thymus (Adkins, 1991).

Phenotypically and functionally, peripheral $CD4^+$ lymphocytes derived from foetal murine thymic precursors have been shown to differ from murine adult-derived cells, and whilst the peripheral $CD4^+$ cells derived from murine foetal thymic precursors have a high potential for Th2 function (a property retained up to 7 days post birth in intact neonates and adoptively transferred adult hosts), they similarly have a high potential for Th1 function which due to peripheral influences is down-modulated leading to reduced antigen-specific Th1 activity (Adkins, 2003). Differences in intracellular molecular processes, such as the signalling cascades leading to cytokine production following TCR activation by antigen, may account for the differential responses of murine foetal- and adult-derived cells (Adkins, 2003).

1.9.2 Immunophenotypic features of cord blood lymphocytes

Differences in the relative T-cell values and immunophenotypic features between cord blood and adult peripheral blood T-cells are reported. Although the differences may be due to a disparity in the methods used they include:

(i) Greater absolute lymphocyte counts in cord blood than in adult peripheral blood (D'Arena *et al*., 1998).

(ii) Predominant $CD45RA^+$ or naïve phenotype in the foetus and neonate (80-90% of the peripheral $CD4^+$ T-cells) compared with adult cells (40-60%) (D'Arena *et al*., 1998).

(iii) Low level of expression (less than 10% of the T-cells at birth) of the CDw29 surface marker, (a marker found after stimulation on memory T-cells), increasing over the first year of life (Arkachaisri and Ballow, 1999).

(iv) Differences in the percentage of $CD4^+$ and $CD8^+$ cells and the ratio of $CD4^+/CD8^+$ of cord blood leukocytes (CBL) and adult peripheral blood leukocytes (PBL). While some researchers report the percentage cell number and the ratio to be similar (D'Arena *et al*., 1998), others have found the percentage and absolute numbers of $CD4^+$ T-cells to be higher in neonates than in adults whilst those of $CD8^+$ T-cells are lower in neonates than adults, increasing through the first 5 years with the ratio of $CD4^+/CD8^+$ high during the perinatal period (4.9:1) reaching adult values (2:1) at 4 years (Arkachaisri and Ballow, 1999).

(v) CD38, a transmembrane glycoprotein and a marker of activation and immaturity, has been reported to be present on virtually all cord T-cells compared to approximately half of adult T-cells (D'Arena *et al*., 1998).

(vi) The level of expression of class II molecules and the IL-2 receptor are lower on cord blood than adult monocytes (Racadot *et* al., 1993; Paloczi 1999) and the expression of adhesion molecules are either similar to (CD11a, CD54, CD2), weaker (CD58) or increased (L-selectin) (Racadot *et al*., 1993).

(vii) The levels of expression of the α/β-TCR, CD3, CD4, CD5, CD8 and CD28 proteins on foetal and neonatal α/β T-cells are reportedly similar to adult cells (Lewis, 1998).

(viii) Low expression levels of the ligand CD40 (CD40L) in neonatal T-cells (less than 30% on neonatal $CD45RA^+$ T-cells) compared to levels found in adults (more than 80% of adult $CD45RA^+$ T-cells) (Nonoyama *et al*., 1995). CD40L is an early T-cell activation antigen that plays an important role in determining T-cell dependent immune responses, with reduced production contributing to decreased antigen-specific immunity mediated by Th1 effector cells and B-cells in the neonate (Lewis, 1998).

1.9.2.1 Proliferative responses

The first PHA responses by thymocytes are observed at 10 weeks gestation and 3 to 4 weeks later in the spleen and peripheral blood, and for concanavalin-A (con-A) at 13 to 14 weeks gestation in the thymus and at 14 to 18 weeks gestation in the foetal spleen (Arkachaisri and Ballow, 1999). IL-2 receptor expression, IL-2 synthesis and proliferation is less in cord blood $CD4^+$ naïve T-cells than in adult cells (Gerli *et al*., 1989). Studies however suggest that in response to antigens, superantigens or alloantigens, neonatal T-cells proliferate and produce IL-2 similarly to adult T-cells (Lewis, 1998), whilst T-cell responses (proliferation, IL-2 mRNA and IL-2 production) to mitogenic antibodies such as anti-CD3 or anti-CD2 are significantly lower than adult T-cell responses (Hassan and Reen, 1997; Schelonka and Infante, 1998b). While other researchers report that mitogenic responses of cord blood T-cells from healthy, term neonates are comparable to those of adults, proliferative responses to stimuli such as tetanus toxoid have been found to be absent until 13 to 26 months of age and to influenza A virus until 36 months while PBL from children older than 36 months demonstrate responses similar to those observed in adults, suggesting that responses to tetanus toxoid may reflect the fact that infants have been vaccinated with tetanus toxoid while the development of memory T-cells specific for influenza A virus would depend on natural

exposure and would be expected to lag behind the potential to respond to tetanus toxoid (Clerici *et al*., 1993b). Other studies report the magnitude of the proliferative response of cord blood T-cells to alloantigen to be greater than or equal to that of adult peripheral blood (Risdon *et al*., 1994).

In primary mixed leukocyte reactions (MLRs) cord blood mononuclear cells (CBMC) and T-cells show a normal response to activation by alloantigens that is indicative of normal TCR activation and proliferative capacities (Roncarolo *et al*., 1994). Normal levels of accessory molecules (contribute to T-cell response amplification) such as CD28 antigen, LFA-1 and CD40L have also been reported in these cells (Roncarolo *et al*., 1994). MLR responses of CBL have however been reported to be reduced compared to those of adult peripheral blood until 12 months of age (Arkachaisri and Ballow, 1999).

1.9.2.2 Stimulation induced cytokine profiles of cord blood

Although reports differ with respect to cord blood cytokine profiles there is some consensus amongst researchers that dependent on the stimuli used for activation neonatal cytokine data can differ (Perez-Cruz *et al*., 2000; Roncarolo *et al*., 1994). While polyclonal activation using mitogens or antibodies to cell surface receptors expressed on T-cells is commonly used in studies to assess the immunological capability of the neonate, this approach, whilst being rapid and providing information of immune potential differs with respect to being nonphysiological (Trivedi *et al*., 1997; D'Arena *et al*., 1999). Neonatal cytokine data should possibly be interpreted with care since the methods chosen for activation can result in different responses. Superantigen-mediated activation elicits T-cell activation through MHC class II binding, T-cell responses to recall antigens are proliferative responses and responses to alloantigens do not require priming but do require HLA-mediated antigen recognition (Trivedi *et al*., 1997). Polyclonal and antigen-mediated activation of cells can elicit distinct intracellular signalling pathways and consequently different patterns of cytokine responses (Cantrell, 1996). Nonetheless, cytokine profiles from cord blood T-lymphocytes are considered distinct from those of adult peripheral blood (Paloczi, 1999; D'Arena *et al*., 1999). Table 1.4 indicates the ability of cord blood T-lymphocytes to produce numerous cytokines/chemokines in response to various stimuli.

Table 1.4 Ability of cord blood T-lymphocyes to produce cytokines/chemokines using different stimuli for activation

Cytokine/ Chemokine	Stimulation	Cell source	Comments	Reference(s)
Cytokines that regulate and mediate specific immunity				
IL-2	Mitogen/ alloantigen	Th0, Th1, some CTL	Decreased potential to produce	Paloczi, 1999; D'Arena *et al*., 1999
IL-4	Mitogen/ alloantigen Polyclonal	Th2	Decreased potential to produce Levels comparable to[1] / decreased[2] compared to healthy adults	D'Arena *et al*., 1999 [1]Chipeta *et al*., 2000, [2]Vigano *et al*., 1999
IL-5	Polyclonal	Th2	Decreased potential to produce	Vigano *et al*., 1999
IL-10	Polyclonal	Th2, macrophages	Levels comparable to[1] / decreased[2] compared to healthy adults	[1]Chipeta *et al*., 2000; [2]Vigano *et al*., 1999
IFN-γ	Mitogen/ alloantigen Polyclonal	Th1, CTL	Decreased potential to produce Levels comparable to[1] / decreased[2] compared to healthy adults	Paloczi, 1999; D'Arena *et al*., 1999 [1]Chipeta *et al*., 2000; [2]Vigano *et al*., 1999
TGF-β	Polyclonal	T-cell, macrophages, other	Decreased potential to produce	Arkachaisri and Ballow, 1999
IL-12	Polyclonal		Levels comparable to healthy adults	Chipeta *et al*., 2000
Pro-inflammatory cytokines				
IL-6	Polyclonal	T-cell, endothelial cell, macrophages	Decreased potential to produce	Vigano *et al.,* 1999
TNF-α	Polyclonal	Th1, some Th2, some CTL	Levels comparable to[1] / decreased[3] compared to healthy adults	[1]Chipeta *et al*., 2000; [3]Arkachaisri and Ballow, 1999
Haematopoietic growth factors				
IL-3	Polyclonal	Th1, Th2 some CTL	Decreased potential to produce	Vigano *et al*., 1999
GM-CSF	Polyclonal -	Th1, some Th2, some CTL	Decreased potential to produce Levels measurable in cord blood of term neonates but undetectable in adults	Arkachaisri and Ballow, 1999 Laver *et al*., 1990
Chemokines				
CCL3	Polyclonal Phorbol 12-myristate 13-acetate	T-cell, monocytes, mast cells, fibroblasts	Decreased potential to produce Potential to produce similar Decreased (14-19%)[4] or higher[5] cord blood levels compared to adult levels	Arkachaisri and Ballow, 1999 Hariharan *et al*., 2000 [4]Chang *et al*., 1994; [5]Sullivan *et al*., 2002
CCL4	Phorbol 12-myristate 13-acetate	Monocytes, macrophages, neutrophils, endothelium	Potential to produce similar	Hariharan D 2000
CCL5	Phorbol 12-myristate 13-acetate -	T-cell, endothelium, platelets	Decreased potential to produce* Preterm/term neonatal plasma/serum levels similar to adult levels	Hariharan *et al*., 2000 Sullivan *et al*., 2002

* Reduced levels of CCL5 in cord blood have been attributed to the deficiency of $CD8^+/CD45RO^+$ cells, with $CD45RO^+$ cells predominantly responsible for CCL5 production in cord blood (Hariharan *et al*., 2000).

Responses in cord blood $CD4^+$ and $CD8^+$ T-cells activated with TCR stimulation have been reported to be comparable with those of healthy adults (Chipeta *et al*., 2000). These authors suggest that apart from being naïve, the neonatal cellular immune system is mature enough to mount competent immunological responses under appropriate antigenic stimulation. The antigen dosage (Sarzotti *et al*., 1996) and type of APC (Ridge *et al*., 1996) involved were found to be critical for an effective and appropriate immune response in neonates. It has been suggested that the differences in cytokine production between cord blood and peripheral blood may be due to an intrinsic deficiency in the capacity to produce these cytokines (Lewis, 1998). Many of these deficiencies may be attributed to reduced transcription of cytokine genes due to reduced mRNA expression, as is the case for IL-4 and IFN-γ, (Lewis, 1998) or due to post-transcriptional mRNA instability (as observed with IL-3, GM-CSF (Cairo *et al*., 1991; Lee *et al*., 1993; Buzby *et al*., 1996), macrophage-colony stimulating factor (M-CSF) (Suen *et al*., 1994), IL-12 (Lee *et al*., 1996) and IL-15 (Qian *et al*., 1997)) in activated CBMC (Suen *et al*., 1998).

1.9.2.3 Cord blood lymphocyte chemokine receptor expression

While some researchers suggest that cord blood lymphocytes express measurable but lower levels of most cytokine receptors compared to adult cells, with the selective expression of cytokine receptor levels by some neonatal cell types suggesting that the regulation of receptor expression is complex and possibly regulated by activation (Zola *et al*., 1995), other researchers have demonstrated that peripheral blood T-cells and cord blood T-cells exhibit distinct chemokine responsiveness via a different chemokine receptor repertoire (Sato *et al*., 2001). Age-related changes in CXCR4 and CCR5 expression have been reported in infants that are born to HIV-1 seropositive mothers, with CXCR4 expression being higher in cord blood than in adult blood and CCR5 expression lower but gradually increasing with age (Shalekoff *et al*., 2004).

1.9.3 Cord blood dendritic cells

Lymphoid dendritic cells (DCs), although capable of stimulating all T-cells are uniquely required for the stimulation of cord blood T-lymphocytes that bear the isoform CD45RA (Inaba and Steinman, 1984). Studies suggest that cord blood DCs are functionally immature (Hunt *et al*., 1994). Reduced expression of intercellular adhesion molecules-1

(ICAM-1; CD54) and MHC class I and class II antigens on cord blood DCs has been suggested to account for the relative inefficiency of cord blood DCs to activate T-cells (Hunt *et al*., 1994; Trivedi *et al*., 1997; Petty and Hunt, 1998). Cytokine-mediated events involving GM-CSF, IL-4 and TNF-α can result in the differentiation of $CD14^+$ monocytes into functionally mature $CD83^+$ DCs (Zhou and Tedder, 1996). Thus, the inefficiency of cord blood DCs to stimulate T-cells may be a consequence of the failure of cells producing these cytokines or relate to a maturational inability of DCs to respond to the influence of these cytokines (Petty and Hunt, 1998).

1.9.4 Neonatal antigen-presenting cells

Studies suggest that the APC function in neonates is immature (Trivedi *et al*., 1997). A reduced number and/or function of neonatal APC may be related to the limited ability of neonates to mount an immune response and to their high susceptibility to tolerance (Bona and Bot, 1997). The antigen-presenting functions of CBL and PBL from healthy children have been reported to be age-dependent, with the antigen-presenting function of CBL and PBL from children 13 months or older being found to be capable of presenting alloantigen whilst PBL from children younger than 13 months are not (Clerici *et al* 1993b).

1.9.5 Mature antigen-specific T-cells

Neonates immunized with various antigens can mount immune responses, indicating that mature T-cells exist at the earliest stages of postnatal development suggesting quantitative differences, such as the total number of naïve antigen specific T-cells, could account for the difference in immune responsiveness of neonates and adults (Bona and Bot, 1997).

1.10 Interaction of HIV-1 with the immune system

A dynamic interaction between HIV-1 and the immune system exists during infection (Ibegbu *et al*., 1994). During clinical latency, HIV-1 replicates inducing destruction of $CD4^+$ T-cells and immature cells in the bone marrow, thymus and lymph nodes where T-cells are produced (Kitchen *et al*., 1997). In response, the immune system induces the proliferation of T-cells with the result that during this stage of the infection the $CD4^+$ T-cell number remains relatively constant. With the development of AIDS, HIV-1 infection

destroys T-cells in the periphery by direct infection and killing of the cells and through hyperactivation of the immune system suggesting a homeostatic dysregulation with gradual wasting of T-cells resulting in T-cell depletion (Hazenberg *et al*., 2000; Hazenberg *et al*., 2000b). The loss of T-helper cell functions which is observed in HIV-1 infected individuals long before a decline in the number of circulating $CD4^+$ T-cells indicates that factors other than $CD4^+$ T-cell depletion contribute to T-cell dysfunction (Clerici *et al*., 1989). Major T-cell defects following HIV-1 infection include a higher rate of apoptosis following interaction with APC and a decreased secretion of type-1 cytokines (Bailer *et al*., 1999). The immunopathology of HIV-1 infection includes immune defects in T-cell cytokine secretion, resulting in decreased antigen-specific responses (Bailer *et al*., 1999). Progress in the field of HIV-1 and SIV pathogenesis has revealed that mucosal tissues, primarily the gut-associated lymphoid tissue, which harbours the majority of T-lymphocytes in the body (Mowat and Viney, 1997), is an important target for HIV-1, a major site of viral replication and $CD4^+$ T-cell depletion (Schneider *et al*., 1995) and a frequent site of AIDS-related opportunistic infections and neoplasms (Chui and Owen, 1994). This tissue may therefore be a major viral reservoir even in patients receiving HAART. A study using SIV-infected rhesus macaques has suggested that although most infected monkeys are rapidly depleted of mucosal $CD4^+$ T-cells in the acute phase, those that could partially reconstitute mucosal $CD4^+$ T-cells progressed more slowly to AIDS than those unable to mount a reconstitutive response (Picher *et al*., 2004). Brenchley *et al*., (2006) hypothesize that the extent of immune deficiency after the acute phase, particularly the capacity to control potential pathogens at mucosal surfaces, determines the rate of immune activation and therefore the rate of progression to AIDS.

1.10.1 HIV-1 in primary T-cells and the role of the cell cycle

The main targets of infection by HIV-1 are cells bearing the CD4 receptor and one or both co-receptors, CXCR4 or CCR5, such as T-lymphocytes and monocyte-derived macrophages. HIV-1 preferentially infects $CD4^+CD45RO^+$ (memory) cells (Helbert *et al*., 1997; Blanco *et al*., 2002), with this subset of $CD4^+$ lymphocytes being lost earlier in HIV-1 infection than the $CD4^+CD45RA^+$ (naïve) cells (Helbert *et al*., 1997). $CD4^+CD45RO^+$ cells are reportedly hyper-responsive by virtue of enhanced expression of cytokine receptors and adhesion molecules (Wallace and Beverley, 1990).

T-cell activation as well as induction of specific host cellular factors is required for the productive infection of primary $CD4^+$ T-cells (Kinoshita *et al*., 1998). Whilst productive HIV-1 infection depends on progression of the cells through the G_{1b} phase of the cell cycle (Korin and Zack, 1998), the G_{1b} phase is insufficient to confer competency for completed reverse transcription (Kinoshita *et al*., 1998). It has thus been speculated that productive HIV-1 infection occurs under certain cellular activation states that may coincide with certain phases of the cell cycle and which may involve access to nucleotides or other cellular factors required for completion of reverse transcription (Kinoshita *et al*., 1998).

1.10.2 HIV-1 infection of quiescent/nondividing cells

Researchers showing that HIV-1 is able to infect and propogate in resting lymphocytes have challenged the view that cellular activation is a requirement for productive infection and replication of HIV-1 (Bukrinsky *et al*., 1991). Reports regarding the replication of HIV-1 in quiescent $CD4^+$ T-cells are controversial, with researchers describing that: (i) the RNA is fully transcribed in activated lymphocytes and only partially transcribed in resting lymphocytes with the later only becoming fully transcribed if the cell is activated within 1 to 2 days (Zack *et al*., 1990), (ii) HIV-1 RNA is completely transcribed to cDNA in resting cells, but that integration of the HIV-1 DNA is blocked due to the lack of viral DNA ATP-dependent import into the nucleus (Bukrinsky *et al*., 1991; Bukrinsky *et al*., 1992), (iii) HIV-1 can infect and establish a complete, stable form of viral DNA in primary $CD4^+$ T-lymphocytes *in vitro* but that transcription is blocked in the absence of activation (Spina *et al*., 1995), (iv) highly purified human quiescent $CD4^+$ T-lymphocytes in the G_0/G_1 state of cell replication, which lack an activated-cell phenotype, can be infected by HIV-1 but cannot be stimulated to produce virus (Tang *et al*., 1995). Studies suggest that HIV-1 gene expression can be detected in naïve, resting T-cells *in vivo* but that *in vitro* these cells are resistant to productive infection (Eckstein *et al*., 2001). Notably, that HIV-1 is able to infect non-dividing cells such as macrophages and quiescent T-cells has implications for their role in AIDS pathogenesis (Spina *et al*., 1995). More recently it has been hypothesized that latently infected cells with integrated HIV-1 DNA may be generated when lymphoblasts that are in the process of reverting to a resting state become infected (Persaud *et al*., 2003). When latently infected cells subsequently encounter the relevant antigen, they become permissive for virus gene expression and

virus production (Persaud *et al*., 2003). Latently infected cells may be maintained by intrinsic stability, as well as by the process of proliferative renewal if they do not become susceptible to HIV-1 induced cytopathic effects or host cytolytic mechanisms during this process (Persaud *et al*., 2003). Important to this hypothesis is the fact that (i) resting memory cells have a long life span and (ii) latently infected cells can persist even when viral replication is fully suppressed by antiretroviral drugs (Persaud *et al*., 2003).

1.10.3 Latent infection of $CD4^+$ T-cells

Latently infected cells are thought to arise when productively infected $CD4^+$ T-lymphoblasts escape immune effector mechanisms and the cytopathic effects of the virus, and revert to a resting memory state in which there is little or no expression of viral genes (Piersen *et al*., 2000). In these memory cells HIV-1 is considered to be in a stable post-integration form (Chun *et al*., 1997). R5 viruses can enter resting memory $CD4^+$ T-cells that express sufficient levels of CCR5 (Blankson *et al*., 2000) and remain in a labile pre-integration form. Activation of these resting $CD4^+$ T-cells before the pre-integration complex becomes non-functional will result in productive infection (Zack *et al*., 1990; Bukrinsky *et al*., 1991; Spina *et al*., 1995). Despite HAART the presence of residual latently infected resting $CD4^+$ T-cells can serve as a potential source of reactivation of viral replication (Chun *et al*., 1998). Studies showing that resting T-cells treated with cytokines, can be infected with replication-competent HIV-1 has led to the suggestion that cytokine signals may play an important role in HIV-1 infection of resting T-cells *in vivo* (Unutmaz *et al*., 1999).

1.10.4 Integrated or unintegrated HIV-1 proviral DNA

The expression of critical cellular components, including cell surface receptors, transcription factors necessary for the expression of the viral genome and other factors necessary for viral replication, can be affected both by the state of differentiation of the host cell and by the stage of the host cell cycle.

Reverse transcription of the viral RNA is initiated in the cytoplasm and leads to the formation of a linear, double-stranded DNA molecule containing the viral genes and long terminal repeat (LTR) sequences at each end. Full-length linear DNA is observed

approximately 3 to 4 hours post-infection (Vandegraaff *et al*., 2001; Kok *et al*., 2001). The linear viral DNA molecule is actively transported to the nucleus and integrated into the host cell genome. Linear viral DNA that integrates into the host genome forms the provirus. Integration of viral DNA into chromosomal DNA has been observed 8 hours post-infection and this process has been found to be complete by 72 hours post-infection (Kok *et al*., 2001). Integration preferentially occurs in cells with actively replicating DNA and at transcriptionally active sites. HIV-1 DNA has been shown to integrate at a higher frequency near *Alu* repeat sequences in the human genome (Kok *et al*., 2001).

Time course analysis of the appearance of viral DNA forms in the nucleus following a single round of infection indicates that the linear viral DNA molecules are rapidly transformed soon after entry into the nucleus. The concentration of linear DNA in the nucleus remains relatively constant and low, while the unintegrated circular forms and integrated provirus accumulate with time suggesting that once the linear viral DNA enters the nucleus, it either stably integrates into the host DNA or forms circular molecules which can no longer integrate (Farnet and Haseltine, 1991). The high copy number of unintegrated viral DNA that occurs in several monomeric and multimeric forms in infected cells during the course of acute infection is considered to be the result of multiple events of infection in individual cells (Montagnier, 1999). The unintegrated DNA can remain within the cytoplasm as a linear form or circularize by homologous intramolecular recombination to yield a covalently closed circular form with one LTR or circularize with two LTRs (Bukrinsky *et al*., 1993; Pauza *et al*., 1994). The circular forms of viral DNA have been shown to appear at 8 hours post-infection, with the 2-LTR species constituting a minor population compared to the 1-LTR and linear forms over the course of infection (Vandegraaff *et al*., 2001). Although these viral DNA forms are unable to integrate and support viral replication, they may play a significant role in viral cytopathogenesis (Bukrinsky *et al*., 1993).

1.11 Immune activation

Effective immune responses are triggered and controlled by a variety of immunocompetent cells including T-helper cells and by signalling molecules such as cytokines, chemokines and other mediators of inflammation (Widner *et al*., 2000). In most diseases, host immune reaction involves both the Th1 and Th2 cells. The Th1 cells play an important role in protective cell-mediated immune responses, delayed type

hypersensitivity, macrophage activation, the production of opsonizing antibodies and are critical for the eradication of intracellular pathogens (Szabo *et al*., 2003; Gerosa *et al*., 1996) producing IFN-γ, IL-2, IL-12 (Fakoya *et al*., 1997) and TNF-α and -β (type-1 cytokines). Th2 lymphocytes on the other hand, stimulate the activation of eosinophils, basophils, B-lymphocytes and the generation of antibodies and are essential for the elimination of extracellular organisms e.g. helminthes and nematodes and produce IL-4, IL-5, IL-6, IL-10 and IL-13 (type-2 cytokines) (Szabo *et al*., 2003; Gerosa *et al*., 1996) Type-1/type-2 cytokines are reciprocally regulated (Fakoya *et al*., 1997) and during the course of diseases this cross-regulation has been suggested to be responsible for the change of the pattern of the immune response (Clerici and Shearer, 1993c). Plasma activation markers are products of cytokine activity and represent the biologic consequences of increased cytokine production and cellular responses (Fahey *et al*., 1998). Excessive immune activation has been associated with impaired immune function (Fahey *et al*., 1998).

1.11.1 Neopterin – a marker of cell-mediated immune responses

Neopterin (6-D-erythro-trihydroxypropylterin) is a pyrazino-pyrimidine compound derived from guanosine triphosphate (GTP) within the biosynthetic pathway of tetrahydrobiopterin (BH_4), which is an essential cofactor for tyrosine and tryptophan hydroxylation, and plays an important role in the biosynthesis of catecholamines and serotonin (Nichol *et al*., 1985). The biological significance and mode of action of neopterin are unknown (Fuchs *et al*., 1988) however findings point to neopterin playing a role in the cytotoxic armature of stimulated monocytes/macrophages (Widner *et al*., 1998). Neopterin is a low molecular weight compound (253 Da) and within the circulation its diffusion rate and biological stability are high (Widner *et al*., 2000). *In vitro*, large amounts of neopterin are produced by human monocytes/macrophages upon stimulation with IFN-γ (Huber *et al*., 1984). *In vivo*, increased neopterin concentrations in human serum and plasma, which indicate activation of the cell-mediated immune response, are observed in response to inflammatory disorders including autoimmune diseases, allograft rejection, certain types of malignancies, viral, bacterial or protozoal infection (Huber *et al*., 1984; Melmed *et al*., 1989). In essence therefore the *in vitro* and *in vivo* production of neopterin is associated with immune responses with increased concentrations representing early stages in T-cell responses and the interplay between T-

cells and macrophages (Fuchs *et al*., 1988). Serum and urinary neopterin levels have been shown to correlate to stage of disease and prognosis (Fahey *et al*., 1990; Fuchs *et al*., 1988), and in patients with HIV-1 infection represent a relation to AIDS defining infections and other pathology rather than the amount of virus (Fahey *et al*., 1998). Elevation of neopterin levels appears to be independent of changes in $CD4^+$ T-cells, but there is a significant inverse correlation between neopterin levels and $CD4^+/CD8^+$ T-cell ratios and absolute $CD4^+$ T-cell numbers in AIDS related complex (ARC) and AIDS patients which is weak in asymptomatic individuals (Fuchs *et al*., 1988). A close correlation exists between the amount of neopterin produced by activated monocytes/macrophages, the release of reactive oxygen species (Murr *et al.,* 1999) and IFN-γ activity (Huber *et al*., 1984). Neopterin concentrations can therefore be regarded as an indirect estimate of the degree of oxidative stress emerging during cell-mediated immune responses (Murr *et al*., 1999).

1.11.2 β_2-microglobulin – a potential regulator of the immune system

β_2-microglobulin (β_2-m) is present in serum in low concentrations (2 μg/ml) (Bernier and Conrad, 1969) and was initially isolated from urine of patients with renal tubular disorders (Berggard and Bearn, 1968) which suggests that tubular re-absorption is an important step in the catabolism of this protein (Bernier and Fanger, 1972). β_2-m has been shown to be a 12 kDa 99 amino acid polypeptide that forms the light chain of class I MHC molecules. β_2-m possesses an Ig-like domain and non-covalently associates with the 44 kDa classical (HLA-A, -B and –C) and 40 kDa non-classical (HLA-E, -F and –G) class I MHC heavy chains as well as 43 to 49 kDa non-classical heavy chains (CD-1). The association of β_2-m with the MHC class I heavy chain is essential for the stable expression of these molecules (Gobin *et al*., 2003). β_2-m is necessary for the functioning of molecules central in antigen presentation, IgG transport and iron metabolism (Gobin *et al*., 2003). The classical MHC class I molecules are expressed on all nucleated cells and are crucial in the presentation of antigen-derived peptides, commonly viruses, to $CD8^+$ cytotoxic T-lymphocytes as well as being important in protection against NK cell-mediated cytotoxicity (Osmond *et al*., 1991). Circulating β_2-m is generated during HLA turnover. Several cytokines, such as TNF-α, IFN-α, IFN-β and IFN-γ up-regulate the transactivation of β_2-m. Inflammation or infection is therefore associated with the coordinate cytokine-regulated expression of the genes involved in the MHC class I

antigen presentation pathway including β_2-m and MHC class I molecules (Hallermalm *et al*., 2001). Stimulation of lymphoid cells increases β_2-m production and T-cells are more potent producers than B-cells (Kin *et al*., 1979). Raised serum levels of β_2-m have been reported in a number of disease states including renal failure (Wibell *et al*., 1973), autoimmune disorders such as rheumatoid arthritis (Manicourt *et al*., 1978), leukemias and lymphomas (Simonsson *et al*., 1980), HIV-1 infection and AIDS (Hofmann *et al*., 1990). While some researchers have shown that monocytes can be stimulated by β_2-m to secrete high levels of proinflammatory cytokines TNF-α, IL-1, IL-6, IL-8 and IL-10 (Xie and Yi, 2003) others have shown that β_2-m can inhibit the growth of, and induce apoptosis or necrosis in tumor cells (Mori *et al*., 1999). β_2-m can initiate inflammatory processes by inducing apoptosis or necrosis resulting in the released enzymes and cytokines acting as chemoattractants for mononuclear cells (Xie and Yi, 2003). β_2-m appears to trigger a cascade of signalling events that exert a negative effect on the immune system, including impaired antigen-presentation capacity of DC (Xie *et al*., 2003).

1.11.3 L-selectin – involvement in specific immune responses

L-selectin (CD62L) is a glycoprotein which is constitutively expressed on the cell surface of granulocytes, lymphocytes, monocytes, haematopoietic progenitors and thymocytes (Schleiffenbaum *et al*., 1992; Spertini *et al*., 1991; Tedder *et al.,* 1990; Catalina *et al*., 1996; Wang *et al* 1997b). Lymphocytes re-circulate from the blood to lymphoid and extra-lymphoid sites in order to detect antigen within tissues and for immune surveillance (Gowans and Knight, 1964). This movement is regulated by the interaction between adhesion receptors on the surface of lymphocytes (homing receptors) and their ligands on specialized postcapillary endothelial cells (Gallatin *et* al*.,* 1983). The recruitment of selective leukocyte subtypes can be controlled by the differential expression of adhesion molecules and chemokine receptors with the possibility being that adhesion molecules trigger signals for enhanced chemokine receptor expression and function (Ding *et al*., 2003). L-selectin plays a role in the migration of lymphocytes into peripheral lymph nodes and sites of chronic inflammation and of neutrophils into sites of acute inflammation. L-selectin mediates the initial interaction of circulating leukocytes with endothelial cells that produces a characteristic 'rolling' of the leukocytes on the endothelium leading, through the interaction of other adhesion molecules, to cell

extravasation (Kourtis *et al*., 2000). Interaction with APC is enhanced by lymphocyte stimulation through L-selectin (Gonzalez-Amaro and Sanchez-Madrid, 1999). L-selectin plays an important role in specific immune responses. In L-selectin deficient mice T-cells do not home to peripheral lymph nodes, primary T-cell responses to antigen are impaired and cutaneous delayed-type hypersensitivity responses do not occur (Xu *et al*., 1996; Steeber *et al*., 1996).

L-selectin is shed by proteolytic cleavage from the surface of leukocytes after activation resulting in an increased detection of soluble (s) L-selectin in the plasma (Kourtis *et al*., 2000). sL-selectin retains bioactivity and at high concentrations can inhibit binding of lymphocytes to endothelium suggesting a possible role in modulating this binding *in vivo*. Studies suggest that levels of sL-selectin in biological fluids may be elevated or lowered in subjects with a variety of pathological conditions, indeed, a significant positive correlation between sL-selectin levels and HIV-1 viral load has been demonstrated in paediatric infection and adults with AIDS (Kourtis *et al*., 2000).

1.12 Factors involved in immunomodulation

1.12.1 IL-7

IL-7 is a cytokine produced by the stromal cells of the thymus and the bone marrow, by keratinocytes and by intestinal epithelium (Hofmeister *et al*., 1999) and functions as an important regulator in the development, proliferation and homeostatic maintenance of naïve (Tan *et al*., 2001) and antigen-stimulated mature T-cells, both through peripheral expansion and through thymus-dependent mechanisms (Hofmeister *et al*., 1999). IL-7 is considered to be essential for the viability and proliferation of immature thymocytes (Kees and Ford, 1999). IL-7 also acts through a variety of mechanisms to increase the proliferative response and survival of mature T-cells following encounter with antigen (Fry and Mackall, 2001). A very recent study has shown that IL-7 signalling is sufficient to phenotypically and functionally prime human $CD4^+$ naïve T-cells (Managlia *et al*., 2005). Although IL-7 has recognized functions in B-cell lymphopoiesis it is not essential for the development of B-cells in humans (Fry and Mackall, 2002). Circulating levels of IL-7 increase in response to T-cell depletion, suggesting that IL-7 plays a role in thymic

dependent T-cell regeneration and peripheral expansion of mature T-cells (Fry and Mackall, 2001).

1.12.1.1 Role of IL-7 in HIV-1 infection

Most circulating peripheral T-lymphoctes are in the G_0 state and the detection of HIV-1 in naïve T-cells *in vivo*, albeit at significantly lower levels than in the memory T-cell subset, raises the question regarding the mechanism controlling HIV-1 infection in the former population (Jaleco *et al*., 2002). IL-7 has been suggested to induce HIV-1 replication in naturally infected PBMC (Smithgall *et al*., 1996) by inducing a state of permissiveness in quiescent T-lymphocytes (Ducrey-Rundquist *et al*., 2002) and even in naïve $CD4^+$ T-cells (Steffens *et al*., 2002) or by inducing expression of latent virus (Scripture-Adams *et al*., 2002). Induced expression of latent HIV-1 by IL-7 has little effect on naïve and memory phenotypes (Scripture-Adams *et al*., 2002; Jaleco *et al*., 2002). The involvement of IL-7 as a cellular cofactor of HIV-1 induced T-cell permissiveness occurs at the level of transcription rather than being the result of a direct interaction with the preintegration complex (Ducrey-Rundquist *et al*., 2002). *In vitro* studies suggest that IL-7 might sustain the survival of mature $CD4^+$ thymocytes as well as favour HIV-1 replication in these cells (Chene *et al*., 1999). The positive regulation of CXCR4 by IL-7 in mature $CD4^+$ thymocytes correlates with their capacity to favour the replication of X4 versus R5 HIV-1 variants compared with intermediate thymocytes or PBMC (Schmiitt *et al*., 2003). IL-7 may induce CXCR4 expression on resting $CD4^+$ memory T-cells *in vitro* (Llano *et al*., 2001; Scripture-Adams *et al*., 2002). The emergence of CXCR4 syncytium-inducing (SI) variants of HIV-1 during advanced stages of infection associated with the increased levels of plasma IL-7 and the depletion of $CD4^+$ T-cells has resulted in the suggestion of a possible relationship between IL-7 levels and the emergence of SI isolates (Llano *et al*., 2001). However, IL-7 has been shown to stimulate T-cell reconstitution without increasing viral replication in asymptomatic SIV-infected macaques (Nugeyre *et al*., 2003). Furthermore, an inverse correlation between $CD4^+$ T-cell depletion and increased circulating levels of IL-7 has been described (Llano *et al*., 2001; Scripture-Adams *et al*., 2002), with higher circulating levels of IL-7 being associated with a greater viral load (Ducrey-Rundquist *et al*., 2002), a correlation not observed in children perinatally infected with HIV-1 (Chiappini *et al*., 2003). Higher IL-7 levels produced in response to

T-cell depletion have been associated with increased thymic rebound in adult HIV-1 infected patients undergoing HAART (Ruiz-Mateos *et al*., 2003).

1.12.2 GM-CSF

GM-CSF is produced in a paracrine manner that is, GM-CSF is produced and acts locally (Gasson, 1991) by activated (but not resting) T-cells of both the Th1 and Th2 phenotype (Chan *et al*., 1986; Shannon *et al*., 1997). GM-CSF stimulates the proliferation, maturation and function of myeloid progenitor cells, giving rise to neutrophils, eosinophils and monocytes (Gasson, 1991). At higher concentrations, and/or in synergy with other cytokines, it promotes the proliferation of erythroid progenitors and megakaryocytes *in vitro* (Gasson, 1991). In response to specific activating signals and chemokines GM-CSF can be synthesized by a variety of cell types including T-lymphocytes, macrophages, mast-cells, endothelial cells and certain fibroblasts (Gasson, 1991). Gene knock-out experiments suggest that GM-CSF, IL-3 and IL-5 are not essential in maintaining 'steady-state' populations of granulocytes, macrophages and eosinophils but are important for the production of these cells in response to infection and inflammation (Shannon *et al*., 1997; Guthridge *et al*., 1998). In the peripheral blood GM-CSF enhances host defense cell function by promoting many functional activities of mature effector cells such as neutrophils, monocytes, macrophages and dendritic cells. Neutrophils are affected both directly and indirectly by GM-CSF with direct effects including membrane ruffling, inhibition of neutrophil migration, degranulation, increased expression of cell surface adhesion proteins (Weisbart *et al*., 1985), changes in receptor expression for f-Met-Leu-Phe and IgA (Weisbart *et al*., 1987) and effects on cytoskeleton and cell shape (Gasson, 1991). Indirect or 'priming effects' resulting in the enhanced ability of the neutrophil to respond to a secondary triggering stimuli include enhanced chemotaxis, increased superoxide production, changes in Ca^{2+} flux and pH, enhanced cytotoxic and phagocytic activity and enhanced production of leukotrienes in neutrophilic and eosinophilic granulocytes (Gasson, 1991).

1.12.2.1 Functional role of the GM-CSF receptor

The GM-CSF receptor consists of two subunits, alpha (GMRα - CD116) and beta (β_c - CDw131) (Dabusti *et al*., 2001). Whilst the low molecular weight α subunit of 60 to 80

kDa binds GM-CSF with low affinity (K_d=1-5 nM), the β subunit has no intrinsic binding affinity for GM-CSF but interacts with the α subunit to form a high affinity receptor (120 kDa; K_d=40-100 nM), functioning to facilitate ligand acquisition and stabilize ligand binding (Niu *et al*., 1999) and playing a fundamental role in signal transduction in responsive cells (Dabusti *et al*., 2001). Whilst the α-subunit is unique to the GM-CSF receptor, β_c is shared with the receptors for IL-3 and IL-5 (Armitage, 1998). Receptor dimerization (the formation of a complex of GM-CSF:GMRα:β_c) is essential for receptor activation signalling (Guthridge *et al*., 1998). Specific regions of the intracytoplasmic domain of the α subunit have been found to play an essential role in GM-CSF mediated cell proliferation, survival and differentiation (Matsuguchi *et al*., 1997) suggesting that these processes may be controlled in complex ways. Ligand bound GM-CSF receptors are rapidly internalized and unoccupied receptors are recycled to the cell surface (Gasson, 1991). It is not known whether receptor internalization is required for or associated with signal transduction (Okuda *et al*., 1991). Receptor internalization appears to be a general property of haematopoietin receptors, either in response to binding of a specific ligand or in response to activation of a receptor for another CSF (receptor transmodulation) (Okuda *et al*., 1991).

1.12.2.2 Role of GM-CSF in HIV-1 infection

There is little consensus amongst studies regarding GM-CSF production during HIV-1 infection with reports suggesting a progressive decline in GM-CSF production from the early stages of HIV-1 infection (Re *et al*., 1992) whilst normal or even high levels of GM-CSF have been reported (Hober *et al* 1993). Furthermore, the mechanism by which GM-CSF affects HIV-1 replication in macrophages or promonocytic lines is also controversial since some researchers report GM-CSF to increase HIV-1 entry and replication by up-regulating CCR5 (Wang *et al*., 1998) while others report suppressed CCR5 and CD4 expression on macrophages and reduced HIV-1 entry with GM-CSF (Di Marzio *et al*., 1998). Other studies suggest that HIV-1 infection of macrophages by primary X4 and R5X4 isolates is inhibited by GM-CSF, an effect associated with down-regulation of surface CXCR4 expression (Wang *et al*., 2001). Although the differential regulation of HIV-1 replication has been suggested to relate to the maturation state of the monocytes and thus their state of permissiveness (Kedzierska *et al*., 1998), an *in vitro* study has found GM-CSF to inhibit HIV-1 replication in a dose-dependent manner regardless of the

HIV-1 strain, source of GM-CSF, stage of monocyte-derived macrophage maturation or timing of GM-CSF exposure in relation to HIV-1 infection (Kedzierska *et al*., 2000). GM-CSF may affect HIV-1 replication through stimulation of the binding of host-cell transcriptional factors to the HIV-1 LTR resulting in transactivation or increased HIV-1 transcription (Nabel and Baltimore, 1987). The conserved membrane proximal region and tyrosine residues on the β_c subunit of GM-CSFR have also been shown to upregulate the HIV-1 LTR through modulation of the NF-κB and Sp1 binding sites by multiple signalling pathways (Watanabe *et al* 2002).

1.13 Cytokines and chemokines and their role in the immune system

Cytokines function to stimulate, or suppress cellular proliferation and differentiation and to modulate immune function (Paul and Seder, 1994). Cytokines play an important role both in response to infection and in the development of a functional immune system (Holloway and Shannon, 2001). Cell-specific expression patterns of cytokine gene transcription are established during the differentiation of specific cells of the immune system with cytokine expression occurring after exposure of a cell to a specific set of signals (Holloway and Shannon, 2001).

Chemokines are a superfamily of small (8-10 kDa), inducible, secreted pro-inflammatory cytokines (Hariharan *et al*., 2000). All chemokines are structurally similar, having at least three β-pleated sheets and a C-terminal α-helix (Rollins, 1997). Most chemokines have, in conserved positions, at least four cysteines. The relative positions of the cysteine tandem defines four classes of chemokines, the CXC or α, CC or β, C or γ and CX3C or δ chemokine classes (Rossi and Zlotnik, 2000). Chemokines bind to and activate G-protein coupled 7-transmembrane domain receptors (Hariharan *et al*., 2000; Rossi and Zlotnik, 2000). The biological functions of chemokines and their receptors are numerous and include lymphoid organ development, lymphoid trafficking, Th1/Th2 development, cell recruitment, inflammation, wound healing, angiogenesis/angiostasis and metastasis (Rossi and Zlotnik, 2000). The CC chemokines have a broad spectrum of action and attract monocytes, lymphocytes, eosinophils, basophils and NK cells (Rollins, 1997; Kakkanaiah *et al*., 1998) while functioning to regulate leukocyte trafficking, inflammation, haematopoiesis, antitumour immunity and HIV-1 infection (Rollins, 1997). In contrast, CXC chemokines predominantly recruit neutrophils (Rollins, 1997; Kakkanaiah *et al*.,

1998). Chemokines are a key component of immune surveillance and have unique immunomodulatory properties in that they are capable of finely tuning protective immune responses by recruiting and activating specific cells (Rollins, 1997; Lusso, 2002). Chemokines have been shown to play a pivotal role in the Th1/Th2 polarization of $CD4^+$ and $CD8^+$ T-cell cytokine-secretion patterns (Lusso, 2002).

1.13.1 Modulation of immune function by CXCL12

CXCL12 is an 8 kDa CXC chemokine that plays an important role in immunomodulation, organogenesis and haematopoiesis (Nagasawa *et al*., 1996; Tachibana *et al*., 1998; Zou *et al*., 1998). CXCL12 is produced by bone marrow stromal cells (Aiuti *et al*., 1997) and is expressed by thymus medullar epithelial cells (Zaitseva *et al*., 2002), secondary lymphoid organs (Rossi and Zlotnik, 2000) and is broadly expressed in cells of both the immune (except blood leukocytes) (Soriano *et al*., 2002) and central nervous system (Zaitseva *et al*., 2002). In the thymus, CXCL12 is a chemoattractant for immature T-cells and thymocyte subsets, possibly playing a role in the regulation of the intrathymic migration that occurs during T-cell development (Rossi and Zlotnik, 2000) as well as in the elimination of apoptopic thymocytes (Zaitseva *et al*., 2002). CXCL12 produced by the bone marrow is reported to be a chemoattractant for T-cells, peripheral blood lymphocytes (Bleul *et al*., 1996a), $CD34^+$ haematopoietic progenitors (Aiuti *et al*., 1997), mature B-cells (D'Apuzzo *et al*., 1997), megakarocytes, and pre- and pro-B-cell lines (D'Apuzzo *et al*., 1997). Naïve and memory B-cells in secondary lymphoid organs are responsive to CXCL12 (Rossi and Zlotnik, 2000). The CXCL12-CXCR4 interaction is essential for the maturation of $CD34^+$ stem cells in the bone-marrow environment, for B-cell lymphopoiesis and myelopoiesis and for the physiological trafficking of monocytes, neutrophils and peripheral blood lymphocytes and their endothelial transmigration as indicated by intracellular Ca^{2+} concentration changes and chemotaxis (Marechal *et al*., 1999; Soriano *et al*., 2002). The constitutive expression of CXCL12 in a range of tissues suggests that its function is one of lymphocyte recirculation and in basal recruitment of monocytes that is, normal replenishment and turnover of tissue mononuclear phagocytes rather than in inflammation (Bleul *et al*., 1996b).

The amino terminus of CXCR4 plays a critical role in the initial binding of CXCL12 and the interaction of CXCL12 with the second extracellular loop of CXCR4 results in the

activation of downstream signalling pathways (Doranz *et al*., 1999). The activation of several pathways and functions e.g. chemotaxis and ERK/MAP kinase cascade activation, has been suggested to result from the internalization of the CXCR4 receptor that is triggered by CXCL12 (Roland *et al*., 2003).

1.13.1.1 Role of CXCL12 and its receptor CXCR4 in HIV-1 infection

The chemokine receptor for CXCL12, CXCR4 serves as a fusion co-receptor for T-cell tropic and dual tropic HIV-1 strains (Feng *et* al., 1996). The effects of CXCL12 on HIV-1 pathogenesis are complex, including both the inhibition of X4 viral entry and the stimulation of proviral gene expression as noted by the increased infectivity of CCR5-tropic HIV-1 strains (Marechal *et al*., 1999). Delayed progression to AIDS has been reported in some individuals homozygous for the genetic polymorphism in the untranslated region (UTR) of the gene coding for CXCL12 (CXCL12 3'A/3'A) (John *et al*., 2000) but this genotype has also been associated with rapid progression to AIDS with prolonged survival after diagnosis of AIDS (van Rij *et al*., 1998). The precise role of CXCL12 as well the CXCL12 3'A allele in HIV-1 disease remains unclear (John *et al*., 2000). A report suggests that CXCL12 3'A homozygosity is associated with low plasma CXCL12 levels in uninfected individuals and was not related to long-term progression (Soriano *et al*., 2002). Furthermore, a low frequency of the CXCL12 3'A allele in exposed uninfected individuals is coupled with high CXCL12 plasma levels and low CXCR4 expression (Soriano *et al*., 2002). High plasma CXCL12 levels and low CXCR4 expression on T-lymphocytes is a feature of long-term non-progression (Soriano *et al*., 2002). In advancing disease CXCR4 expression increases and in more advanced stages of HIV-1 infection is accompanied by a decrease in plasma CXCL12 levels (Soriano *et al*., 2002).

Perinatal HIV-1 transmission is associated with a maternal heterozygous CXCL12 genotype (CXCL12 3'A/wt) and while infant CXCL12 3'A heterozygous status has been significantly associated with maternal heterozygosity, heterozygous infants are not at increased risk of infection (John *et al*., 2000). The CXCL12 3'A mutation in association with another maternal cofactor may be responsible for increased perinatal HIV-1 transmission (John *et al*., 2000).

1.13.2 Modulation of immune function by CCL3, CCL4 and CCL5

CCL3 encodes a 92 amino acid residue precursor protein with a 22 amino acid residue signal peptide that is cleaved to generate the secreted mature protein. CCL4 encodes a precursor protein with a 23 amino acid residue signal peptide that is cleaved to generate a 69 amino acid residue, non-glycosylated mature protein. The mRNAs for CCL3 and CCL4 are not expressed in unstimulated cells, but expressed after activation of T-cells, B-cells, monocytes and mast cells. CCL3 is produced by neutrophils, Langerhans cells, astrocytes, endothelial cells, fibroblasts and smooth muscle cells and attracts predominantly $CD8^+$ T-cells while CCL4 attracts $CD4^+$ cells, although there is some overlap between subsets in response to both chemokines (Taub *et al*., 1993; Schall *et al*., 1993). CCL3 is a monocyte chemoattractant and has differential chemoattractant and pro-adhesive effects on T-lymphocytes, DCs, NK cells, cytotoxic cells, B-cells, basophils and eosinophils. It has been identified as a stem cell inhibitor that can inhibit the proliferation of haematopoietic progenitor cells both *in vitro* and *in vivo*. CCL4 attracts and activates monocytes less efficiently than CCL3 (Fahey *et al*., 1992) and has no activity on DCs (Sozzani *et al*., 1995) and minimal activity on NK cells (Taub *et al*., 1995).

The CC chemokine, CCL5 encodes a highly basic 91 amino acid residue precursor polypeptide with a 23 amino acid residue hydrophobic signal peptide that is cleaved to generate the 68 amino acid residue mature protein. CCL5 has a complex influence on the biology of a variety of cell types being a chemoattractant of monocytes, unstimulated $CD4^+/CD45RO^+$ memory T-cells and stimulated $CD4^+$ and $CD8^+$ T-cells with naïve and memory phenotypes. Other cellular targets for CCL5 include eosinophils, basophils, NK cells and DCs (Rollins, 1997). CCL5 binds to and activates several 7-transmembrane G protein-coupled receptors (GPCRs), namely CCR1, CCR3 and CCR5 (Roscic-Mrkic *et al*., 2003). CCL5 is a chemokine with a distinct physiologic role and a unique mode of regulation (Rollins, 1997). In addition to chemotaxis, CCL5 acts as an antigen-independent activator of T-cells *in vitro*, mediating a spectrum of cellular responses such as calcium channel opening, cytokine release and IL-2 receptor expression and T-cell proliferation (Bacon *et al*., 1995).

1.13.2.1 Role of CCL3, CCL4, CCL5 and the receptors CCR5 and CXCR4 in HIV-1 infection

The general consensus is that T-cell-line tropic strains (T-tropic viruses) use the CXCR4 molecule as co-receptor and are inhibited by CXCL12, the natural ligand for this receptor while macrophage tropic (M tropic viruses) use CCR5 as co-receptor. CCL3, CCL4 and CCL5 have been implicated *in vivo* as the major HIV-1 suppressive factors produced by activated $CD8^+$ T-cells (Cocchi *et al*., 1995), inhibiting infection of lymphocytes with macrophage-tropic HIV-1 (R5) isolates by interaction with the CCR5 receptor (Lehner 2002) and receptor downregulation (Amara *et al*., 1997). Cytokines and chemokines modulate CCR5 and CXCR4 availability which could therefore influence the replicative potential of R5 and X4 HIV-1 strains (Kinter *et al*., 2000). It is likely that the interaction of HIV-1 with chemokine receptors induces cellular signalling events that play an important role in HIV-1 pathogenesis. The level of expression of specific chemokine receptors on the cell surface correlates with the susceptibility of cells to HIV-1 infection (Wang *et al*., 1998). The expression of CXCR4 and CCR5 on T-cells are upregulated by exposure to IL-2 and PHA (Bleul *et al*., 1997).

The role of CC-chemokines *in vivo* remains unclear with studies finding moderately elevated levels of CC-chemokines in HIV-1 asymptomatic subjects (Kinter *et al*., 1996) and a correlation between production of CCL3, CCL4 and CCL5 and reduced viral load (Ferbas *et al*., 2000). While some studies suggest that individuals with high concentrations of these factors remain uninfected despite repeated exposure to HIV-1 (Hariharan *et al*., 2000), other studies demonstrate no correlation between CC-chemokine levels and non-progression (Clerici *et al*., 1996; Scala *et al*., 1997). It has been suggested that the chemokines CCL3, CCL4 and CCL5 may play a role in protection from HIV-1 infection since uninfected newborns of HIV-1 infected mothers have increased expression of these chemokines compared with HIV-1 infected newborns (Wasik *et al*., 1999).

Individuals with a 32 base pair deletion in CCR5 have been shown to have significantly increased levels of CCL3, CCL4 and CCL5 (Paxton *et al*., 1996). Individuals homozygous for this mutation (~1% of Caucasians) are less susceptible to HIV-1 infection (Samson *et* al., 1996; Berger *et al*., 1999). Heterozygous Δ32 CCR5 occurs in ~18% of Caucasians (Liu *et al*., 1996) and is associated with reduced expression of

CCR5, reduced entry of R5 HIV-1 strains, decreased viral load and delayed progression of AIDS (Dean *et al*., 1996)

1.13.3 Modulation of immune function by IL-10

The cDNA for interleukin (IL)-10 encodes a peptide of 178 amino acid that forms an 18 kDa polypeptide (Vieira *et al*., 1991). It exhibits extensive homology to BCRF1, an open-reading frame in the Epstein-Barr virus genome (Moore *et al*., 1990). IL-10 is produced by T- and B-cells, monocytes and keratinocytes (Chehimi *et al*., 1996) and is produced relatively late following activation of T-cells or monocytes compared to other cytokines which suggests that IL-10 may play an important role in inhibiting T-cell and macrophage activation (Moore *et al*., 1993). IL-10 is able to regulate its own production by a negative feedback mechanism (de Waal Malefyt *et* al., 1991a). Furthermore, IL-10 inhibits cytokine production (IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF and G-CSF) by monocytes at a transcriptional level (de Waal Malefyt *et al*., 1991a) and reduces antigen-specific proliferative responses of T-cells and $CD4^+$ Th1- or Th2-like T-cell clones by inhibiting the APC by monocytes through the downregulation of constitutive, as well as IFN-γ or IL-4 induced, class II MHC expression on monocytes (de Waal Malefyt *et al*., 1991b). IL-10 is therefore recognised for its ability to downregulate several major functions of Th1 cells and macrophages as well as controlling Th2-mediated inflammatory processes (de Waal Malefyt *et al*., 1991b).

1.13.3.1 Role of IL-10 in HIV-1 infection

The bifunctional nature of IL-10, that is, the ability to suppress immune and inflammatory responses and reduce antigen-specific proliferation and cytokine production of T-cells and function as a B-cell stimulatory cytokine, has been suggested to have beneficial and detrimental effects on the course of HIV-1 infection and AIDS (Blazevic *et al*., 1996; Breen, 2002). Ultimately, the regulation of IL-10 during HIV-1 infection is unclear and conflicting data is reported with regards to the constitutive production of IL-10 and changes in plasma/sera levels during infection (Chehimi *et al*., 1996; Emilie *et al*., 1994). Notably, a recent study has suggested that IL-10 may play a protective role in diminishing oxidative stress during immune activation (Stephensen CB *et al*., 2005). It is interesting that IL-10 can differentially modulate CCR5 and CXCR4 expression in different cell

types specifically, IL-10 has been described to (i) upregulate CCR5 expression and function in human monocytes (Houle *et al*., 1999) but (ii) downregulate CCR5 expression in $CD4^+$ T-lymphocytes (Patterson *et al*., 1999) (iii) downregulate CXCR4 expression on $CD4^+$ T-lymphocytes and correspondingly regulate CXCL12 induced $CD4^+$ T-lymphocyte chemotaxis (Jinquan *et al*., 2000) but (iv) upregulate CXCR4 expression in primary monocytes-macrophages (Wang *et al*., 2001).

Data has shown that the HIV-1 gp120 can potently induce IL-10 in resting PBMC cultures (Ameglio *et al*., 1994). Subsequent research has shown that HIV-1 and its gp120 protein can induce IL-10 in monocytes and monocyte-derived macrophages (Borghi *et al*., 1995). Other HIV-1 proteins that have been found to induce IL-10 production in monocytes but not T- or B-lymphocytes include the Nef protein (Tangsinmankong *et al*., 2000), the Tat protein which induces IL-10 production in peripheral blood monocytes in a dose and time-dependent manner (Badou *et al*., 2000) and the Rev protein (Blazevic *et al*., 1996).

1.14 Effects of antiretroviral drugs on host-cell function

Kuhn *et al*. (2001a) demonstrated that HIV-1 specific T-helper cell responses are associated with protection against intrapartum and breastfeeding HIV-1 transmission. However, these cellular immune responses were found to be substantially reduced among exposed uninfected infants whose mothers were treated with short-course regimens of AZT-3TC (Kuhn *et al*., 2001c). This raises the question of whether certain antiviral drugs have other less beneficial effects for the immune response of infants. *In vitro* experiments using clinically relevant concentrations of AZT have demonstrated diminished responsiveness of PBMC to mitogen, decreased numbers of proliferating cells in G_1, S and G_2 + M phases of the cell cycle, DNA content and [^{3}H]-thymidine uptake, reduced RNA and protein content in the cultures and inhibition of cell growth. These findings suggest that AZT may adversely affect immune functions that are dependent on proliferation or RNA and protein synthesis, such as cytokine production (Heagy *et al*., 1991). AZT has also been shown to have no effect on resting PBMC but to substantially inhibit the growth of CTL clones (Nishimura *et al*., 1998). *In vitro* studies using the HIV-1 protease inhibitor Indinavir have found cell-cycle progression of PBMC of HIV-1 infected and HIV-1 uninfected individuals to be inhibited leading the researchers to

suggest that protease inhibitor effects could potentially modulate effects independently of antiviral activity against HIV-1 (Chavan *et al*., 2001).

Numerous studies have examined the effects of ART on immune responses among HIV-1 infected individuals and propose that early intervention may reverse most of the immune defects induced by HIV-1 infection whilst delayed initiation of ART may result in sustained functional immune impairment (Montoyo and Landay, 2003). Drug treatment is associated with a significant increase in total, naïve and memory $CD4^+$ T-cells, reduction of total and activated $CD8^+$ T-cells and activated $CD4^+$ T-cells, restoration of the proliferative response to anti-CD3/CD28, antigenic and allogeneic stimulation and the preservation of the HIV-1 specific Th1 responses (Blazevic *et al*., 2000; Al-Harthi *et al*., 2000b). Drug treatment is also associated with reduced expression of CCR5 on cells (Nicholson *et al*., 2001) a rapid reduction of activation markers, downregulation of proinflammatory cytokines and reduced apoptosis which precedes reduction in virus (Bisset *et al*., 1998 Andersson *et al*., 1998; Autran *et al*., 1997; Lederman *et al*., 1998). Other immunological changes associated with antiretroviral drug treatment of HIV-1 exposed uninfected individuals includes reduced detection of HIV-1 specific CTL responses (D'Amico R *et al*., 1999) reduced proinflammatory cytokines, TNF-α, IL-2 and IFN-γ (Tovo, 2000). Two studies however reject any direct effects of antiretroviral agents (AZT or AZT-3TC), independent of retroviral inhibition, on proliferation and redistribution of T-lymphocytes (Puro and Ippolito, 2000) or that AZT decreases T-lymphocyte subset counts of uninfected infants treated to prevent perinatal HIV-1 infection (de Martino *et al*., 1998).

1.15 Aims of the study

In South Africa, the current intervention to reduce MTCT of HIV-1 is based on the HIVNET 012 regimen which demonstrated that a single-dose of NVP given to the mother in labour and a dose administered to the infant soon after birth reduced transmission by half (Guay *et al*., 1999; Jackson *et al*., 2003). An infant born to an HIV-1 infected mother is therefore exposed to the dose of NVP given to the mother since this drug rapidly cross the placenta, and receives another dose following birth. In those cases where the mother does not take the oral dose of NVP, the infant receives PEP within 72 hours of delivery. The aims of this study therefore were two-fold. Firstly, to investigate *in vitro* the

replication of a primary HIV-1 isolate, using primary cell culture, under conditions where NVP or AZT were added at different times relative to HIV-1 infection, to understand how the timing of administration of single-dose antivirals influences viral infection outcome. Secondly, to describe, using samples from newborn infants of HIV-1 seropositive mothers who did or did not receive single-dose NVP, mechanisms by which short-course antiretroviral therapy modulates immune responses *in vivo*.

Specific objectives:

1. To examine the effect of NVP or AZT (added at different times prior or subsequent to infection) on the growth of a primary isolate M502L used to infect primary cell cultures.
2. To establish the immunological consequences of being born to an HIV-1 seropositive mother and of exposure to single-dose NVP given to the mother at the onset of labour.
3. To examine if immune factors (immune activation markers, cytokines, chemokines and HIV-1 specific T-helper responses) can predict infection outcome in the infant and to establish if differences exist in immune responsiveness that might distinguish infants that develop T-helper envelope responses from those who do not.
4. To determine the consequences of HIV-1 exposure or infection on thymic output of infants born to drug-naïve mothers.

CHAPTER TWO

MATERIALS AND METHODS

2.1 *In vitro* studies

2.1.1 Isolation of PBMC from donor blood

Normal donor buffy coats (leukocyte-enriched whole blood, from HIV-1 negative donors), also known as a leukopacks, were obtained from the South African Blood Transfusion Service (SABTS), Johannesburg and processed within 12 hours. Plasma from the donor units was collected and screened for the presence of HIV-1 and hepatitis B antibodies.

The blood was transferred into 50 ml Falcon centrifuge tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and centrifuged at 478 g for 10 min, 4°C using a Sigma 4K15 centrifuge (Wirsam Scientific, South Africa). The buffy coat cells were aspirated from each gradient tube and transferred to a 50 ml Falcon centrifuge tube and diluted with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS), pH 7.0. The mixture was carefully layered onto Ficoll-Paque^{TM} Plus (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 850 g (no brake) for 30 min at room temperature. The top layer comprising of diluted plasma and platelets was removed to just above the buffy coat and discarded as hazardous waste liquid. The cells at the interface between the PBS and the Ficoll were collected, transferred to a 50 ml Falcon centrifuge tube and washed three to four times with PBS by centrifuging at 212 g for 10 min, 4°C (to remove platelets). Contaminating red blood cells were lysed using ammonium chloride (0.15 M NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.0). The cell suspension, containing lysed RBC was washed a further three times with PBS by centrifuging the suspension at 212 g for 10 min, 4°C. Viable cell numbers were quantified using an Improved Neubauer haemocytometer after the cell suspension was diluted at a ratio of 1:1 with 0.4% trypan blue (Sigma, Steinheim, Germany). After the last wash the cells were resuspended in RPMI 1640 medium (Gibco, Invitragen Life Technologies NY, USA) supplemented with 2mM L-glutamine (stock 200 U/ml) (Sigma, Steinheim, Germany), 10% heat inactivated foetal calf serum (FCS) (Gibco, Invitragen Life Technologies NY, USA), either with or

without 3% recombinant human IL-2 (stock 200 U/ml) (Roche Diagnostics Corporation, Indianapolis, USA) and either with or without 5 μg/ml PHA, a lectin from *Phaseolus vulgaris* (stock 500 μg/ml) (Sigma, Steinheim, Germany), depending on the protocol for which they were to be used, to a final cell concentration of 1-5x10^6 cells/ml in T125 cm^3 flasks (Corning Inc., NY, USA). All cell cultures were maintained in a 5% CO_2 incubator (humidified) at 37°C.

2.1.1.1 Unstimulated and stimulated normal donor PBMC

Unstimulated lymphocytes were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures grown for extended time periods were supplemented with the addition of 3% recombinant human IL-2 (growth medium).

Prior to HIV-1 infection, PBMC were stimulated by incubation in medium containing 5 μg/ml PHA (PHA medium). Briefly, PBMC from a donor (2x10^6 cells/ml) were incubated in medium containing RPMI 1640 supplemented with 2 mM L-glutamine, 20% heat inactivated FCS, 3% recombinant IL-2 and 5 μg/ml PHA for 24 to 48 hours (depending on protocol). Donor cells were then washed and resuspended in growth medium.

2.1.2 Isolation of a primary HIV-1 isolate 98ZA502c3 (M502L)

PBMC from an HIV-1 seropositive patient (UNAIDS sponsored clinical trial of short-course AZT-3TC (Kuhn *et al.,* 2001c)) were separated from heparinized blood using Ficoll-Paque™ Plus. Qualitative HIV-1 cultures were set up using plastic flasks and 1-5x10^6 patient PBMC and an equal number of 2 day old PHA-stimulated PBMC from healthy donors and incubated at 37°C in 5% CO_2. Half the medium was replaced with fresh growth medium every 3rd day and with fresh growth medium containing freshly stimulated PBMC every 7th day. p24 antigen was measured by enzyme linked immunosorbent assay (ELISA) (Coulter Corp., Hileah, FL, USA) in cell supernatants on days 14, 21 and 28. The isolate was determined as CCR5-utilising by an ability to grow in CCR5-expressing osteosarcoma cell lines and an inability to grow in CXCR4-expressing cell lines (RA, Cilliers). The isolate was named according to WHO style nomenclature and included the following: year and country of identification, unique laboratory

identification, and relevant clone number (Korber *et* al., 1994) i.e. 98ZA502c3 accession number AY158534 (Hunt *et al*., 2003). For simplification we refer to this isolate as M502L.

M502L has been shown to replicate well in both PBMC and CBMC (C.T. Tiemessen, unpublished data).

2.1.2.1 Virus stock titration

The 50% tissue culture infective dose ($TCID_{50}$) of the M502L virus stock was determined by endpoint dilution using serial five-fold dilutions (ranging from 1:5 to 1:390 625) in a Costar 96-well U-bottomed microtitre plate (Corning, Inc, NY, USA). For precision, three replicate wells were used per dilution. The virus stock was serially diluted in 100 µl RPMI 1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20% heat inactivated FCS and 3% IL-2. 100 µl ($2x10^5$ cells/well) PHA-stimulated PBMC were added to each well and PBS was added to the outer wells to minimize evaporation. Cultures were incubated at 37°C in a 5% CO_2 incubator overnight. Cells were washed to remove free virus and resuspended in fresh medium. This ensured that p24 antigen detected at the time of harvest was due to new virus production. On day 3, 100 µl of medium was removed from each well and replaced with fresh medium. On day 7, 200 µl of the supernatant was removed from each well and assayed for p24 antigen production. Wells were scored positive when the p24 antigen concentration in the culture supernatant was twice that of the corresponding control well. Wells were scored negative when the corrected p24 antigen concentration was lower than or equal to the control well value at that dilution. The $TCID_{50}$ was defined as the amount of virus stock at which 50% of the inoculated wells were positive, as determined by the method of Spearman and Karber (Lane, 1999). The value obtained represents the reciprocal of the virus titre in terms of infectious doses per unit volume.

2.1.2.2 Viral infection

M502L infection was accomplished by incubating $2x10^5$ PBMC with ~ 2000 $TCID_{50}$ (multiplicity of infection (moi) of ~ 0.01) and ~ 1000 $TCID_{50}$ (moi of ~ 0.005) per ml for 1.5 hours at 37°C, 5% CO_2. Cells were washed twice with 10 ml RPMI 1640 medium

prior to culturing to remove residual free virus and resuspended in growth medium. An aliquot of the final wash step was assayed for HIV-1 p24 antigen to ensure that cell-free virus was effectively removed and also to establish a baseline for HIV-1 p24 antigen determinations. All infections were standardized using ELISA assays to the p24 gag protein of HIV-1. Polycationic substances such as DEAE-dextran and hexadimethrine bromide (Polybrene), which enhance adsorption of HIV-1 onto cells in culture, were not used in any infection experiments.

2.1.3 Antiretovirals

NVP and AZT were obtained from the AIDS Research and reference Reagent program (Division of AIDS, NIAID, NIH, USA). Stocks of AZT were prepared in phosphate buffered saline whilst stocks of NVP were prepared in dimethylsulfoxide (DMSO) to improve its solubility. The final concentration of DMSO in cultures was always 0.034%.

2.1.3.1 Antiviral effect of NVP and AZT added pre-infection or post-infection to unstimulated and PHA-stimulated PBMC

PBMC isolated from healthy donors were isolated as described in section (2.1.1) and were split into two equal portions and used for experiments to investigate the antiviral effects of NVP or AZT added either pre-infection or post-infection (p.i.) to unstimulated/stimulated PBMC. Donor cells were resuspended in growth medium at a concentration of $2x10^6$ cells/ml but inoculated at a concentration of $2x10^5$ cells/well. Cells used to investigate NVP or AZT effects prior to infection were cultured in the presence of 10 μM NVP or 20 μM AZT for different time periods up to 24 hours before they were infected with 2000 $TCID_{50}$ of M502L for 1.5 hours, 37°C, 5% CO_2. Cells used to examine the effect of NVP or AZT p.i. were infected with 2000 $TCID_{50}$ of M502L for 1.5 hours, 37°C 5% CO_2 and 10 μM NVP or 20 μM AZT were added at different time periods p.i. up to 24 hours. In both instances the infected cells were washed three times with RPMI medium to remove unabsorbed virus and cultured in the absence/presence of stimuli in growth medium. Depending on the protocol, cell culture supernatants were either collected for p24 antigen determinations 7 days after infection or NVP or AZT addition, or in the case of rescue experiments, cultures were washed to remove the antiretroviral drugs from the medium and the cells were resuspended in PHA medium to

activate cells. p24 antigen production was then monitored on day 14 using p24 ELISA. Inhibitory effects were expressed as percentage inhibition i.e. % inhibition=[(p24-Ag control – p24-Ag of NVP or AZT treated cultures)/p24-Ag control] x 100.

2.1.4 Determination of p24 antigen production

p24 antigen production from culture supernatants was determined using the p24 Coulter ELISA system according to the manufacturer's instructions. p24 cutoff values ranged from 5-6.43 pg/ml, thus p24 antigen concentrations falling below the first concentration on the standard curve, namely 7.8 pg/ml, were considered negative.

2.2 *In vivo* studies

2.2.1 Study populations

2.2.1.1 Demonstration of antiretroviral therapy (DART) and post-exposure prophylaxis (PEP) cohorts

The study population included HIV-1 seropositive and HIV-1 seronegative women delivering live-born infants at Chris Hani Baragwanath Hospital (CHBH), Soweto, South Africa, from the period April 2001 to October 2002. Women who had not received any antiretroviral drugs prior to the infant's birth either for prevention of mother-to-child HIV-1 transmission or for HIV-1 treatment (n=124) were recruited as part of a post-exposure prophylaxis (PEP) trial (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-naïve usually because they received no prenatal care or had attended prenatal clinics where HIV-1 testing was not available at the time. Their infants were randomized to receive either AZT or NVP to reduce the risk of vertical transmission. Drug-exposed HIV-1 seropositive women (n=78) were recruited from among women delivering at the hospital around the same time who had attended prenatal clinics at CHBH, had received HIV-1 counselling and testing there and had accepted NVP as part of a demonstration of antiretroviral therapy (DART) initiative. NVP was given as previously described (Guay *et al*., 1999) 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. A control group of HIV-1 seronegative women (n=30) was recruited at this site over the same time period.

The infants of these mothers were followed prospectively after birth to determine their HIV-1 status until 3 months or until at least 4 weeks after all breastfeeding had stopped. HIV-1 DNA PCR was performed on infant peripheral blood samples collected at 6 weeks of age (Roche Diagnostic Systems, Inc, New Jersey, USA). To establish the timing of infection, samples collected on the day of birth were tested if the 6 week PCR was positive. A positive result on the day of birth was used to infer intrauterine transmission (IU) and a negative result at birth with a positive result at 6 weeks or later was used to infer intrapartum transmission (IP). Children testing HIV-1 negative at 6 weeks or older were included as exposed-uninfected (EU). Children born to HIV-1 seronegative women (control group) were not followed after birth.

We selected for this nested case-control study, all 18 HIV-1 infected infants (4 IU and 14 IP) and a random sample of 63 uninfected infants from the cohort of 124 infants born to HIV-1 seropositive mothers who did not receive any antiretroviral drugs before delivery (PEP) (Table 2.1). We also selected all 7 HIV-1 infected infants (4 IU and 3 IP) and a random sample of 54 uninfected infants from the cohort of 78 infants born to HIV-1 seropositive mothers who received single-dose NVP before delivery (DART) (Table 2.1). Samples were insufficient for this analysis for one of the IP infected infants (PEP group).

Table 2.1 HIV-1 seropositive mothers and their infants enrolled in the PEP and DART study trial. The mother-child pairs selected for the nested case-control study are indicated as are the control mother-child pairs

PEP and DART groups			
	PEP	**DART**	**Total**
Negative (EU)	106	71	177
IP	14	3	17
IU	4	4	8
Total	124	78	202
Nested case control study group			
Negative (EU)	63	54	117
IP	14	3	17
IU	4	4	8
Total	81	61	142
Control group			
Negative (uninfected)			30

* Frequency of IU or IP infection in PEP and DART infants
IP PEP 14/124 (11.3%), IU PEP 4/124 (3.2%) Total 18/124 (14.5%)
IP DART 3/78 (3.8%), IU DART 4/78 (5.1%) Total 7/78 (9.0%)

2.2.1.1.1 Infants demonstrating HIV-1 specific cellular immune responses

For the investigation using infants that demonstrated HIV-1 specific immune responses to envelope (Env) peptides we selected all 18 HIV-1 infected infants (4 IU and 14 IP) and the random sample of 63 uninfected infants from the cohort of 124 infants born to HIV-1 seropositive mothers who did not receive any antiretroviral drugs before delivery (PEP). Three were excluded from the EU infant group as T-helper cell reactivity had not been determined. Samples were insufficient for this analysis for one of the IP infected infants (PEP group). As only one IU infected infant was found to have Env responses this infant was excluded from the analysis on the basis of sample size representation for this group. Ten control infants were also excluded due to T-helper cell reactivity not having been determined.

2.2.1.2 Anonomously collected cord blood samples

We selected for a study to determine thymic output of newborns, 76 anonymously collected cord blood samples from infants of HIV-1 infected women that did not receive any antiretroviral treatment. These samples were collected to supplement the UNAIDS-sponsored clinical trial of short-course zidovudine-lamivudine (AZT-3TC) (Kuhn *et al*., 2001c). Routine antiretroviral treatment was not the standard of care at the time this study was conducted. HIV-1 antibody tests showed that 29 mothers were HIV-1 seropositive and 47 HIV-1 seronegative.

As these infants were not followed prospectively after birth, timing of infection was determined on the basis of HIV-1 RNA PCR values, that is, infants were considered EU if the viral loads (VL) in cord blood plasma were less than 400 copies/ml while infants were considered to have become infected IU if viral loads were greater than 400 copies/ml. Based on VL, 25 infants (<400 copies/ml) were considered EU while 4 were considered infected IU (VL >400 copies/ml).

2.2.2 Blood samples

Small aliquots (3-5 ml) of cord blood were obtained by cordocentesis immediately after delivery of the placenta, and 10 ml peripheral blood obtained from each mother within 24 hours of delivery. Blood samples were drawn into ethylenediamine tetra-acetic acid

(EDTA) tubes. Mononuclear cells (adult peripheral and cord blood) and plasma were processed by Ficoll-Paque^{TM} Plus gradient centrifugation within 24 hours. Plasma (mother and infant) was stored at -70°C and mononuclear cell samples (mother and infant) were stored in liquid nitrogen until testing.

2.2.3 Cord blood mononuclear cell (CBMC) isolation

Mononuclear cells were isolated from cord blood by density centrifugation on Ficoll-Paque^{TM} Plus at 1 000 g for 30 min at room temperature. The plasma layer was removed and centrifuged at 850 g to remove platelets using a Sigma 4K15 centrifuge, aliquoted and stored at -70°C. The CBMC layer was removed and washed twice in PBS. Contaminating erythrocytes were lysed using 0.15 M NH_4Cl, 10 mM $KHCO_3$ and 1 mM sodium EDTA, pH 7.0. The cell suspension containing lysed cells was washed a further two times in PBS and the viable cell numbers determined by trypan blue exclusion. CBMC resuspended to a final concentration of $3x10^6$ cells/ml in RPMI containing 1% L-glutamine were used in the IL-2 bioassay. Cells were either resuspended in DMSO/FCS at $1\text{-}5x10^6$ cells/ml or as a cell pellet and stored in liquid nitrogen.

2.2.4 Determination of mothers' infection status

HIV-1 antibody tests (Abbott Laboratories, Abbott Park, Illinois, USA) were conducted on mothers who were unaware of their HIV-1 status but arrived at Chris Hani Baragwanath Hospital, Soweto, South Africa to deliver their infants.

2.2.5 Qualitative virus determination

Standard diagnostic DNA PCR tests (Roche Diagnostic Systems, Inc, New Jersey, USA) were carried out on infants at <48 hours, 6 weeks, and 3 months.

2.2.6 Quantitation of maternal and infant viral loads

Plasma HIV-1 RNA levels were measured using the Roche Amplicor RNA Monitor assay (Roche Diagnostic Systems, Inc, New Jersey, USA) with a lower detection limit of 400 RNA copies/ml.

2.2.7 Quantitation of mothers' CD4 T-cell counts

CD4 T-cell counts were determined using the commercially available FACSCount System from Becton Dickinson (San Jose, CA).

2.2.8 Enzyme linked immunosorbent assays

Maternal and infant (cord blood) plasma levels of the immune activation factors β_2-m and sL-selectin were determined using the commercially available Quantikine ELISA assays kits obtained from R&D Systems Inc (Minneapolis, MN, USA) as described by the manufacturer. β_2-m levels were determined using undiluted plasma, whereas sL-selectin determination required a 100-fold dilution of the samples. Neopterin was quantitated in undiluted plasma using an Immunotech ELISA system (Beckman Coulter, France). The minimum detectable dose of β_2-m is less than 0.2 μg/ml, for sL-selectin less than 0.3 ng/ml and for neopterin 0.2 ng/ml.

Maternal and infant (cord blood) plasma levels of GM-CSF were determined using the commercially available high sensitivity (h)GM-CSF Biotrak ELISA system (Amersham Biosciences UK Ltd). Samples were tested undiluted for GM-CSF with the minimum detectable level of GM-CSF being 0.1 pg/ml.

IL-7 levels were determined undiluted using the commercially available Quantikine high sensitivity R&D ELISA system (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The minimum detectable level for IL-7 being less than 0.1 pg/ml.

Levels of CXCL12 were determined undiluted using the commercially available a Quantikine R&D ELISA system (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The minimum detectable level of CXCL12 is 18 pg/ml.

Maternal and infant (cord blood) plasma levels of IL-10 were determined using the commercially available high sensitivity Biotrak ELISA system (Amersham Biosciences UK Ltd). IL-10 levels were determined from plasma samples diluted 10-fold with the minimum detectable dose being 1 pg/ml.

CCL3, CCL4 and CCL5 cord blood plasma levels were determined using the commercially available Quantikine ELISA assays kits obtained from R&D Systems Inc (Minneapolis, MN, USA) as described by the manufacturer. CCL5 levels were determined from infant plasma samples diluted 50-fold. CCL3 and CCL4 levels were determined from undiluted infant plasma samples. The minimum detectable dose of CCL3 is less than 10 pg/ml, for CCL4 less than 11 pg/ml and for CCL5 less than 2 pg/ml.

2.2.9 IL-2 production and ELISA assay

CBMC, ($2x10^5$ cells/well) were unstimulated or stimulated with a cocktail of five synthetic HIV-1 envelope (Env) peptides (shown in Appendix D, Table 1) at a final concentration of 2.5 μM to measure HIV-1 specific responses and with PHA (positive control) used at a final concentration of 12.5 μg/ml. For each stimulus, cultures were run in duplicate. After 1 hour incubation at 37^0C, pooled human serum (10%) was added to each well. In addition, anti-IL-2 receptor antibody (monoclonal anti-TAC) was added to the cultures at a concentration of 2 mg/ml to block IL-2 consumption. After 7 days incubation at 37^0C in a moist, 5% CO_2 atmosphere, culture supernatants were harvested and stored at -20^0C until testing. IL-2 was quantitated in culture supernatants using the Quantiglo human IL-2 ELISA system (R&D Systems) with a chemiluminescent detection step, to allow maximum sensitivity. Results are expressed as the ratio of IL-2 produced among the stimulated cultures to that of unstimulated cultures. All ratios >3 were considered as a positive response to a particular stimulus.

The selection of the HIV-1 stimulus (HIV-1 gp120 peptides T1, T2 and TH4.1 of conserved regions, and P18 MN and P18 IIIB of variable regions) was based on previous

studies which have identified these peptides to be broadly immunogenic across MHC haplotypes (Cease *et al.*, 1987; Hale *et al.*, 1989; Berzofsky *et al.*, 1991). T-helper cell responses to these peptides have also been documented in several, independent populations of HIV-1 EU individuals (Clerici *et al.*, 1991; Clerici *et al.*, 1992; Clerici *et al.*, 1994a; Mazzoli *et al.*, 1997; Kuhn *et al.*, 2001a).

2.2.10 The *in vitro* ability of stimulated cord blood cells to produce haematopoietic growth factors

The ability of CBMC ($3x10^6$ cells/ml in RPMI medium containing 1% L-glutamine) to produce GM-CSF, CXCL12 and IL-7 following a 24 hour stimulation in response to medium (unstimulated), a cocktail of synthetic HIV-1 Env peptides (final concentration of 2.5 μM) or PHA (final concentration 12.5 μg/ml) was determined using (h)GM-CSF Biotrak, CXCL12 Quantikine and IL-7 Quantikine high sensitivity ELISA systems, according to the manufacturer's instructions. Cell supernatants were diluted 10-fold for GM-CSF produced in response to medium and Env and 100-fold for responses to PHA. Two-fold dilutions of the cell supernatants were carried out for CXCL12 and IL-7 levels determinations in response to medium, Env and PHA.

2.2.11 DNA preparation and quantitation

DNA was isolated from $3x10^6$ CBMC using the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany). Cell pellets were resuspended in 200 μl PBS and DNA extracted according to the manufacturer's instructions. The concentration of total DNA (μg/ml), for each sample, was determined spectrophotometrically by measuring the absorbance at 260 nm using a Spectronic 1001 spectrophotometer (Bausch and Lomb).

2.2.12 Real-time PCR for the quantitation of single joint T-cell rearrangement excision circles

Thymic T-cell production was evaluated by measuring single joint TRECs (sjTRECS) using real-time PCR based on SYBR-Green fluorescence. One ng of DNA from each sample was amplified using a PCR master mix consisting of 1xSYBR green buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTP, 0.05 μM each of forward,

5'CCTCTGTCAACAAAGGTGAT-3' and reverse, 5'-GTGCTGGCATCAGAGTGTGT-3' primers (L. Al-Harthi, Rush University), 0.01 U/μl AmpErase uracil-N-glycosylase (UNG) and 0.02 U/μl AmpliTaq Gold DNA polymerase (SYBR green PCR core reagents purchased from PE Biosystems, Foster City, CA, USA). PCR cycling included an initial step at 50°C for 2 min and 95°C for 10 min to activate AmpErase UNG then 50 cycles of DNA amplification at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec using a 5700 Sequence Detection System (Applied Biosystems). Cloned sjTRECs at various copies (0, 500, 5 000, 50 000, 500 000 and 5 000 000) were co-amplified with the samples to generate a standard curve from which the DNA concentration from the samples was extrapolated using GeneAmp SDS software (PE Applied Biosystems). DNA samples were amplified in duplicate and the data expressed as TREC copies/μg DNA.

2.2.13 Statistical analysis

The paired-samples *t*-test was used to test for differences between means for results from the *in vitro* study.

Concentrations of the activation markers, immunomodulatory and immunoregulatory cytokines/chemokines were compared between the groups using the non-parametric Mann-Whitney U test. Spearman's rank correlation coefficient was used to describe associations between viral load and the factors. The significance of differences between mothers' and infants' immune factors was tested using the Wilcoxon signed rank test for paired data. Fisher's exact test was used to compare the proportions (frequencies) of envelope responder (Env^+) and non-responder (Env^-) infants across the PEP study cohort. Statistical analyses were performed using SPSS software (version 11.0, SPSS Inc., Chicago, IL). All statistical tests were two-tailed and considered significant at $p<0.05$.

CHAPTER THREE

Varied replication ability of M502L, a primary HIV-1 isolate, in the presence of NVP and AZT in primary cultures

3.1 Introduction

At any one time only a small fraction of the circulating $CD4^+$ T-lymphocytes in the peripheral blood are in the process of division with the vast majority in either a quiescent (G_0) or a post-mitotic state (Crabtree, 1989). Thus, the majority of the potential target cells ($CD4^+$ T-lymphocytes) in the peripheral blood of HIV-1 infected individuals are in a resting (G_0) state (Zack, 1995; Davis *et al*., 2001). T-cell activation as well as induction of specific host cellular factors is required for the productive infection of primary $CD4^+$ T-cells (Kinoshita *et al*., 1998). Peripheral blood mononuclear T-cells are functionally and phenotypically heterogeneous and can be divided into naïve ($CD4^+CD45RA^+$) and memory ($CD4^+CD45RO^+$) subsets. Naïve T-cells have been shown to be mildly resistant to HIV-1 infection *in vitro* and to harbour less virus in infected patients *in vivo* (Schnittman *et al*., 1990, Cayota *et al*., 1993) whilst memory cells are reportedly hyper-responsive by virtue of enhanced expression of cytokine receptors and adhesion molecules (Wallace and Beverley, 1990). It has been suggested that the milieu of the cells quantitatively influences the support for viral replication and although unstimulated naïve cells have a significantly smaller capacity for HIV-1 replication than memory cells, the production of growth factors may enhance the production from naïve cells without the need to trigger cell cycle entry (Eckstein *et al*., 2001). Furthermore, the possibility exists that cellular events triggered by virus-associated factors, such as auxiliary proteins, might induce a replication-permissive state (Eckstein *et al*., 2001). The ability of HIV-1 to infect non-dividing cells undoubtedly plays an important role in viral pathology *in vivo*, particularly with respect to newborn infants where 80-90% of peripheral $CD4^+$ T-cells express the naïve phenotype compared with 40-60% in adult cells (D'Arena *et al*., 1998).

Although the HIVNET 012 trial, (single-dose NVP given to both mother and infant, to reduce HIV-1 transmission from mother-to-child), gave new hope to developing counties in their fight against the spread of HIV-1, the emergence of resistance stains in mother and child pairs (Flys *et al*., 2005) has put the use of this antiretroviral agent into question. While the use of antiviral prophylaxis that includes 3 or more drugs from at least two

classes has reduced the transmission rate in industrialized countries to <2% (Mofensson and McIntyre, 2000), short-course NVP intervention has been shown to lower the rate of transmission of HIV-1 to between 10.6% and 13% (Ayouba *et al*., 2003) but not to reduce it to the levels achieved when using multiple drug regimens. The mechanisms affording protection from MTCT of HIV-1 using short-course regimens are poorly understood. However, the fact that a subgroup of infants with appreciable cord blood plasma viral loads and exposed to single-dose NVP-AZT remain uninfected whilst others exposed to single-dose antiviral prophylaxis become infected (C.T. Tiemessen, unpublished data) leads one to question whether (i) HIV-1 is transmitted to the infant but is unable to replicate due to the cellular milieu or the immature status of the infants' immune system, or (ii) whether single-dose antiretroviral drugs act directly on the virus by preventing reverse transcription.

In an attempt to understand how the timing of administration of short-course antiretroviral therapy (ART) affects viral infection outcome in MTCT of HIV-1 (simulating the situation where infants are exposed to the mothers dose of NVP: DART and where infants are only exposed to NVP following birth: PEP), we used a primary HIV-1 isolate (M502L), that has demonstrated good growth characteristics in both PBMC as well as CBMC, to infect blood mononuclear cells isolated from different healthy adult donors. The ability of this virus to replicate when NVP or AZT were added either prior or subsequent to the infection was assessed in PHA-stimulated cells. Furthermore, we attempted to address the question of whether virus can be rescued from 'unstimulated' cells following a short exposure of NVP or AZT, when added in the above two scenarios. To reflect the true *in vivo* setting as closely as possible experiments were designed that used infection of cultures of healthy adult donor PBMC with a primary HIV-1 isolate (M502L) rather than using cell lines or laboratory strains of HIV-1. Furthermore, the drug concentrations used related to the highest plasma therapeutic ranges measured from cord blood (10 μM[*]) following a single-dose NVP (200 mg) given to the mother at the onset of labour and concentrations of AZT that would potently inhibit HIV-1 replication, namely 20 μM AZT.

[*]Footnote information on page 73

*For mothers given NVP on the day of delivery mean neonatal NVP plasma concentrations at 24 hours have been determined to be 1.39 mg/L (5.2 μM). In mothers treated during pregnancy, a mean NVP concentration of 3.42 mg/l (12.8 μM) has been determined in cord blood (Taylor *et al*., 2000). Median cord plasma AZT concentrations of 0.7 mg/L (2.6 μM) (range 0.16 mg/L (0.6 μM) to 2.27 mg/L (8.5 μM)) have been reported from a study conducted on HIV-1 infected mothers receiving ART therapy for clinical indications and that received AZT intravenously during labour (Chappy *et al*., 2004).

3.2 Results

3.2.1 Identifying the growth and inhibitory conditions for M502L which best mimic the *in vivo* setting where an HIV-1 seropositive mother at the onset of labour is given a single-dose NVP or the infant is given PEP

3.2.1.1 p24 Antigen production at different multiplicities of infection

The maternal viral load at delivery may be useful for determining the risk of transmission (Sperling *et al*., 1996; Cao *et al*., 1997; Mofenson, 1997; Mofenson *et al*., 1999) however it does not predict transmission. While some infants born to HIV-1 infected mothers with a high viral load remain uninfected, others born to mothers with a low viral load become infected. It is therefore difficult to predict what level of maternal HIV-1 exposure a newborn infant has had. In order to optimize our *in vitro* assay a number of experiments were set-up using PHA-stimulated PBMC from a healthy donor that were infected at different moi. Figure 3.1 demonstrates M502L growth in PHA-stimulated PBMC from a healthy donor at various moi. High multiplicities of infection (moi) (0.1 and 0.05) yielded the highest levels of p24 antigen (p24-Ag). Since the total amount of p24-Ag measured in culture may be affected by cell death, replication kinetics of the virus (including release of non-infectious virions) and multiple rounds of virus replication, we chose, based on the p24-Ag levels and protocols used to assess antiviral agents for activity against HIV-1, to infect donor PBMC for all subsequent experiments with a moi of 0.01 (~2000 $TCID_{50}$) (or 0.005 (~1000 $TCID_{50}$), where specified). While this moi did not yield the highest p-24 Ag levels we selected this moi as (i) it yielded levels of p24-Ag *in vitro* that could be measured using an ELISA (quantitatively measured virus replication of the HIV-1 primary isolate M502L) and (ii) viral replication could be inhibited by the drug concentrations selected (in the higher range of those measured in cord blood plasma following maternal dosing).

3.2.1.2 Kinetics of M502L growth in PHA-stimulated and unstimulated PBMC

We examined the growth kinetics of M502L (~2000 $TCID_{50}$, moi ~0.01) in PHA-stimulated (Figure 3.2i and 3.2ii) and unstimulated PBMC (Figure 3.2iii and 3.2iv) following viral adsorption for 1.5 hours (Figure 3.2i and 3.2iii) and 24 hours (Figure 3.2ii

and 3.2iv). Peak p24-Ag levels were measured in PHA-stimulated PBMC after 14 days of infection. Although higher p24-Ag levels were measured following a 24 hour adsorption period at day 14, higher p24-Ag levels were not measured at all sampling points (days p.i.) which suggested that there was no significant advantage to incubating PBMC with M502L beyond 1.5 hours. As expected, M502L was unable to replicate effectively in unstimulated cultures (unstimulated PBMC infected immediately after isolation) after either a 1.5 or 24 hour adsorption period (Figure 3.2iii and Figure 3.2iv). Based on this data a standard incubation period of 1.5 hours (~2000 $TCID_{50}$, cell concentration $2x10^5$ cells/well) was adopted for subsequent experiments.

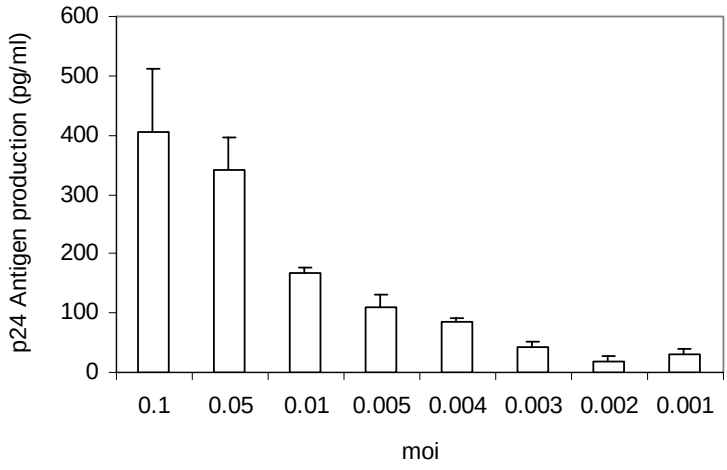


Figure 3.1 Growth of M502L in PHA-stimulated PBMC (24 hours) from a healthy donor following infection at different moi for 1.5 hours at 37°C. Cultures were given fresh growth medium on day 3. On day 7 supernatants were harvested and p24-Ag production measured by ELISA. Bars denote the p24-Ag production measured (expressed as mean values of triplicate samples ± SD).

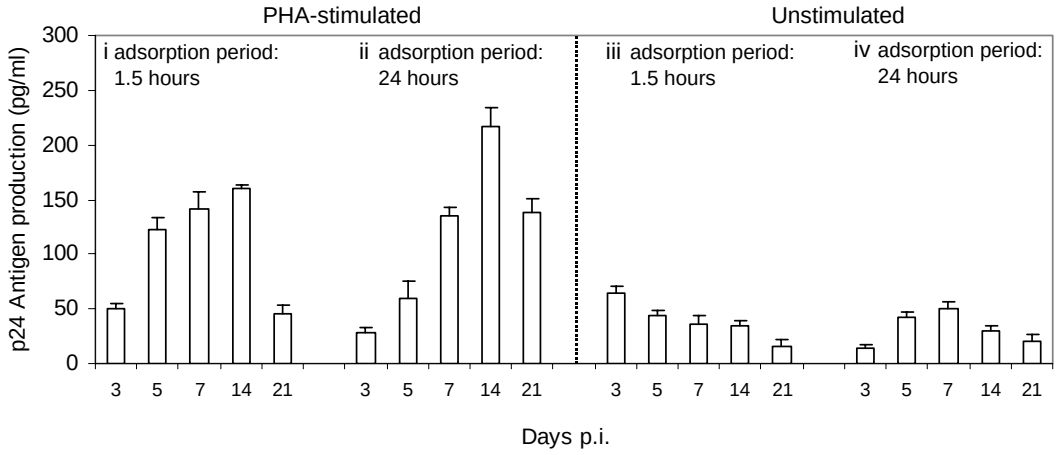


Figure 3.2 Growth kinetics of M502L (~2000 $TCID_{50}$) following infection of PHA-stimulated (i and ii) and unstimulated PBMC (iii and iv) for 1.5 hours (i and iii) or 24 hours (ii and iv). Cultures were given fresh medium on days 3, 10, and 17 and fresh donor cells (stimulated or unstimulated) were added on days 7 and 14. Bars represent the mean p24-Ag produced by triplicate samples ± SD.

3.2.1.3 Inhibition of HIV-1 infection by addition of NVP or AZT

Figure 3.3 depicts the inhibitory effect of increasing concentrations of NVP and AZT on M502L growth. Increasing concentrations of NVP (0-10 μM) and AZT (0-50 μM) were associated with an increased inhibition of viral replication. At the higher concentrations, p.i. addition of NVP and AZT, (10 μM NVP and 50 μM AZT), were more effective in inhibiting growth than pre-infection addition of NVP or AZT (Table 3.1). Since we were trying to mimic the *in vivo* scenario where mothers are given a single 200 mg dose of NVP at the onset of labour, it was decided to select a NVP concentration which reflects levels measured from cord blood. Mean neonatal plasma and cord blood concentrations of 5.2 μM to 12.8 μM have been reported from mothers treated with NVP on the day of delivery or during pregnancy, respectively (Taylor *et al*., 2000), while a mean cord blood AZT concentration of 2.6 μM has been measured from infants whose mothers received ART at the time of delivery and who received intravenous AZT (2mg/kg) during labour (Chappuy *et al*., 2004). *In vitro* protocols assessing anti-HIV-1 activity tend however to use drug concentrations as high as 10 μM. Ten μM AZT has also been used in an *in vitro* study to examine the phosphorylation rate of AZT in foetal and maternal PBMC (Agbaria *et al*., 2003). For experimental purposes therefore we considered (i) 10 μM NVP to adequately reflect concentrations to which an infant would be exposed and (ii) 20 μM

AZT to effectively inhibit HIV-1. In order to assess the efficiency of 10 μM NVP and 20 μM AZT in inhibiting replication of M502L in PHA-stimulated and unstimulated healthy donor PBMC, we incubated cells with NVP or AZT either prior (pre-infection) or subsequent to infection (p.i.) and compared M502L growth to that of NVP or AZT unexposed cells infected with M502L (control cells). Infection of PHA-stimulated PBMC with M502L followed 24 hours later with the addition of either NVP (Figure 3.4ii, Table 3.2,) or AZT (Figure 3.4iv, Table 3.2) reduced the ability of M502L to replicate but did not completely inhibit M502L growth (Table 3.2). NVP or AZT added pre-infection (Figure 3.4iii and 3.4v, respectively, Table 3.2) inhibited M502L growth but not to levels achieved when the drugs were added p.i.. Overall, addition of NVP or AZT either p.i. or pre-infection reduced M502L growth in PHA-stimulated PBMC. As expected and as demonstrated in section 3.2.1.2 unstimulated donor PBMC supported low levels of M502L replication. These results suggest that some activation of PBMC occurs through the isolation procedures and may be responsible for low grade replication of virus. It is interesting to note that in unstimulated cultures NVP or AZT added pre-infection were more effective in preventing M502L growth than if added p.i. (Figure 3.5; Table 3.2).

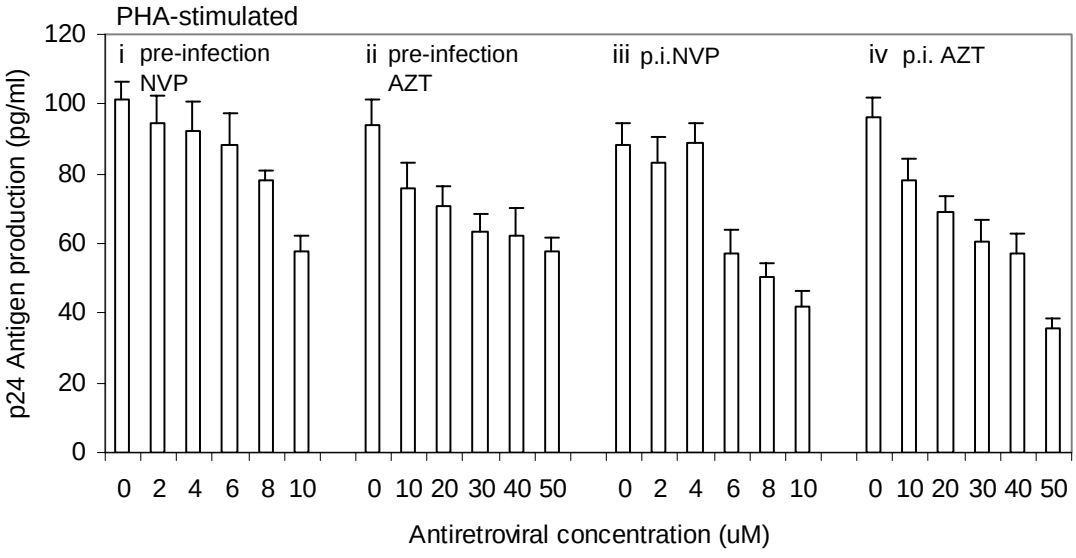


Figure 3.3 Inhibitory effect of increasing concentrations of NVP and AZT on M502L determined after 7 days of culture. The mean p24-Ag levels (pg/ml) of triplicate samples is presented (± SD).

Table 3.1 Percentage inhibition (relative to HIV-1 controls) of M502L replication in PHA-stimulated PBMC following addition of increasing concentrations of NVP and AZT either pre-infection or p.i.

Drug concentration (μM)	Inhibition (%)	
NVP	**p.i.**	**pre-infection**
2	6	6
4	-0.6	8
6	35	13
8	43	23
10	53	43
AZT		
10	19	19
20	28	25
30	37	32
40	41	34
50	63	39

Shaded figure indicates no inhibition but stimulation (not significant)

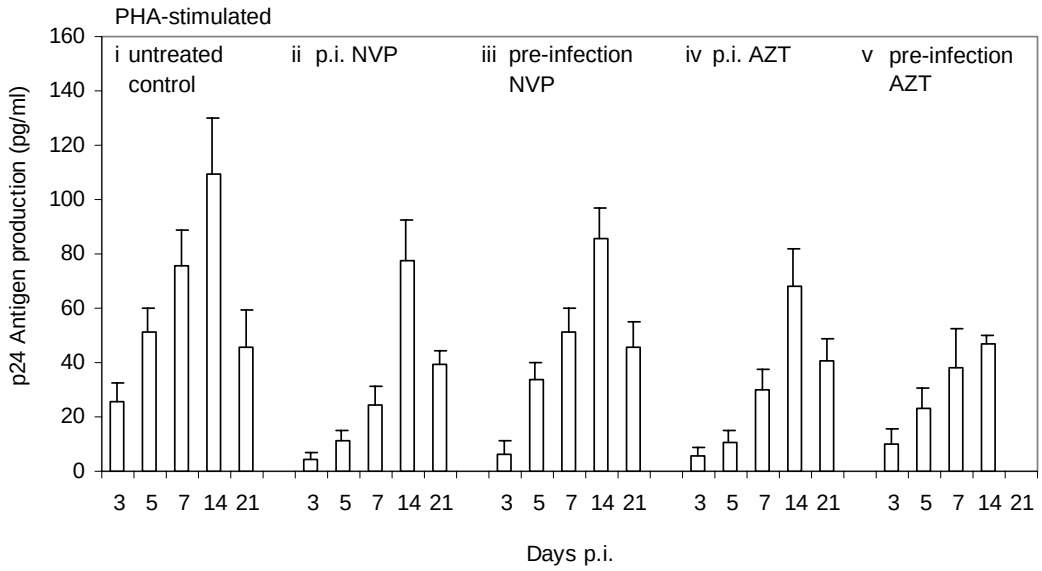


Figure 3.4 The ability of 10 μM NVP or 20 μM AZT to inhibit the replication of M502L in PHA-stimulated PBMC when added either pre- or post-infection. The mean p24-Ag production of triplicate samples (± SD) is presented for PBMC infected with M502L only (i), PBMC infected for 1.5 hours and NVP added 24 hours p.i. (ii), PBMC incubated for 24 hours with NVP added pre-infection (iii), PBMC infected for 1.5 hours and AZT added 24 hours p.i. (iv) and PBMC incubated for 24 hours with AZT added pre-infection (v) over a period of 21 days. Cultures were given fresh growth medium on days 3, 10, and 17 and fresh donor cells (PHA-stimulated) added on days 7 and 14. The 21 day sample (v) was not tested.

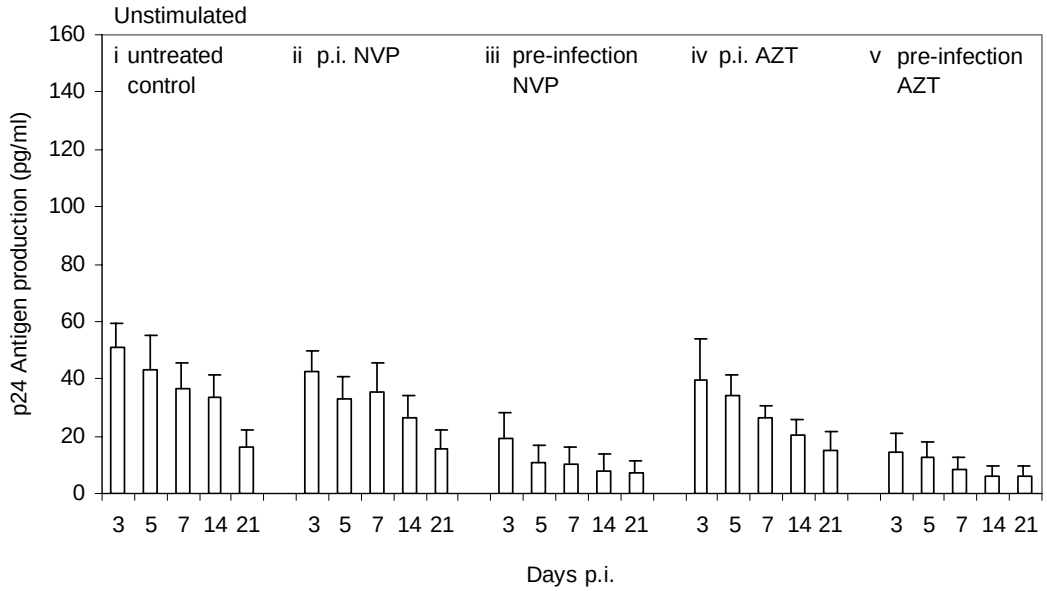


Figure 3.5 The ability of NVP or AZT to inhibit the replication of M502L in freshly isolated PBMC (no stimulation with PHA), when added either pre- or post-infection. The mean p24-Ag production of triplicate samples (± SD) is presented for PBMC infected with M502L only (i), PBMC infected for 1.5 hours with M502L and NVP added 24 hours p.i. (ii), PBMC incubated for 24 hours with NVP added pre-infection (iii), PBMC infected for 1.5 hours with M502L and AZT added 24 hours p.i. (iv) and PBMC incubated for 24 hours with AZT added pre-infection (v) over a period of 21 days. Cultures were given fresh growth medium on days 3, 10, and 17 and fresh donor cells (unstimulated) added on days 7 and 14.

Table 3.2 Percentage inhibition (relative to HIV-1 controls) of M502L replication in PHA-stimulated and unstimulated PBMC following addition of 10 μM NVP or 20 μM AZT either pre-infection or p.i.

Days p.i.	Inhibition (%)			
	p.i. NVP	pre-infection NVP	p.i. AZT	pre-infection AZT
PHA-stimulated PBMC				
3	83	76	79	61
5	78	33	79	55
7	68	32	61	49
14	29	22	38	57
21	14	0	11	ND
Unstimulated PBMC				
3	16	62	22	71
5	23	75	21	71
7	4	73	29	78
14	21	76	40	83
21	4	56	8	65

ND – not determined

3.2.1.4 Effect of drugs on cell viability in the absence of HIV-1 infection

To determine to what extent NVP or AZT affected the viability of healthy donor PBMC in the absence of HIV-1 infection, the viability of stimulated PBMC from two separate donors (Figure 3.6A and 3.6B, respectively) was evaluated using trypan blue exclusion staining following a week of culture in the presence of increasing concentrations of antiviral drugs. Cell viability in donor 1 did not differ significantly (p>0.05) with respect to different drug treatments or drug concentrations. In donor 2 only 20μM AZT was found to significantly affect cell viability (p=0.015). Differences between donors at the higher drug concentrations (NVP and AZT) were significant (15 μM NVP p=0.001; 15 μM AZT p=0.021 and 20 μM AZT p=0.008).

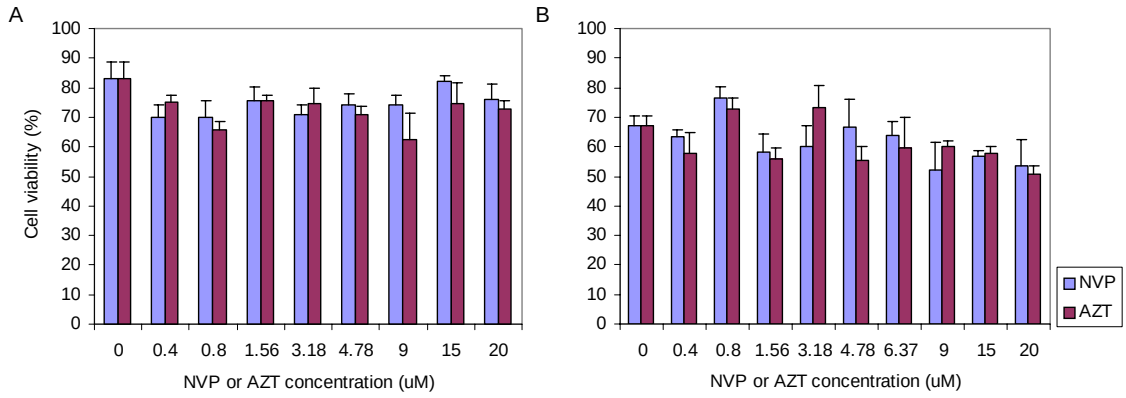


Figure 3.6 Effect of increasing concentrations of NVP and AZT on the viability of PHA-stimulated PBMC. The viability of the PBMC was determined after 7 days of growth in the presence of the antiretroviral drugs. The bars denote the percentage of viable cells at each antiretroviral concentration tested (expressed as mean values of triplicate samples ± SD).

All donors (A-G) used in subsequent experiments were tested for effects of NVP and AZT on cell viability. All showed similar cell viability profiles to donor A indicating that the concentrations selected for study, (10 μNVP and 20 μM AZT), did not significantly affect cell survival and so did not confound interpretation of drug effects on HIV-1 replication.

3.2.2 *In vitro* studies to determine how timing of addition of NVP or AZT influences growth of M502L

Figure 3.7 schematically represents the *in vitro* experimental approach we adopted to simulate the *in vivo* setting where an infant is exposed to maternal HIV-1 and single-dose NVP either because the mother is given a dose at the onset of labour (DART) or the infant is given a dose of NVP within 24 hours of birth (PEP).

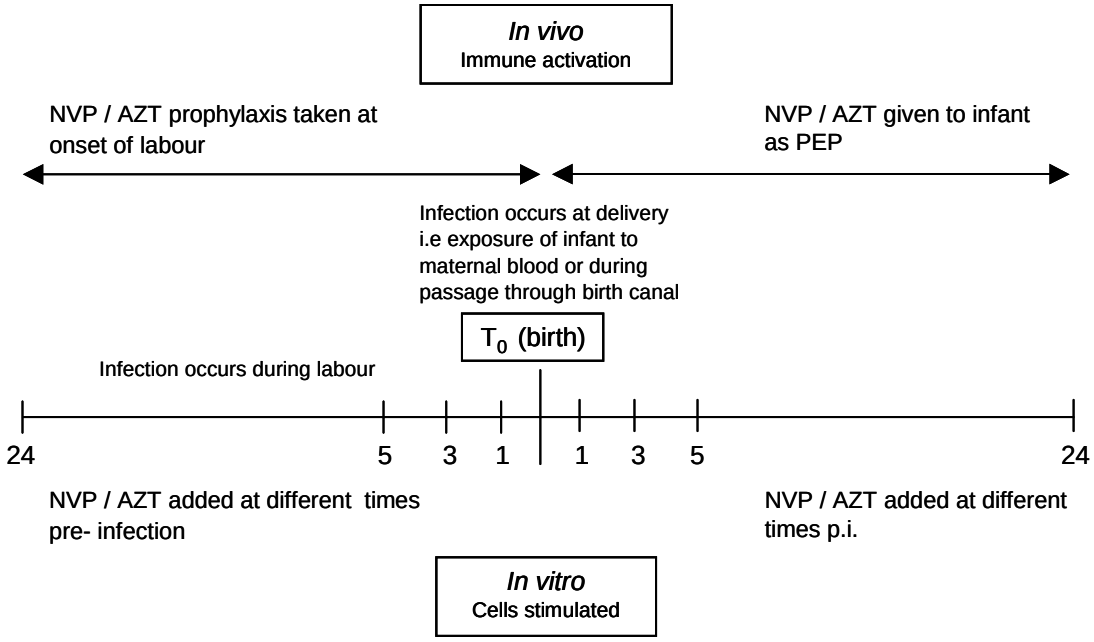


Figure 3.7 Schematic diagram depicting the *in vivo* timing of ART administration to either the mother (at the onset of labour: DART) or to the infant (within 24 hours of birth: PEP) and our *in vitro* experimental approach to mimic the *in vivo* setting. The times of addition of NVP or AZT have been arbitrarily selected for this diagram and merely indicate addition pre-infection (within 24 hours) (that is, mother is given NVP at onset of labour) or p.i. (within 24 hours) (that is, infant given PEP 24 hours after birth).

3.2.2.1 Replication kinetics of M502L and drug inhibition of HIV-1 replication vary in PBMC isolated from different donors

The ability of the primary HIV-1 isolate M502L to replicate in PBMC and the ability of NVP or AZT to inhibit replication was highly variable as shown in Figure 3.8 to Figure 3.11.

3.2.2.1.1 Donor A

Figure 3.8 demonstrates the replication kinetics of M502L in PHA-stimulated PBMC at a moi of 0.01 (Figure 3.8A) and at a moi of 0.005 (Figure 3.8B) as well as the ability of 10 μM NVP and 20 μM AZT to inhibit replication in these cultures over a period of 7 days. M502L infected at a moi of 0.01 replicated with great efficiency in donor A with NVP (10 μM) and AZT (20 μM) inhibiting M502L by 26% and 44%, respectively, by day 4 and by 64% and 77%, respectively, by day 7 (Table 3.3, Figure 3.8A,). This inhibition was significant (Table 3.3). Growth of M502L at the lower moi (0.005) was not as efficient as observed with the higher moi (0.01) and addition of NVP and AZT resulted in only slight insignificant reductions in replication measured 4 days after treatment, with the greatest reductions measured 7 days after treatment (Table 3.3, Figure 3.8B), however this inhibition was not statistically significant. What these results do highlight is the importance of the input moi in establishing productive infection of cultures.

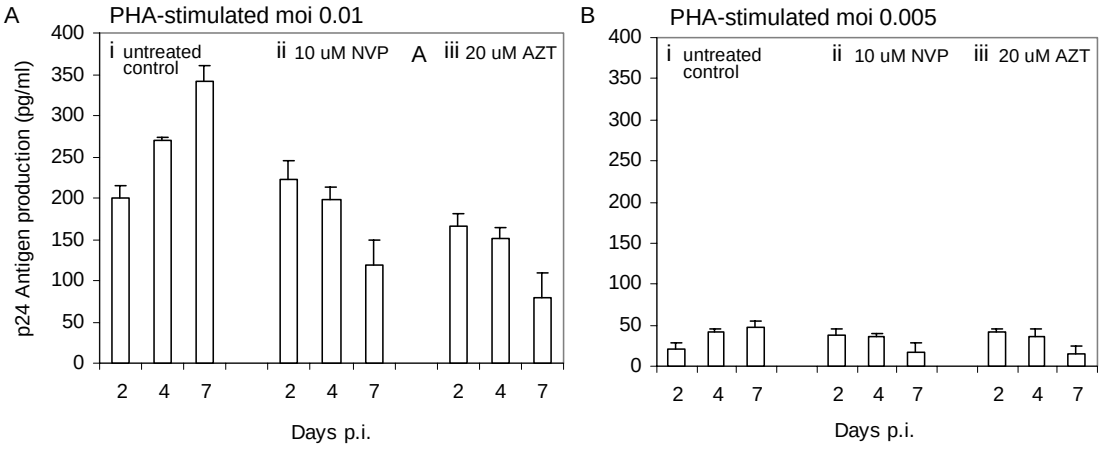


Figure 3.8 Replication kinetics of M502L in PHA-stimulated PBMC from donor A. PBMC were infected for 1.5 hours at a moi of 0.01 (Figure A) and a moi of 0.005 (Figure B). The ability of 10 μM NVP (ii) and 20 μM AZT (iii) to inhibit M502L replication were compared to uninfected control cultures (i). The bars represent the mean p24-Ag production of triplicate samples (± SD) measured on different days p.i..

Table 3.3 Percentage inhibition (relative to controls) of M502L (moi 0.01 and 0.005) in PHA-stimulated PBMC isolated from a donor A and treated with either NVP (10 μM) or AZT (20 μM)

Days p.i.	Inhibition (%) and statistical significance (*P*)			
	NVP	***P****	**AZT**	***P****
PHA-stimulated PBMC (moi 0.01; ~2000 $TCID_{50}$)				
2	-11	>0.05	17	>0.05
4	26	0.013	44	0.004
7	64	0.001	77	0.005
PHA-stimulated PBMC (moi 0.005; ~1000 $TCID_{50}$)				
2	-85	>0.05	-100	>0.05
4	11	>0.05	11	>0.05
7	64	>0.05	67	>0.05

Shaded figures indicate no inhibition but stimulation.

* Sample means were compared using the paired-samples *t*-test. Statistical tests were two-tailed and significant at $p<0.05$

3.2.2.1.2 Donor B

Figure 3.9 and Table 3.4 presents the growth (p24-Ag production after 7 days) of M502L in donor B following addition of NVP or AZT at specific time periods pre-infection and p.i.. Addition of NVP and AZT p.i. had no significant effect on inhibiting M502L growth or enhancing it. Pre-infection NVP however significantly enhanced M502L growth if added 5 to 21 hours prior to infection. Pre- and post-infection AZT had no effect on M502L growth as no statistical significance was demonstrated between treated and control cultures (Table 3.4).

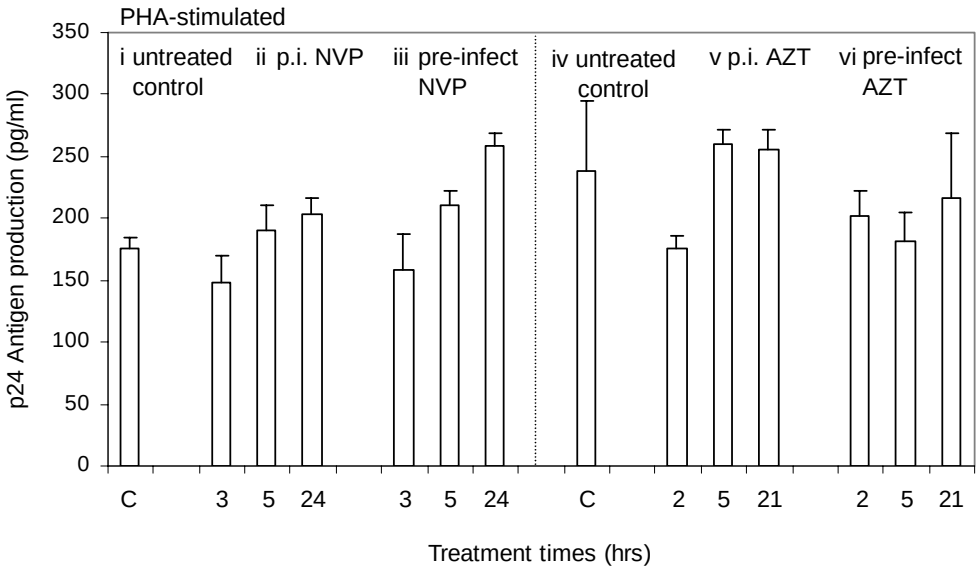


Figure 3.9 Replication of M502L in PHA-stimulated PBMC from donor B. PBMC treated at different time periods with NVP or AZT p.i. (ii or v) and pre-infection (iii or vi) were compared to untreated controls (i and iv). The bars represent the mean p24-Ag production of triplicate samples (± SD) after 7 days of culture.

Table 3.4 Percentage inhibition (relative to HIV-1 controls) of M502L replication in PHA-stimulated PBMC from donor B following addition of 10 μM NVP or 20 μM AZT either prior to or p.i.

Treatment times (hours)	**Inhibition (%) and statistical significance (*P*)**			
p.i.	**NVP**	***P****	**AZT**	***P****
2 (AZT) 3 (NVP)	15	>0.05	10	>0.05
5	-9	>0.05	-20	>0.05
24 (NVP)	-16	>0.05	-48	>0.05
pre-infection				
2 (AZT) 3 (NVP)	26	>0.05	15	>0.05
5	-10	0.005	24	>0.05
21 (AZT) 24 (NVP)	-7	0.001	9	>0.05

Shaded figures indicate no inhibition but stimulation

* Sample means were compared using the paired-samples *t*-test. Statistical tests were two-tailed and significant at $p<0.05$

3.2.2.1.3 Donor C

Interestingly, only AZT added immediately after high input infection (moi 0.01) significantly inhibited M502L growth (Figure 3.10Aiii, Table 3.5). At this moi (0.01) AZT significantly stimulated M502L growth when added 6 hours p.i. (Figure 3.10Aiii, Table 3.5) or for any length of time up to 24 hours, if added pre-infection (Figure

3.10Avi, Table 3.5). NVP added 0 to 6 hours before infection significantly enhanced 502L growth (Figure 3.10v, Table 3.5).

Addition of NVP or AZT either pre- or post-infection with a lower moi (0,005) was unable to inhibit viral replication. In fact, all treatments at this lower moi resulted in significant viral growth (Table 3.5).

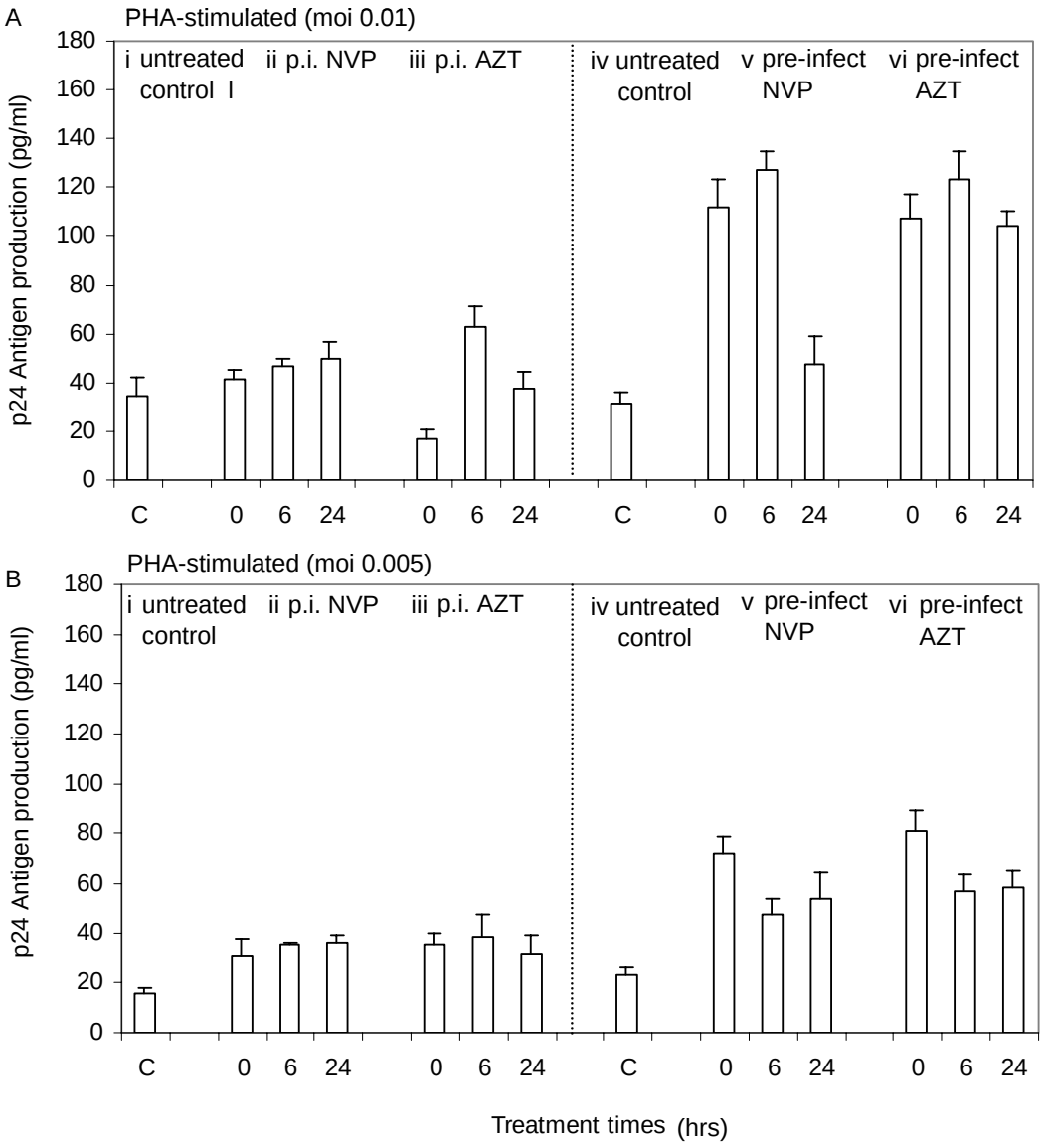


Figure 3.10 Growth of M502L in PHA-stimulated PBMC from donor C infected at a moi of 0.01 (A) and at a moi of 0.005 (B). PBMC were infected either prior (ii and iii) or subsequent (v and vi) to either addition of 10 μM NVP or 20 μM AZT at different time periods (0, 6 or 24 hours pre or p.i.) and compared to M502L replication in antiretroviral untreated cultures (i and iv). The bars represent the mean p24-Ag production of triplicate samples (± SD) following 7 days of culture.

Table 3.5 Percentage inhibition (relative to HIV-1 controls) of M502L replication in PHA-stimulated PBMC from donor C following addition of 10 μM NVP or 20 μM AZT either prior to or p.i.

Treatment times (hours)	Inhibition (%) and statistical significance (*P*)			
PHA-stimulated (moi 0.01; ~2000 $TCID_{50}$)				
p.i.	**NVP**	***P****	**AZT**	***P****
0	-20	>0.05	51	0.028
6	-35	>0.05	-82	0.027
24	-44	>0.05	-9	>0.05
pre-infection				
0	-253	0.011	-237	0.01
6	-300	0.004	-289	0.007
24	-50	>0.05	-228	0.001
PHA-stimulated (moi 0.005; ~1000 $TCID_{50}$)				
p.i.				
0	-97	0.035	-124	0.036
6	-127	0.004	-145	0.032
24	-131	0.022	-103	0.031
pre-infection				
0	-208	0.014	-249	0.004
6	-104	0.047	-146	0.026
24	-133	0.019	-153	0.003

Shaded figures indicate no inhibition but stimulation

* Sample means were compared using the paired-samples *t*-test. Statistical tests were two-tailed and significant at $p<0.05$

3.2.2.1.4 Donor D

In the case of donor D, where only NVP was added either pre-infection or p.i., yet another response to infection and NVP addition was demonstrated (Figure 3.11, Table 3.6). NVP added immediately after infection significantly reduced M502L growth, while later addition of NVP p.i. (5 and 24 hours) resulted in significant enhancement of growth (Figure 3.11ii, Table 3.6). Pre-infection addition of NVP in this donor was more effective in preventing M502L replication especially if cultures were treated with NVP up to 5 hours pre- infection (Figure 3.11 iv, Table 3.6)

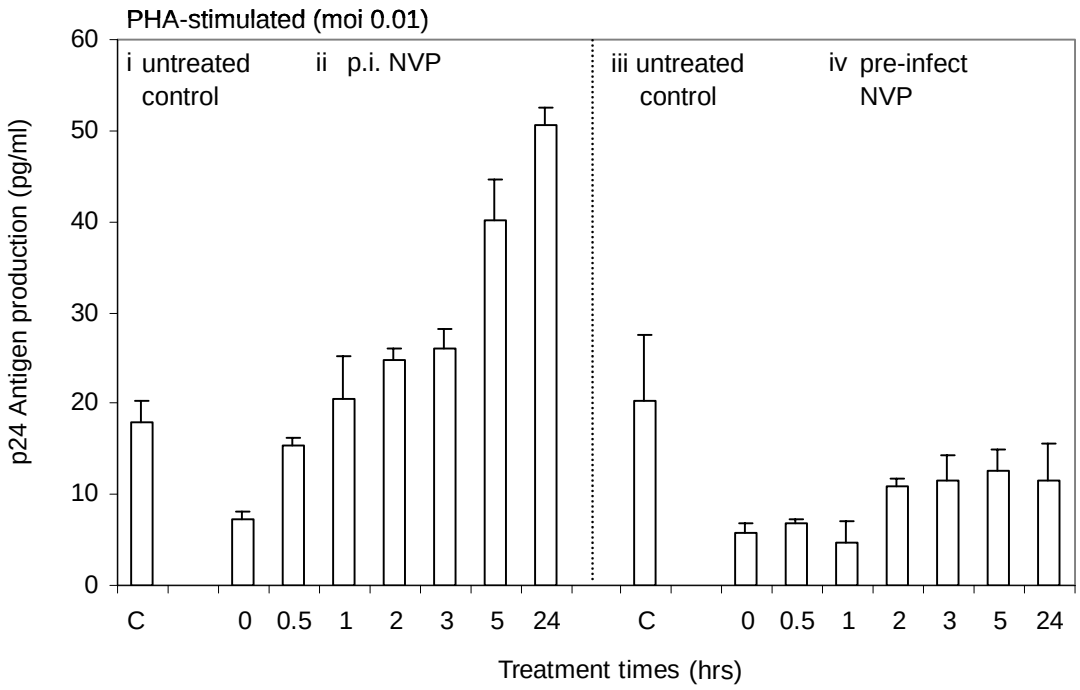


Figure 3.11 Growth of M502L in PHA-stimulated PBMC from donor D infected at a moi of 0.01, with 10 μM NVP added at different time periods (0, 0.5, 1, 2, 3, 5 and 24 hours) p.i. (ii) or pre-infection (iv). Growth was compared to M502L (NVP untreated) cultures (i and iii). The bars represent the mean p24-Ag production of triplicate samples (± SD) following 7 days of culture.

Table 3.6 Percentage inhibition (relative to HIV-1 controls) of M502L replication in PHA-stimulated PBMC from donor D following addition of 10 μM NVP or 20 μM AZT at different time periods either pre-infection or p.i.

Treatment times (hours)	**Inhibition (%) and statistical significance (*P*)**	
p.i. NVP	**Inhibition (%)**	***P****
0	59	0.008
0.5	15	>0.05
1	-150	>0.05
2	-38	>0.05
3	-45	>0.05
5	-123	0.032
24	-182	0.001
pre-infection NVP		
0	68	0.012
0.5	62	0.004
1	74	0.001
2	40	0.027
3	36	>0.05
5	29	0.04
24	36	>0.05

Shaded figures indicate no inhibition but stimulation

* Sample means were compared using the paired-samples *t*-test. Statistical tests were two-tailed and significant at $p<0.05$

3.2.2.1.5 Summary of M502L growth in different donors and inhibitory effects of NVP and AZT

Clearly, these results demonstrated significant differences between donors both with respect to their ability to support replication of a primary HIV-1 isolate as well as their ability to limit replication of the virus, based on timing of NVP or AZT addition relative to time of infection. The results are summarized in Table 3.7.

Table 3.7 Summary of M502L growth and ability of NVP (10μM) and AZT (20μM) to inhibit HIV-1 replication in different donors. Only statistically significant data are presented

Donor	Virus growth mean p24-Ag (pg/ml) (day 7)	Effect of drug on viral growth in PHA-stimulated cultures (day 7) (% inhibition or enhancement of viral growth (++))				Comments
	Untreated (control)	NVP pre	NVP p.i.	AZT pre	AZT p.i.	
A	342	ND	↓ (26-64)	ND	↓ (44-77)	moi=0.01; p.i. addition significantly reduced growth from days 4 to 7
	48	ND	NS	ND	NS	moi=0.005
B	186	↑ (+) (5-24 hours)	NS	NS	NS	moi=0.01; NVP p.i.; AZT pre- and p.i. had no significant effect; pre-infection NVP stimulated viral growth
C	33	↑ (++) (0-6 hours)	NS	↑ (++) (0-24 hours)	↓ (51) (0 hours) ↑ (++) (6 hours)	moi=0.01; viral growth only inhibited when AZT is added immediately following infection
	20	↑ (++)	↑ (++)	↑ (++)	↑ (++)	moi=0.005; significant enhancement at all time points (0-24 hours)
D	19	↓ (29-68) (0-5 hours)	↓ (59) (0 hours) ↑ (++) (5-24 hours	ND	ND	moi=0.01; NVP most effective when added prior to infection (up to 5 hours) or only if added immediately after infection (addition after 5 hours p.i. stimulated viral growth)

% Inhibition =[(p24-Ag control – p24-Ag of NVP / AZT treated culture)/p24-Ag control] x 100
ND – not determined
NS – viral growth not significantly inhibited or enhanced (relative to control)
↓ - decreased viral growth (% inhibition)
↑ - increased viral growth (+ = a greater than 25% increase in p24-Ag relative to control; ++ = a greater than 50% increase in p24-Ag relative to control)

3.2.3 *In vitro* studies to determine how timing of addition of NVP or AZT influences the ability to rescue M502L growth from unstimulated PBMC

Given that many infants born to HIV-1 seropositive mothers remain uninfected despite only a single short exposure to antiviral prophylaxis given to the mother at the onset of labour, the question of what the mechanisms are that protect these infants from infection remains unclear. We therefore experimentally attempted to mimic (depicted schematically in Figure 3.12) the scenario where infants are born to HIV-1 seropositive mothers, HIV-1 infects quiescent/non-dividing cells and single-dose NVP is given either at the onset of labour or as PEP. *In vivo*, the timing of when cells might be activated (events during labour, immune development or other infections) may be crucial to the establishment of HIV-1 infection in early life. The aim of the following section of work was to test *in vitro* which time points of PHA-stimulation, in cultures treated prior to or p.i. with NVP or AZT, could result in viral replication.

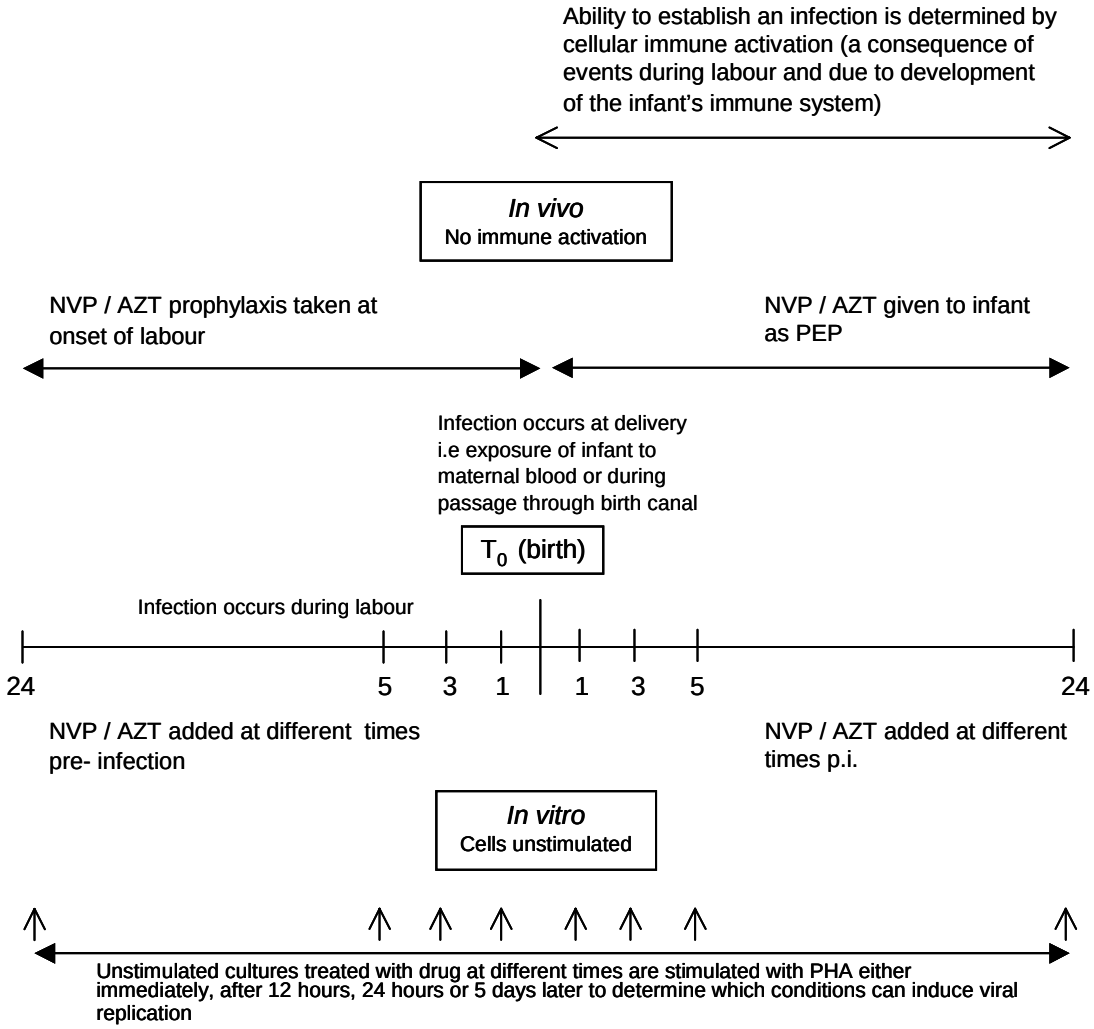


Figure 3.12 Schematic diagram depicting how timing of PHA-stimulation may influence viral infection outcome in unstimulated cultures treated pre or post-infection with NVP or AZT. The times of addition have been arbitrarily selected for this diagram and merely indicate addition pre-infection (within 24 hours) (that is, mother is given NVP at onset of labour) or p.i. (within 24 hours) (that is, infant given PEP 24 hours after birth).

3.2.3.1 Ability to rescue M502L following infection of 'unstimulated' PBMC with M502L

In this section of the work we investigated *in vitro* the conditions under which virus could be rescued from unstimulated PBMC treated with NVP or AZT added at different times. Thus, unstimulated PBMC isolated from three healthy donors (designated E, F and G) were infected with M502L and at specific time points after drug treatment the donor cultures were stimulated with PHA.

3.2.3.1.1 Donor E

Figure 3.13A presents the p24-Ag levels measured from cell culture supernatants collected after 5 days of incubation of PBMC from donor E following addition of NVP or AZT at different time intervals either prior to or subsequent to infection. Statistically, addition of NVP or AZT either pre- or post-infection did not significantly inhibit or enhance viral growth (Figure 3.13, Table 3.8).

Statistically, after PHA-stimulation, (p24-Ag levels measured from culture supernatants after 14 days), of infected cultures 5 days after NVP or AZT pre- or post-infection treatment, only those cultures marked with an asteriks (*) (Figure 3.13B) demonstrated viral growth. We however consider very low levels of virus to have been rescued from the 2 hour p.i. AZT culture only, as all other samples had p24-Ag values less than the cutoff value for the ELISA.

Table 3.8 Percentage inhibition (relative to HIV-1 controls) of M502L replication in unstimulated PBMC (donor E) following addition of 10 μM NVP or 20 μM AZT either pre-infection or p.i.

Treatment times (hours)	**Inhibition (%) and statistical significance (*P*)**			
p.i.	**NVP**	***P****	**AZT**	***P****
0	-0.90	>0.05	-5	>0.05
1	-63	>0.05	-0.2	>0.05
2	-58	>0.05	11	>0.05
3	-6	>0.05	46	>0.05
5	-13	>0.05	9	>0.05
24	3	>0.05	18	>0.05
pre-infection	**NVP**		**AZT**	
1	5.78	>0.05	0	>0.05
2	-50	>0.05	29	>0.05
4	-123	>0.05	-79	>0.05
5	-118	>0.05	-128	>0.05
24	-4	>0.05	-141	>0.05

Shaded figures indicate no inhibition but stimulation

* Sample means were compared using the paired-samples *t*-test. Statistical tests were two-tailed and significant at $p<0.05$

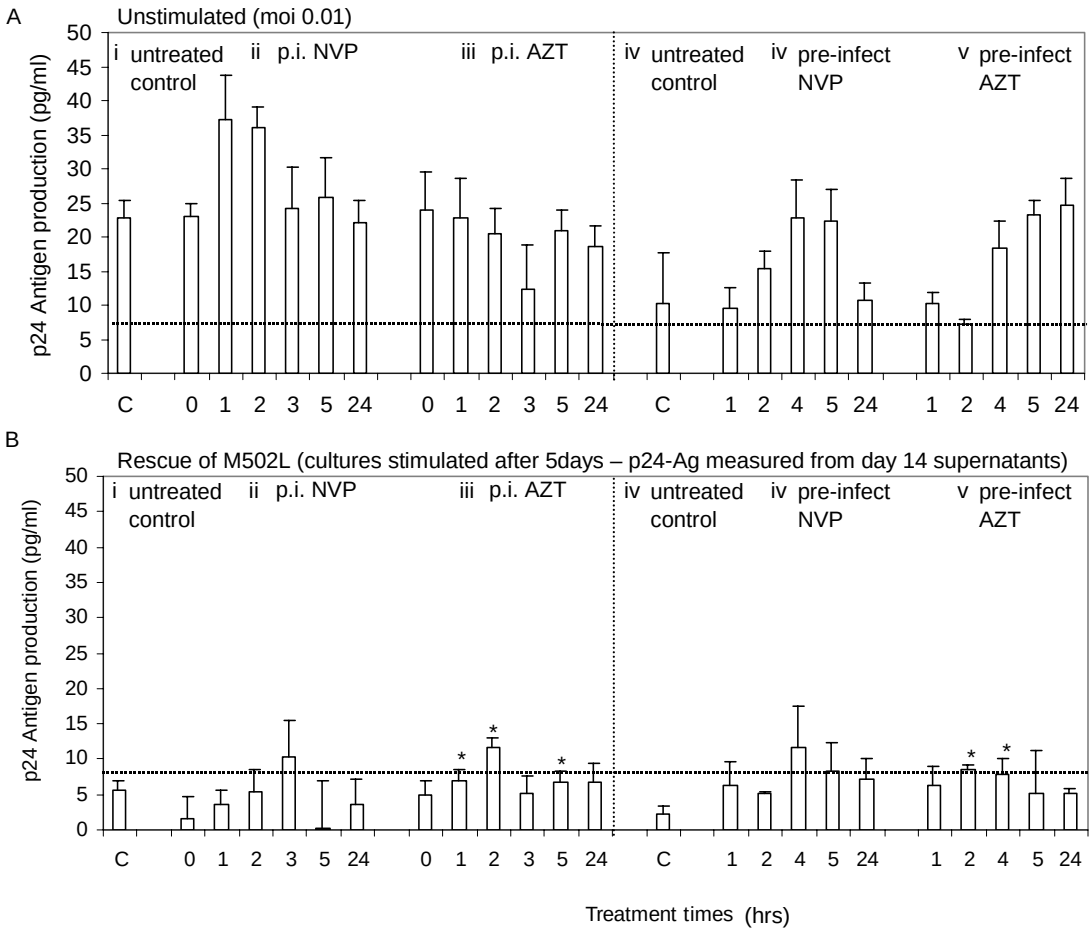


Figure 3.13 The inhibitory effect of NVP or AZT, added at different times to unstimulated PBMC cultures (donor E), either pre- or post-infection (~ 2000 $TCID_{50}$). After 5 days of culture supernatants were harvested and p24-Ag production measured. The PBMC were then stimulated with PHA medium for 24 hours following which the medium was carefully replaced with growth medium and freshly PHA-stimulated donor cells. On day 10 cultures were given fresh growth medium and by day 14 culture supernatants were harvested and p24-Ag production measured by ELISA. The bars denote p24-Ag production of triplicate samples (expressed as mean values ± SD) measured from collected supernatants 5 days after the initial infection and NVP or AZT addition (A) and supernatants collected 14 days after PHA-stimulation (B).The dotted line indicates the p24 cutoff value i.e. values <7.8 pg/ml indicated negative viral growth. The * indicates cultures that demonstrated a statistically significant increase in p24-Ag values relative to the controls (either i or iv).

3.2.3.1.2 Donor F

Levels of p24-Ag measured from culture supernatants collected 48 hours after infection and NVP or AZT addition are presented in Figure 3.14A for donor F. NVP and AZT added either pre- or post-infection had statistically no significant effect on viral growth (Table 3.9). In fact, very low growth of M502L was demonstrated in this donor. Virus could not be rescued from any cultures stimulated 24 hours or 48 hours after treatment with PHA (Figure 3.14 B and C).

Table 3.9 Percentage inhibition (relative to HIV-1 controls) of M502L replication in unstimulated PBMC (donor F) following addition of 10 μM NVP or 20 μM AZT either pre-infection or p.i.

Treatment times (hours)	**Inhibition (%) and statistical significance (*P*)**			
p.i.	**NVP**	***P****	**AZT**	***P****
0	-41	>0.05	-41	0.05
3	4	>0.05	117	>0.05
5	2	>0.05	-9	>0.05
24	24	>0.05	5	>0.05
pre-infection	**NVP**		**AZT**	
1	-50	>0.05	40	>0.05
3	-37	>0.05	-49	>0.05
5	59	below cutoff	8	>0.05
24	73	below cutoff	63	>0.05

Shaded figures indicate no inhibition but stimulation

* Sample means were compared using the paired-samples *t*-test. Statistical tests were two-tailed and significant at $p<0.05$

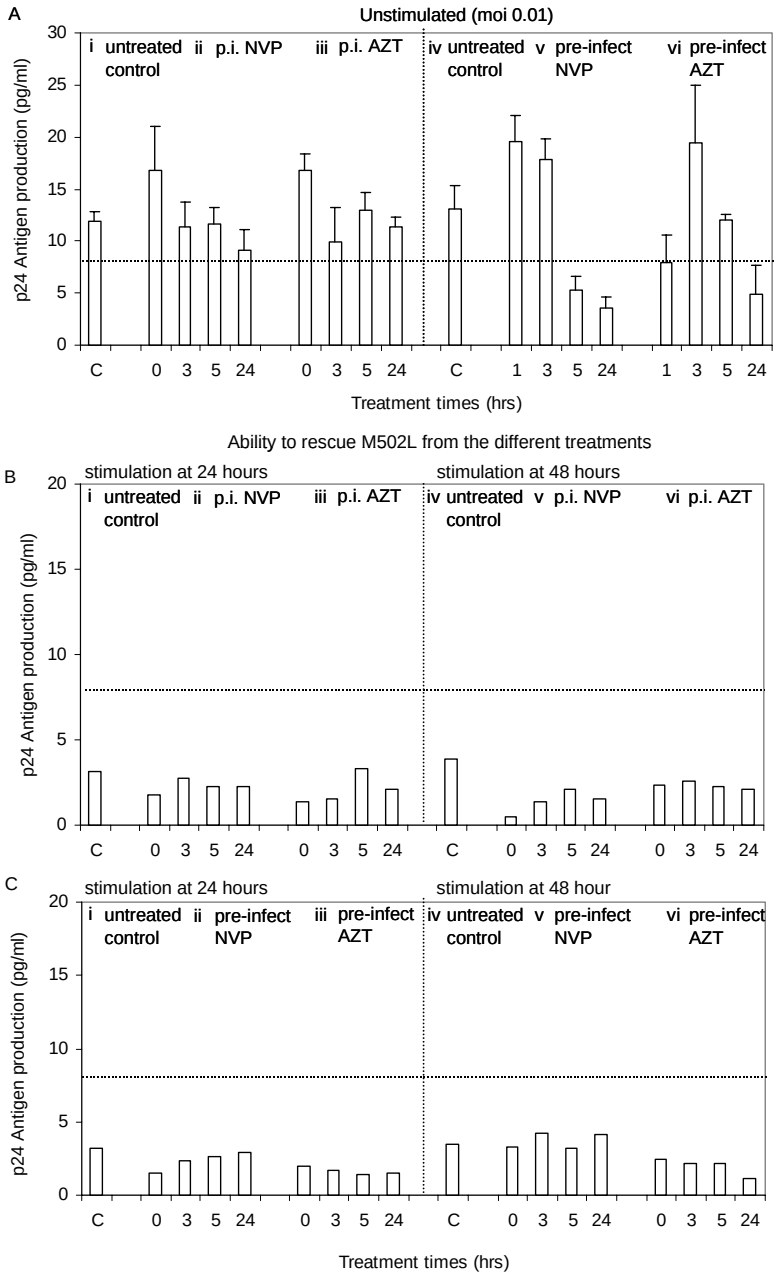


Figure 3.14 The inhibitory effect of NVP or AZT, added at different times to unstimulated PBMC cultures (donor F), either pre- or post-infection (~ 2000 $TCID_{50}$). p24-Ag production measured from supernatants collected 48 hours after the initial infection and NVP or AZT addition (A) and supernatants collected 14 days after infected and NVP or AZT treated cultures were stimulated after 24 hours (Bii and iii and Cii and iii) and 48 hours (Bv and vi and Cv and vi) are presented. PBMC were stimulated with PHA medium for 24 hours following which the medium was carefully replaced with growth medium and freshly PHA-stimulated donor cells. On day 10 cultures were given fresh growth medium and by day 14 culture supernatants were harvested and p24-Ag production measured by ELISA. The bars denote p24-Ag production of triplicate samples (expressed as mean values ± SD) for (A) and the p24-Ag produced by three cultures that were pooled (B and C). The dotted line indicates the p24 cutoff value, that is, values <7.8 pg/ml indicated negative viral growth.

3.2.3.1.3 Donor G

In donor G, NVP (3 hours) and AZT (1 and 3 hours) added pre-infection significantly stimulated viral growth (Figure 3.15Aiv and Av, Table 3.10, (p<0.05)). p24-Ag levels from cultures of other treatment times were not found to be significantly different from those of the control culture (Table 3.10). M502L could be rescued from cultures marked with an asteriks (*) in Figure 3.15B to E. Statistically, M502L could be rescued from cultures treated 5 and 7 hours p.i. with NVP (Figure 3.15Bi) and from 3 hour p.i. AZT cultures stimulated immediately (Figure 3.15Ci). M502L could be rescued from all cultures stimulated 12 hours after NVP and AZT additions p.i (Figure 3.15Bii and Cii). Immediate stimulation of pre-infection NVP and AZT treated cultures resulted in M502L viral growth from the 5 hour NVP (Figure 3.15Di) and from all the AZT treatment cultures (Figure 3.15Ei). M502L could only be rescued from 1 and 5 hour NVP (Figure 3.15Dii) and the 1 hour AZT (Figure 3.15Eii) pre-infection treatment cultures if stimulated 12 hours after the NVP or AZT treatments.

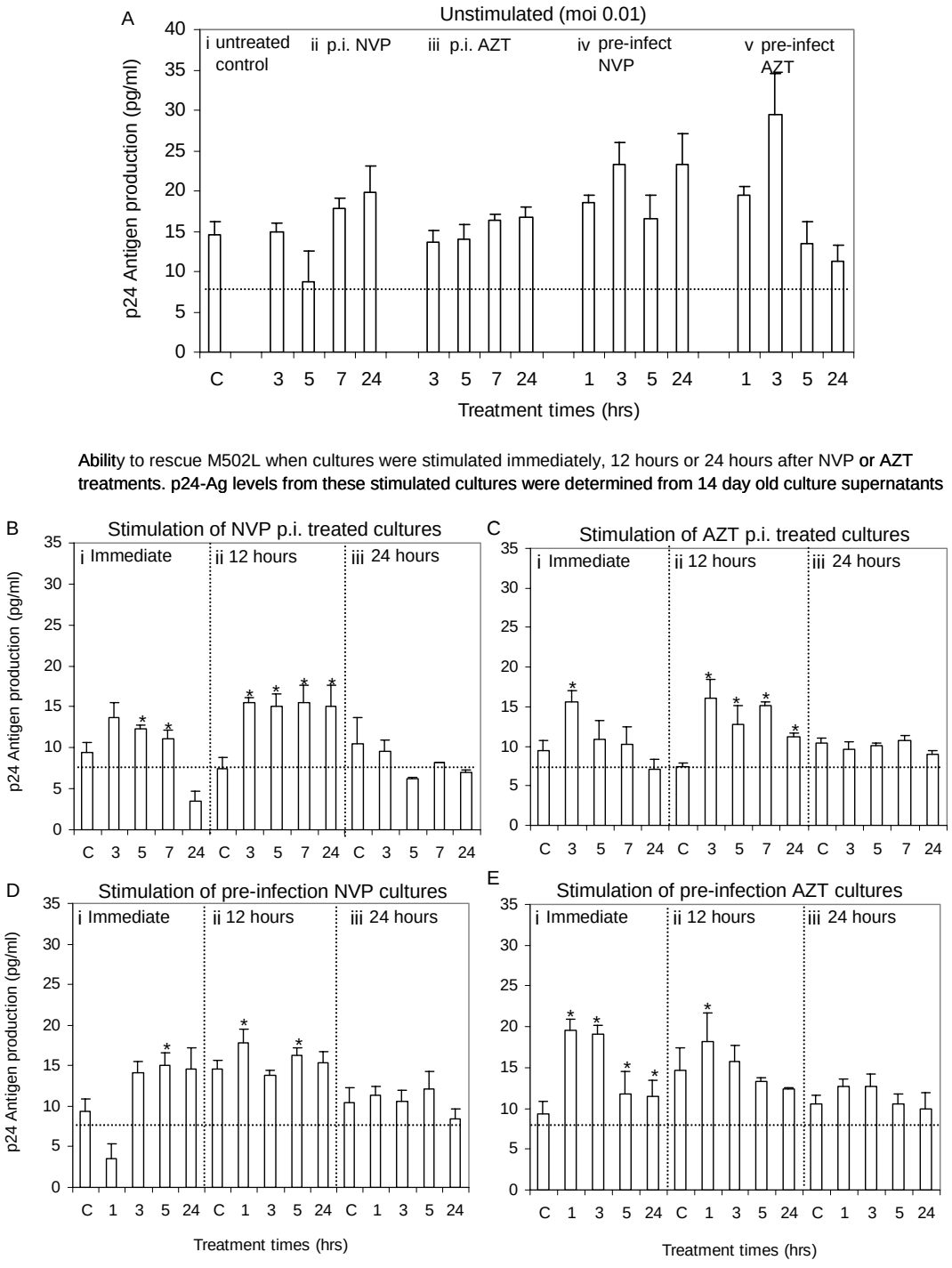
The results from this donor suggest that PHA-stimulation of cultures after NVP or AZT addition either pre- or post-infection can lead to virus replication.

Table 3.10 Percentage inhibition (relative to HIV-1 controls) of M502L replication in unstimulated PBMC (donor G) following addition of 10 μM NVP or 20 μM AZT either pre-infection or p.i.

Treatment times (hours)	**Inhibition (%) and statistical significance (*P*)**			
p.i.	**NVP**	***P****	**AZT**	***P****
3	-2	>0.05	7	>0.05
5	40	>0.05	5	>0.05
7	-21	>0.05	-12	>0.05
24	-35	>0.05	-14	>0.05
pre-infection	**NVP**		**AZT**	
1	-26	0.056	-33	0.042
3	-60	0.035	-101	0.032
5	-13	>0.05	8	>0.05
24	-60	0.053	23	>0.05

Shaded figures indicate no inhibition but stimulation

* Sample means were compared using the paired-samples *t*-test. Statistical tests were two-tailed and significant at p<0.05



Ability to rescue M502L when cultures were stimulated immediately, 12 hours or 24 hours after NVP or AZT treatments. p24-Ag levels from these stimulated cultures were determined from 14 day old culture supernatants

Figure 3.15 The effect of 10μM NVP or 20 μM AZT added at different times to unstimulated PBMC cultures (donor G) either pre-infection or p.i. (~ 2000 $TCID_{50}$). p24-Ag levels from unstimulated cultures is presented (A) as well levels following M502L rescue immediately following treatment (i) after 12 hours (ii) and 24 hours (iii) from cultures treated p.i. with NVP (B) or AZT (C) or pre-infection with NVP (D) or AZT (E). The bars denote p24-Ag production (expressed as mean values of triplicate samples ± SD). The dotted line indicates the p24 cutoff value, that is, values <7.8 pg/ml indicated negative viral growth. The * indicates cultures that demonstrated a statistically significant increase in p24-Ag values relative to the controls.

3.2.3.1.4 Summary of viral growth in unstimulated cultures treated pre- or post-infection with NVP and AZT and the ability to rescue virus by PHA-stimulation at specific time points

Table 3.11 summarizes the virus rescue experiments. The data indicates that donors differ in their ability to harbour HIV-1 and in their responses to antiretrovirals.

Table 3.11 Summary of M502L viral growth in unstimulated donor PBMC following pre- or post-infection addition of NVP or AZT, and time points of PHA-stimulation that resulted in successful viral replication. Only statistically significant data are presented

Donor	Virus growth mean p24-Ag (pg/ml)	Effect of drug on viral growth in unstimulated cultures (% inhibition or extent of viral growth) (treatment time (hours) when growth detected)				Comments
	Untreated (control)	NVP pre	NVP p.i.	AZT pre	AZT p.i.	
E	10-23	NS	NS	NS	NS	moi=0.01; no significant effects demonstrated
F	13	NS	NS	NS	NS	moi=0.01; no significant effects demonstrated; poor growth of M52L
G	15	↑ (++) (3 hours)	NS	↑ (++) (1 and 3 hours)	NS	moi=0.01; NVP or AZT p.i. had no significant effect; early pre-infection addition enhanced viral growth
	Ability to rescue virus - mean p24-Ag (pg/ml) (NVP/AZT treatment times from which M502L could be recovered)					
E	2-5	NS	NS	Below cutoff	↑ (++) (2 hours)	viral growth (>50% of the control) from 2 hour p.i. AZT cultures
F	3-4	0	0	0	0	No viral growth if cultures were stimulated either within 24 or 48 hours
G	9-15	*Immediate* ↑ (+) 5 hours; *12 hour* ↑ (+) 1 and 5 hours	*Immediate* ↑ (+) 5 and 7 hours; *12 hour* ↑ (++) 3-24 hours	*Immediate* ↑ (++) 1-24 hours; *12 hour* ↑ (+) 1 hour	*Immediate* ↑ (++) 3 hours; *12 hour* ↑ (++) 3-24 hours	**Most** viral growth observed if cultures treated with NVP or AZT p.i. were stimulated within 12 hours or if cultures given AZT prior to infection were stimulated immediately after infection

% Inhibition =[(p24-Ag control – p24-Ag of NVP / AZT treated culture)/p24-Ag control] x 100
ND – not determined;
NS – not statistically significant, that is, no statistical difference from control cultures
↑ - increase in viral growth statistically significant (+ = greater than 25% increase relative to control; ++ = greater than 50% increase relative to control)

3.3. Discussion

A high percentage (>87%) of infants that are born to HIV-1 seropositive mothers (given a 200 mg tablet of NVP to take at the onset of labour), and which are given 2 mg/kg NVP syrup within 72 hours of birth, remain uninfected (Ayouba *et al*., 2003). This would imply that short-course antiretroviral regimens are highly effective in reducing the risk of transmission of HIV-1 but do not entirely prevent acquisition of infection. Thus one may question why ART may be unsuccessful in preventing infection in the small percentage of susceptible infants. Productive HIV-1 infection is dependent on T-cell activation (Kinoshita *et al*., 1998). NVP and AZT are recognised for their ability to inhibit HIV-1 replication with NVP binding directly to the HIV-1 reverse transcriptase in a hydrophobic pocket near the polymerization active site thus slowing the rate of DNA synthesis, while AZT is incorporated into the elongating viral DNA chain thus interfering with DNA chain synthesis. It is accepted that infection of mitogen-stimulated and unstimulated PBMC is unlikely to be equivalent to infection of resting or partially activated T-cells *in vivo*, however, the infection of primary cultures using a primary HIV-1 isolate should yield results which more closely reflect the true *in vivo* scenario than if one uses cell lines or laboratory adapted strains of HIV-1. Thus, we wanted to simulate the *in vivo* situation where infants are exposed to HIV-1 only and NVP or AZT, either as a result of the mother being given a single oral dose (e.g. DART cohort) or where the infant receives NVP or AZT as PEP, by assessing *in vitro* the replicative ability of a primary isolate (M502L) in primary culture based on the timing of addition of NVP or AZT. CBMC are relatively immature lymphocyte populations that might serve as more efficient hosts of HIV-1 replication than PBMC, however, viruses isolated on CBMC can be routinely grown on PBMC and vice versa (Salomon *et al*., 1994). Furthermore, these authors report that CBMC and PBMC yield similar results with regard to susceptibility profiles of both wild-type and drug-resistant variants of HIV-1 lending support to our use of PBMC in this study. In further support for the use of adult PBMC in this study, equivalent replication of M502L has been demonstrated in both CBMC and PBMC (C.T. Tiemessen, unpublished data).

Our results have shown that (i) the primary HIV-1 isolate M502L demonstrated good growth characteristics in PHA-stimulated PBMC isolated from healthy donors, (ii) NVP and AZT inhibited M502L in a dose-dependent manner, (iii) 10 μM NVP as well as 20

μM AZT could effectively reduce viral replication in certain donors when added either pre- or post-infection, (iii) over a short time period (7 days), the viability of PBMC from one of the donors tested was affected by the higher NVP or AZT concentrations, (iv) most striking was the degree of variability between PBMC isolated from different donors with respect to viral replication and drug effectivity, suggesting that this phenomenon is likely to exist within patient populations, (v) virus could be recovered from unstimulated cultures of some donors if PHA-stimulation was initiated either immediately or within 12 hours of HIV-1 and drug treatment and (vi) elevated p24-Ag levels were demonstrated in certain donors infected with M502L and exposed to NVP or AZT either pre- or post-infection which suggests that NVP or AZT may play a role in augmenting immune activation *in vitro* and thereby possibly increasing the susceptibility of PBMC to HIV-1 infection. Since immune activation is a characteristic feature of HIV-1 infection, these findings could be explained by antiretrovirals affecting cells in such a way so as to promote an environment more conducive for HIV-1 replication (either through activation of cells or production of cytokines/chemokines). Although the elevated p24-Ag levels might well indicate the rapid emergence of resistant strains we do not believe that our experimental approach selected for resistance as viral growth was monitored over a short time period without multiple passages of virus. Indeed, wild-type HIV-1 isolates have been shown to acquire AZT resistance mutations after 3 to 6 passages with AZT (Reid *et al*., 2005) while the selection for NVP resistance is achieved by repeated passage (for 8 to 15 weeks) of wild-type clinical isolates in CBMC in the presence of increasing concentrations of NVP (Loemba *et al*., 2002).

The fact that we demonstrated variations amongst the donors (when PBMC were stimulated with PHA or 'unstimulated') reflects either (i) variations in cell permissiveness to HIV-1 infection between donors or (ii) variations in the so-called 'unstimulated' cell state. PBMC from different donors may have different abilities to be stimulated by the procedures used to isolate these cells. Ultimately, what we cannot mimic in an *in vitro* scenario is the various states of differentiation, functional activity and replicative activity of cells *in vivo*. Although viral binding and entry may be similar between the donors used it is highly unlikely since the genetic makeup of individuals plays a role in HIV-1 susceptibility. Observed variations have been attributed to immunogenetic diversity (major HLA homozygosity and specific HLA types) and polymorphisms in chemokines, chemokine receptors and cytokine genes (Carrington *et al*., 2001; Telenti *et al*., 2002).

Using a wide array of viral isolates and laboratory strains to infect PBMC Spira and Ho (1995) reported a 40-fold range of differences in infection among individuals. The events that take place once the virus has entered a cell may also differ therefore influencing the titres of the progeny viruses produced. $CD4^+$ $CD45RO^+$ $CD57^-$ PHA-stimulated T-cells from healthy donors (n=128) exhibited a 5-log-unit range in p24-Ag production (Ciuffi *et al*., 2004). These authors also report that for 20 selected donors representing the spectrum of $CD4^+$ T-cell permissiveness, 42% of the total variance in virus production could be attributed to entry factors and 48% to post-entry steps. This would suggest that various viral, intracellular and even host genetic factors affect the disparity that we have observed between donor PBMC which in the host could affect both the spread and pathogenesis of HIV-1.

The benefits of single-dose NVP or AZT exposure to reduce MTCT of HIV-1 are dependent on two important points: (i) that the intrapartum dosing of the antiretroviral is such that it provides sustained inhibitory concentrations throughout labour and delivery, (ii) that the antiretroviral is active (cellular availability). One of the primary goals of administering NVP or AZT intrapartum is to achieve a drug concentration in the foetus at the time of maximum exposure (labour or delivery) that will inhibit the establishment of an infection in the newborn. Whether this can be achieved with every birth is unlikely which therefore places certain infants at risk of acquiring HIV-1. While the $t_{½}$ of AZT in adults averages 1.1 hours, (AZT is rapidly glucuronidated with both the parent compound and the glucuronide being excreted in the urine (Klecker *et al*., 1987)), AZT clearance in premature neonates is low and the $t_{½}$ is prolonged (7.3 hours) due to the hepatic and renal elimination pathways being less well developed (Mirochnick *et al*., 1998a). NVP on the other hand has a mean plasma $t_{½}$ of 30 hours (Podzamczer and Fumero, 2001) however since NVP is metabolized via the hepatic cytochrome P450 enzyme pathway and if NVP prophylaxis is initiated too early rather than intrapartum, plasma concentrations of NVP may not be sustained due to autoinduction of foetal hepatic metabolism (McGowan and Shah, 2000; Taylor *et al*., 2000; Mirochnick *et al* 2001).

Several characteristics of NVP including the quick absorption of NVP after oral dosing and its ability to cross the placenta, together with its long half-life in pregnant women during labour and delivery and in infants (median $t_{½}$ 61.3 hours women and 46.5 hours in infants (Bardsley-Elliot and Perry, 2000) and its activity against cell-free virions and cell-

associated virions (Zhang *et al*., 1996), have resulted in NVP being a drug of choice in reducing the risk of HIV-1 transmission. However, when NVP is administered as a single-dose for treatment of HIV-1 disease, HIV-1 variants with NVP resistance mutations are rapidly selected. HIV-1 variants containing a single mutation sufficient for drug resistance are believed to exist in the viral population of every patient prior to drug exposure. Pharmacological studies show that AZT is taken up effectively and passively transported into the cell, where phosphorylation takes place. The phosphorylation of dideoxynucleosides is a necessary requirement for their antiviral activity (Jacobsson *et al*., 1995) with the extent of inhibition of viral RT depending as much on the interplay of AZT-TP and TTP as on the concentration of their respective intermediates and the degree to which they themselves serve as substrates for the two kinases (Hazuda and Kuo, 1997).

Intervention with single-dose prophylaxis may result in viral suppression however, our study has shown that whilst not all donors whose PBMC were stimulated with PHA would induce transcription of the viral genes, this source of infection cannot be considered irrelevant to disease progression, particularly in the case of newborn infants. Also, the antiviral activity of NVP or AZT tested *in vitro* using PBMC from healthy donors may not adequately reflect the sensitivity to drugs of HIV-1 in HIV-1 exposed infants. That HIV-1 infection results in increased immune activation is well recognised however, that antiviral compounds may directly influence the immune system is not well documented. That these events might influence the level of immune activation may significantly affect viral replication. In newborns, higher immune activation may contribute to an increased risk of becoming infected.

As Salomon *et al*. (1994) demonstrated that the susceptibility profiles between PBMC and CBMC are similar, and if we extrapolate our data to those infants born to HIV-1 seropositive mothers, then the variability accounting for the susceptibility to HIV-1 in PBMC may likewise determine why some infants become infected despite exposure to single-dose NVP or AZT. This would therefore suggest that there may be a group of infants that are at greater risk of becoming infected. The question therefore arises as to how cellular immunity at birth influences the incidence, progression and prognosis of perinatally transmitted infectious diseases such as HIV-1? The infant's ability to develop HIV-1 specific cellular immunity during foetal development may be largely dependent on the time of exposure to HIV-1 and may be influenced by the dose and the phenotype of

the virus i.e. exposure of the foetus to a defective virus may stimulate induction of protective HIV-1 specific cellular responses, whereas exposure to a cytopathic strain of HIV-1 may result in intrauterine HIV-1 infection. Furthermore, *in vitro* studies have suggested that specific cellular characteristics including activation/proliferation and maturation govern the susceptibility of cells to infection with HIV-1. While it is essential to achieve a drug concentration in the infant that is able to inhibit the viral replication it may be considered undesirable that the drug in combination with HIV-1 exposure induces a state of immune activation that might support the establishment of an infection.

Our data has indicated that differences in host-virus-drug interactions between PBMC from different blood donors exist. We acknowledge that the use of primary cells and a primary HIV-1 isolate makes any experimental approach more complex particularly since donor cells vary in their ability to proliferate, their activation status, levels of apoptosis, co-receptor expression and chemokine expression. Furthermore, studies suggest that the bioavailability of NVP or AZT differ between individuals and is determined by biological factors. Cellular functions i.e. host species, cell type and stage in the cell cycle are important factors in the mechanism of action of AZT (Sommadossi, 1993). Interestingly, a large variation in the capacity to phosphorylate AZT in peripheral lymphocytes from different individuals and from the same individual at different times has been reported (Tornevik *et al*., 1991). It may be noteworthy that factors that may contribute to the phosphorylation of AZT include activation of phosphorylation by physiologic factors associated with pregnancy, the stress of childbirth and variability in systemic AZT concentrations (Rodman and Flynn, 1999). Data furthermore suggests that the physiologic processes associated with labour have a large impact on NVP absorption, distribution and elimination – it is suggested that labour decreases NVP bioavailability and clearance (Mirochnick *et al*., 2001). Clearly, it is difficult to extrapolate our data to explain how timing of drug administration influences the viral infection outcome of infants born to HIV-1 seropositive mothers. The role of genetic factors (and probably also viral factors) in determining individual cell susceptibility to HIV-1 is clear. Interestingly, genetic polymorphisms in antiviral genes or in host genes (chemokines, chemokine receptors and cytokines such as those for CCR5, CCR2, CXCR1, CXCL12, CCL3, CCL5, IL-10 and IL-4) could result in differences in the levels of expression or in the functional potential of protein variants and thus lead to differences in permissiveness to HIV-1 infection (Ciuffi *et al*., 2004).

CHAPTER FOUR

Cellular immune activation in the presence and absence of single-dose NVP

4.1 Introduction

Antiretroviral drugs, even if given in short and simple regimens, can dramatically reduce the risk of mother-to-child HIV-1 transmission (Connor *et al*., 1994; Guay *et al*., 1999; Shaffer *et al*., 1999a; Wiktor *et al*., 1999; Dabis *et al*., 1999). The simplest regimen involves only one dose of NVP to the mother at the onset of labour and one dose to the infant within 72 hours of birth (Guay *et al*., 1999; Jackson *et al*., 2003). Although consistently observed, the efficacy of these regimens is surprising since short regimens of monotherapy are expected to have at best minor effects on maternal viral load. Furthermore, the reduction in maternal plasma viral load achieved with even the longest of these regimens explained only a fraction of the transmission reduction due to the regimen (Sperling *et al*., 1996).

There is general consensus that productive replication of the HIV-1 viral genome in $CD4^+$ T-lymphocytes is dependent on cellular activation (Kinoshita *et al*., 1997; Oswald-Richter *et al*., 2004). However, exposure to HIV-1 even in the absence of seroconversion has been shown to induce HIV-1 specific cell-mediated immune responses (Rowland-Jones *et al*., 1997), a process which in its own right would require T-cell activation. If HIV-1 specific cell-mediated immune responses are truly protective, then we have the paradoxical situation that protective immunity can successfully occur in an immune environment that ordinarily may be considered to favour HIV-1 replication. Some antiretroviral drugs, e.g. the protease-inhibitor Indinavir (Chavan *et al*., 2001), have been shown to have immunomodulatory consequences distinct from benefits attributable to control of viral replication. One consequence observed *in vivo* following a short-course AZT-3TC regimen was reduced memory T-cell responses to HIV-1 (Kuhn *et al*., 2001c). These findings raise the question of whether or not there may be immune suppressive effects of antiretroviral drugs given to prevent maternal-infant HIV-1 transmission. Characterization of immunomodulatory influences of antiretroviral drugs may help explain why drugs are effective in reducing transmission.

In this study, we tested the hypothesis that the efficacy of NVP to reduce maternal-infant HIV-1 transmission may in part be a consequence of its ability to reduce immune activation as measured by decreased levels of one or more of the immune activation markers neopterin, β_2-m and sL-selectin, all soluble factors which have been used to quantify levels of T-cell activation *in vivo* (Fahey *et al*., 1990; Fuchs *et al*., 1988).

4.2 Results

4.2.1 Clinical characteristics of HIV-1 seropositive mothers and their infants

Clinical characteristics of the mother-infant participants who were included in this nested case-control study are presented in Table 4.1. Viral loads were significantly higher among mothers who had not received any antiretroviral drugs prior to delivery (PEP) compared to mothers given NVP at the onset of labour (DART).

Table 4.1 Clinical characteristics of the HIV-1 seropositive mothers and their infants

	No antiretroviral drugs given before birth (PEP)	**Single-dose nevirapine given before birth (DART)**	**Total (PEP and DART)**
N	80	61	141
Mean (standard deviation)			
Mothers' $CD4^+$ T-cell count	477 ± 259	Not tested	477 ± 259
Mothers' Age (years)	26 ± 5	26 ± 5	26 ± 5
Infant Birth weight (grams)	2919 ± 453	3039 ± 409	2964 ± 438
Median (IQR[1])			
Mothers' viral load (log_{10})[2]	4.8 (4.1-5.2)	4.4 (3.7-4.9)	4.6 (3.9-5.1)
Percent (n/N)			
Infant sex male	56 (40/72)	50 (29/58)	53 (69/130)
Primiparity	34 (25/74)	23 (13/56)	29 (38/130)
Vaginal delivery	100 (74/74)	91 (49/54)	96 (123/128)
Preterm (<37 weeks)	24 (17/71)	9 (5/55)	17 (22/126)
Infants HIV-1 infected IP[3]	11 (14/124)	4 (3/78)	8 (17/202)
Infants HIV-1 infected IU[3]	3 (4/124)	5 (4/78)	4 (8/202)
Infants ever breast-fed	49 (37/75)	12 (7/58)	33 (44/133)

[1]IQR - Inter-quartile range i.e. 25th and 75th percentiles
[2]Significant differences between PEP and DART groups are indicated (p=0.016)
[3]Transmission rates for PEP and DART study

4.2.2 Infants exposed to or infected with HIV-1 demonstrate greater immune activation at birth than control uninfected infants

In order to establish if exposure to and/or infection with HIV-1 leads to increased immune activation as evidenced by raised levels of cord blood plasma activation markers, infants born to HIV-1 infected mothers were stratified on the basis of their infection outcomes as EU (exposed-uninfected), IP (acquired infection intrapartum, birth HIV-1 PCR negative, 6 week HIV-1 PCR positive) and IU (acquired infection *in utero*, birth HIV-1 PCR positive), and compared to control infants born to HIV-1 uninfected mothers. As markers

are measured in cord blood, the IP infants are uninfected at delivery and so do not indicate consequences of productive infection at this time point.

Neopterin levels were elevated above control levels among infected and uninfected infants born to HIV-1 seropositive mothers regardless of whether or not mothers received antiretroviral drugs (i.e. in both the DART and PEP groups) (Figure 1Ai,iv). Exposure to HIV-1 resulted in moderately elevated levels compared to HIV-1 seronegative controls of β_2-m and sL-selectin that attained significance among exposed-uninfected infants whose mothers received NVP (Figure 1Av-vi).

Infants who became infected during delivery (IP) did not show any difference from EU infants in immune activation markers. In contrast, *in utero* infection (IU) resulted in substantial and significant increases in all the activation markers (Figure 1Ai-vi).

4.2.3 Single-dose NVP contributes to immune activation in infants born to HIV-1 seropositive mothers, particularly among infants infected *in utero*

Having established that infants born to HIV-1 positive mothers have increased immune responsiveness as evidenced by raised levels of plasma activation markers, we next questioned if single-dose NVP might reduce immune reactivity in the presence of exposure or infection with HIV-1. Contrary to our hypothesis, NVP exposure significantly increased neopterin (p=0.002) and sL-selectin (p=0.016) levels of EU infants (Figure 1Bi,iii) as well as increasing neopterin (p=0.021) and β_2-m (p=0.021) levels of infants who were infected *in utero* (IU) (Figure 1Bvii,viii).

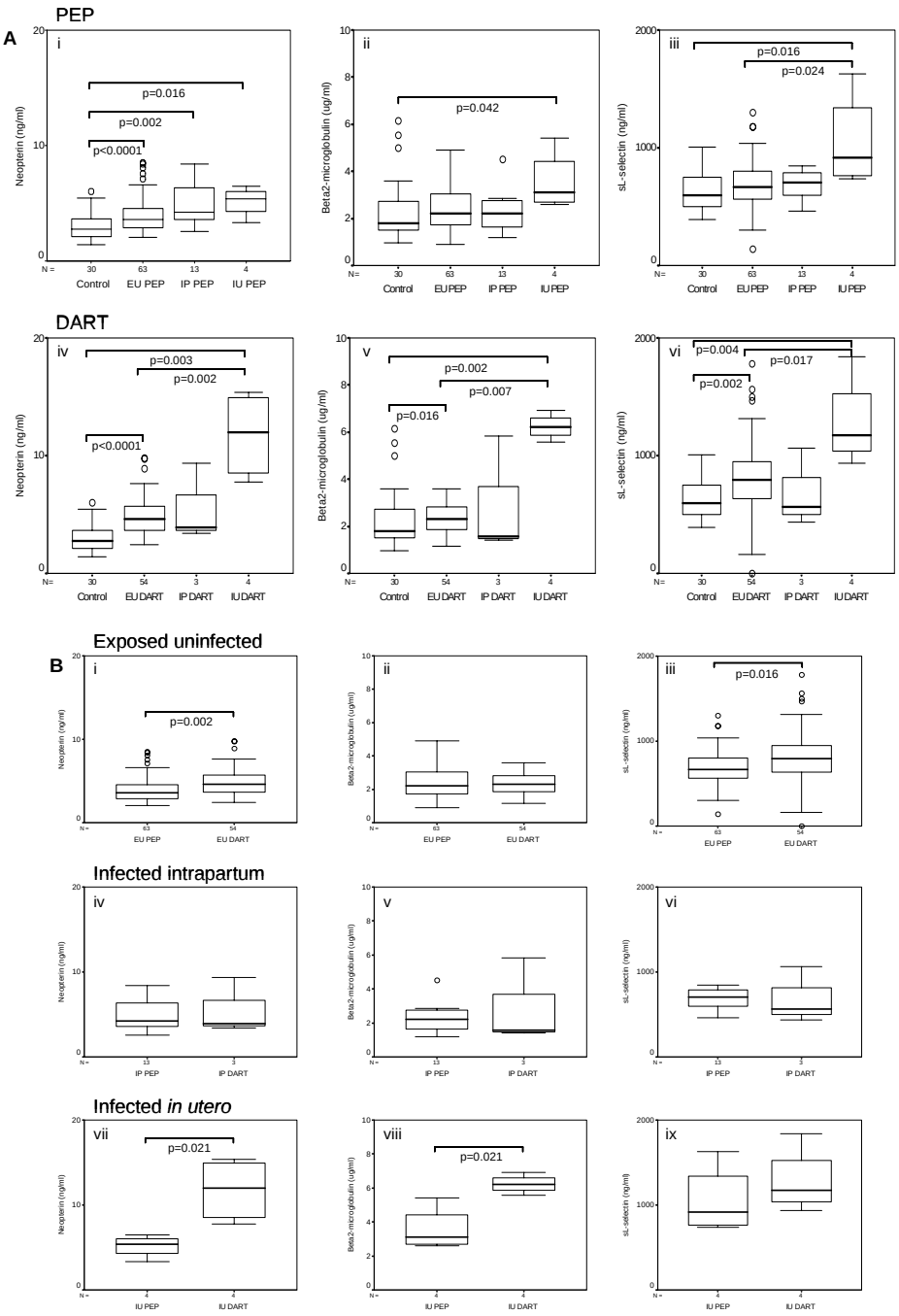


Figure 4.1 Levels of soluble immune activation markers in plasma of infants born to HIV-1 seronegative and HIV-1 seropositive mothers. (A) Levels of neopterin (ng/ml) (i and iv), β_2-m (μg/ml) (ii and v) and sL-selectin (ng/ml) (iii and vi) of infants born to HIV-1 seropositive mothers in the absence (PEP) and in the presence (DART) of a single-dose of NVP, respectively. Immune activation marker levels measured in uninfected (control) infants are included. (B) This panel depicts the effects of single-dose NVP exposure (DART) versus absence of NVP exposure (PEP) on the levels of neopterin (ng/ml), β_2-m (μg/ml) and sL-selectin (ng/ml) of infants that remain uninfected (EU) (i, ii, iii, respectively) or that become infected intrapartum (IP) (iv, v, vi, respectively) or *in utero* (IU) (vii, viii, ix, respectively). Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes) and 10th and 90th percentiles (bars). Significant differences between groups and sample numbers per group are indicated.

4.2.4 Immune activation in the infant is modulated independently of the mother's viral load and is not influenced by gestation period

Given that infants exposed to HIV-1 or infected with HIV-1 demonstrated enhanced immune activation we wanted to ascertain whether the mother's viral load would directly impact on the levels of the immune activation factors of the PEP and DART infants. Only neopterin from the EU DART infants was weakly correlated to maternal viral load (r=0.298, p=0.028). Given that PEP mothers had significantly higher viral loads compared to DART mothers (log_{10}4.78 and log_{10}4.35, respectively) (p=0.016), we might have expected that activation markers would be lower in the NVP-exposed group (DART) in contrast to what we observed. After adjusting for maternal viral load, the association between NVP exposure and higher levels of neopterin (p=0.035) and sL-selectin (p=0.016) remained statistically significant in the EU group indicating that differences in viral load between the groups did not account for the increased immune activation that was observed.

Infants born prior to 37 weeks gestation (preterm infants) might be expected to have a reduced ability to respond to antigen compared to term infants, however, no significant differences in levels of neopterin, β_2-m and sL-selectin levels were observed between preterm or term infants of either the PEP or DART groups (data not shown).

4.2.5 Single-dose NVP does not modulate immune activation markers of IP transmitting and non-transmitting HIV-1 infected mothers

The immune activation markers tended to be higher among HIV-1 infected mothers than among uninfected control mothers (Figure 4.2A). However, in contrast to the findings among the children, NVP exposure was not associated with differences in activation markers among non-transmitting mothers or mothers who transmitted IP (Figure 4.2B). There was however an intriguing increase in neopterin among IU transmitting mothers given NVP (Figure 4.2Bvii).

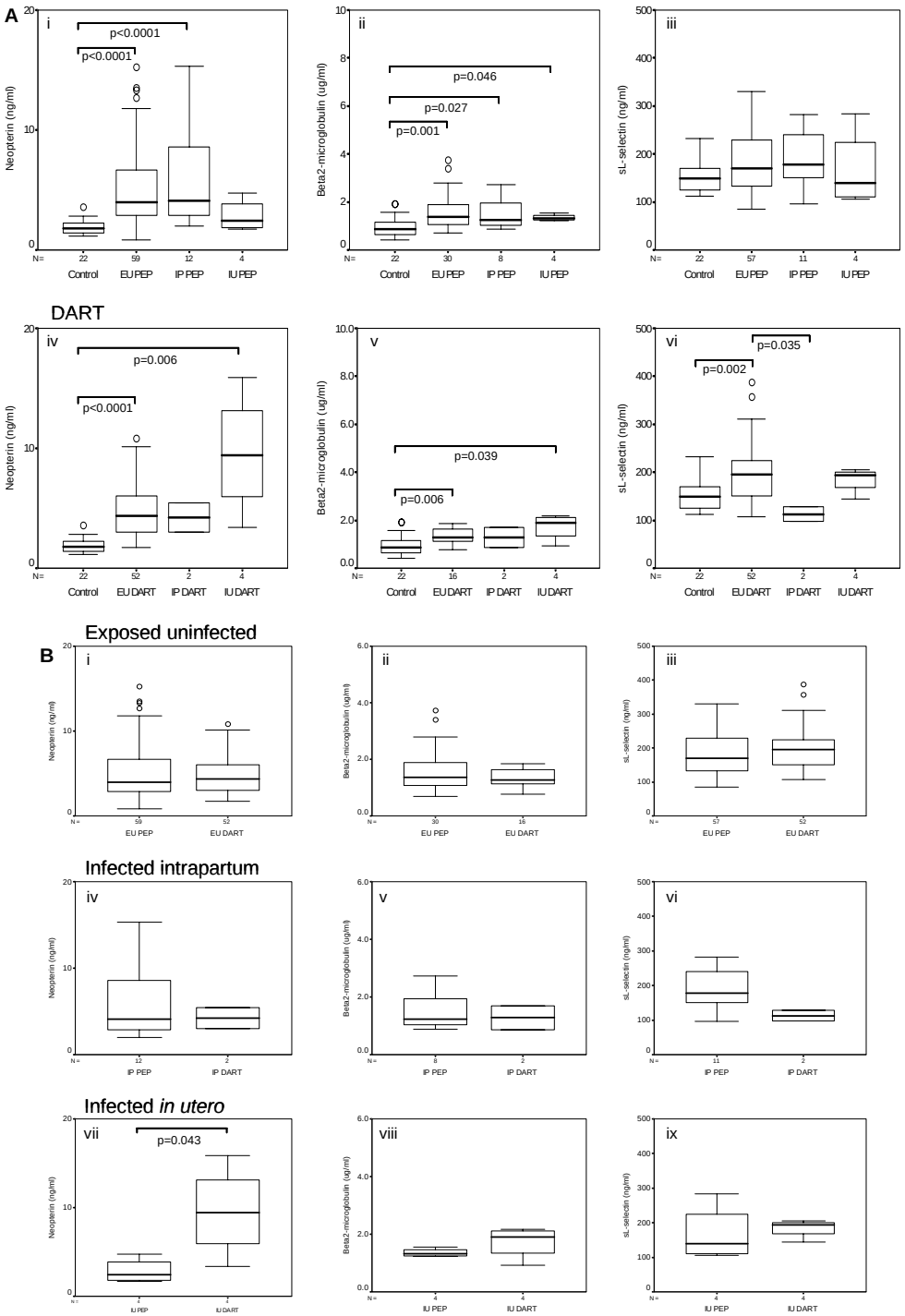


Figure 4.2 Levels of soluble immune activation markers of HIV-1 infected mothers grouped according to infection status of the infant and NVP exposure. (A) Levels of neopterin (ng/ml), β_2-m (μg/ml) and sL-selectin (ng/ml) in uninfected (control) mothers and HIV-1 seropositive mothers who did not receive NVP (PEP group) and those who did receive NVP at the start of labour (DART group), grouped according to infection outcome of the infant (EU, IP, IU). (B) Comparison of the effects on levels of neopterin (ng/ml), β_2-m (μg/ml) and sL-selectin (ng/ml) of single-dose NVP exposure (DART) versus absence of NVP exposure (PEP) in HIV-1 seropositive mothers grouped according to infection status of the infant (EU, IP or IU). Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes) and 10th and 90th percentiles (bars). Significant differences between groups and sample numbers per group are indicated.

4.2.6 Immune activation in the infant is modulated independently of that in the mother

To examine whether immune activation in the infant is modulated independently of the mother, we examined differences between mothers and infants in their immune activation markers. Interestingly, whereas levels of neopterin did not differ appreciably between infants and mothers, mother-child differences are notable for β_2-m and sL-selectin with infants presenting with significantly higher levels of these markers in all groups including the controls. The differences between mothers and infants were largest for infants infected *in utero* in both the PEP and DART groups. Within the control group, there were no significant correlations between mothers' and infants' (n=22) levels of neopterin (r=0.401, p=0.064), β_2-m (r=0.150, p=0.504), or sL-selectin (r=0.068, p=0.764) and infants had significantly raised levels of neopterin (p=0.013), β_2-m (p=0.001) and sL-selectin (p<0.001) (Table 4.2) compared to their mothers further supporting independent modulation of immune activation in the infant.

Table 4.2 Immune activation factor levels in plasma of mother and infant pairs

Infant group		Mother		Infant		
Neopterin (ng/ml)	**n**	**Median**	**IQR**	**Median**	**IQR**	***P****
Control	22	1.77	1.37-2.29	2.63	2.13-3.18	0.013
PEP						
EU	59	4.00	2.69-7.07	3.59	2.85-4.47	0.064
IP	12	4.13	2.78-8.90	4.14	3.17-6.18	0.754
IU	4	2.47	1.77-4.29	5.41	3.81-6.27	0.068
DART						
EU	52	4.34	2.98-5.67	4.62	3.69-5.67	0.387
IP	2	4.22	3.02-5.42	3.64	3.37-3.91	0.655
IU	4	9.44	4.67-14.50	11.96	8.14-15.16	0.465
β_2-microglobulin (µg/ml)						
Control	22	0.9	0.7-1.2	1.7	1.4-2.3	0.001
PEP						
EU	30	1.4	1.1-1.9	2.1	1.5-2.6	0.004
IP	8	1.2	1-2.2	1.9	1.4-4	0.050
IU	4	1.3	1.2-1.5	3.1	2.6-4.9	0.068
DART						
EU	16	1.3	1.1-1.6	2.3	1.9-3.1	0.001
IP	2	1.3	0.9-1.7	1.5	1.4-1. 6	0.655
IU	4	1.9	1.1-2.2	6.2	5.7-6.8	0.068
sL-selectin (ng/ml)						
Control	22	148.7	122.4-172	612	500.4-769.3	0.000
PEP						
EU	57	170.5	131.2-230.6	669.3	567.6-807.9	0.000
IP	11	178.2	134.9-240.8	703.1	568.8-787.6	0.003
IU	4	140.2	108.7-253.5	919	748.6-1483-4	0.068
DART						
EU	52	195.8	150.1-224.8	796	633.9-948.6	0.000
IP	2	112.7	97.7-127.7	497.6	433-562.3	0.180
IU	4	193.9	156.7-203.2	1171	987.5-1682	0.068

IQR - Interquartile range i.e 25th and 75th percentiles

***** Wilcoxon signed rank test for paired data sets. Statistical tests were two-tailed and significant at p<0.05

4.3 Discussion

Previous studies have described non-specific and HIV-1 specific immune responses in uninfected infants born to HIV-1 seropositive mothers (Borkowsky *et al*., 1990; Cheynier *et al*., 1992; Rowland-Jones *et al*., 1993; Aldous *et al*., 1994; De Maria *et al*., 1994; Kuhn *et al*., 2001a; Wasik *et al*., 1999; Clerici *et al*., 2000; Levy *et al*., 1998), indicative of some immune activity *in utero*. Based on prior demonstration of reduced T-helper cell reactivity to HIV-1 envelope peptides in cord blood of infants born to HIV-1 seropositive mothers in the presence of AZT-3TC (Kuhn *et al*., 2001c) and single-dose NVP (Kuhn *et al*., 2003) given to the mother, we hypothesized that other markers of immune activation would accordingly be reduced. This current study was therefore designed using the plasma markers neopterin, β_2-m and sL-selectin to assess the extent of immune activation in infants born to HIV-1 seropositive mothers in the presence (DART group) and absence (PEP group) of single-dose NVP administered to the mother at the start of labour.

Our results have shown that (i) in support of earlier studies, there was substantial immune activity in response to exposure to virus *in utero* (that did not result in infection), or as a result of exposure to other immune consequences of having an HIV-1 infected mother and, as might be expected, this was most elevated in infants infected *in utero,* (ii) in contrast to what we had hypothesized, there was evidence of further increased immune activation in the cord blood of infants exposed to NVP when compared to their drug-unexposed counterparts, and most notably, existing infection at birth was marked by substantially elevated levels of all immune activation markers in the presence of NVP, (iii) levels of peripheral blood immune activation markers were higher in infants than their mothers (HIV-1 seronegative and HIV-1 seropositive), particularly for β_2-m and sL-selectin indicating that their immune systems develop independently of their mothers; (iv) immune activation in the infant was modulated independently of maternal viral load, and (v) elevated immune activation in NVP-exposed HIV-1 infected infants relative to drug-unexposed HIV-1 infected infants (IU group) suggests an apparent synergy between HIV-1 and NVP in increasing overall immune activation. This latter phenomenon was also apparent amongst the IU-transmitting mothers.

How does one explain attenuated HIV-1 specific T-helper cell activity (Kuhn *et al*., 2003) yet increased levels of immune activation markers in plasma through a brief exposure (the

period of labour and delivery) to NVP? That NVP shows consequences of immune stimulation in such a short period of time would suggest that it may mediate its effects predominantly on cells of the innate immune response as these respond rapidly to stimuli. Plasma levels of immune activation markers are indicative of events that have already occurred, whereas detecting recall to HIV-1 peptides (T-helper cell reactivity) involves activation of T-cells following exposure *in vivo* to NVP. Because NVP has already increased activation of cells *in vivo*, as evidenced by increased levels of soluble immune activation markers, T-cells may be more anergic on subsequent stimulation with peptides and therefore unable to respond *in vitro*. This is reminiscent of what occurs in HIV-1 infected individuals where spontaneous release of cytokines is often enhanced because of *in vivo* priming, whereas induced release of cytokines is impaired relative to uninfected controls. Since T-cell responses in newborns are very weak, even a slight reduction in responsiveness would reduce most responses below the level of detection.

Levels of immune activation markers neopterin, β_2-m, and sL-selectin reflect physiologic and pathologic conditions, and in HIV-1 infection the former two are correlates of stage of disease and prognosis (Fahey *et al*., 1990; Fuchs *et al*., 1988; Lifson *et al*., 1992), while increased levels of sL-selectin have been reported in HIV-1 infected infants (Kourtis *et al*., 2000). One could question the extent of maternal transfer of these factors to the infants since studies concur that substances can be transferred from maternal blood to the foetus. There is no documented evidence that passive or active transfer of neopterin (253Da) occurs. Furthermore, foetal neopterin (Radunovic *et al*., 1999) and β_2-m (Cejka *et al*., 1974) concentrations have been reported to change during gestation with neopterin levels being reported to be substantially greater than maternal levels, and not being correlated significantly with paired maternal levels demonstrating that during gestation there is a progressive increase in foetal cell-mediated immunity and monocyte-macrophage activation (Radunovic *et al*., 1999). The immune activation marker levels measured in control uninfected infants of HIV-1 uninfected mothers in this study suggests an association with neonatal immune system development.

Neopterin production is associated with early T-cell responses (Fuchs *et al*., 1988) and serves as an indirect measure of oxidative stress and therefore apoptosis (T-cell anergy) (Widner *et al*., 2000). Elevated levels of β_2-m has been associated with an increased turnover of immune cells especially lymphocytes, however, high concentrations can

trigger a cascade of signaling events that exert a negative effect on the immune system, including impaired antigen-presentation capacity of DCs (Xie *et al*., 2003). Enhanced apoptosis has been described in cord blood T-lymphocytes of HIV-1 exposed newborns (Economides *et al*., 1998), with the one infected newborn tested in this study demonstrating the highest levels of $CD4^+$ and $CD8^+$ apoptosis. It stands to reason that raised levels of these markers would have consequences on the immune capability of newborn infants since increased apoptosis of T-cells, and reduced functional capacity of DCs with a diminished ability to activate T-cells, would compromise antigen-specific T-cell responses. Thus, while exposure to HIV-1 may result in priming of the immune system (non-specific and HIV-1 specific immune responses), the presence of single-dose NVP may be sufficient to further drive the immune system into an anergic/immunodeficient state. Single-dose NVP can mediate its antiviral activity on the one hand by directly binding to the HIV-1 reverse transcriptase, and on the other induce an anergic state in cells that may also be beneficial in preventing replication of HIV-1.

It is intriguing that levels of immune activation markers are higher in infants than in their mothers among all groups, even the HIV-1 seronegative controls. This would support the idea that (i) immune development is accompanied by signs of immune activation, (ii) levels of activation markers in plasma may serve to dampen immune responsiveness early in life, and may account for the general T-cell anergy that exists in early life, (iii) these factors are likely to play very specific roles in immune responsiveness in the infant and are thus unlikely to merely be waste products of immune responses. Furthermore, on the one hand there appeared to be an independence of the infant's developing immune system from the mother; but on the other there were similarities in how infants and mothers responded to common factors in both environments. The mothers' HIV-1 status appears to influence the infants' ability to counteract infectious agents, either through (i) altered maturation of the infants immune responsiveness through exposure to HIV-1 from the mother, or (ii) through deficient signalling that may occur through lack of provision of essential factors to the foetus that may be necessary for early immune development.

Our findings challenge in particular the way one might view the role of immune activation in promoting the establishment and augmentation of HIV-1 replication in the infant. Our data does not argue against the antiviral effects of short-course treatment, rather we propose, in addition, that not all immune activation is necessarily deleterious

and if caused *in vivo* by NVP may well assist infants to prevent establishment of infection. However, for infants already infected when exposed to NVP (i.e. those with *in utero*-acquired infection) the augmented immune activation that occurs in the presence of both NVP and already established infection at birth may well be deleterious. This brings to mind those studies that have indicated that infants who become infected despite perinatal AZT prophylaxis may have a more rapid course of the HIV-1 disease and higher mortality compared with infants who become infected without drug exposure (Italian Registry for HIV infection in Children, 1999; Kuhn *et al*., 2000; de Souza *et al*., 2000; Sutthent *et al*., 2002). However, this has not been observed in all studies (Rich *et al*., 2000; Dabis *et al*., 2001) and may be confounded by the severity of maternal disease. Our data would provide a biological explanation for how this may occur, and whether disease progression is more rapid among infected children with NVP exposure should be investigated. What is further thought-provoking is the effect of NVP on levels of activation markers in the IU transmitting mothers, a phenomenon that did not occur in the absence of NVP (IU PEP mothers). This would suggest the existence of some factor/s unique to this group of mothers that may also relate to why these mothers were more likely to transmit HIV-1 to their foetuses during pregnancy. We propose that NVP is able to synergize with HIV-1 to increase immune activation through either a mechanism whereby NVP directly acts to activate cells harbouring HIV-1, or indirectly by NVP acting on bystander uninfected cells which in turn produce cytokines/factors that increase immune responsiveness or increase HIV-1 replication which in turn generates elevations in levels of activation markers. A question that is raised is what is different about these IU mothers compared to other transmitting (IP) and non-transmitting mothers. The HIV-1 strain infecting these mothers may be much more conducive to increased replication through immunomodulatory effects of NVP, or alternatively, NVP may be metabolized differently in these women resulting in different immunomodulatory effects to the other women. Our *in vitro* data would support that some individuals might have enhanced HIV-1 replication in the presence of NVP, furthermore, this phenomenon appears to be host-dependent (as the same primary isolate was used to infect primary adult PBMC cultures). It will be important to establish if other antiretroviral drugs or drug combinations used to prevent mother-infant HIV-1 transmission also enhance immune activation in some individuals.

In conclusion, this study has demonstrated that HIV-1 exposure and short-course antiretroviral prophylaxis impact on the developing immune system of the infant. Short-course NVP exposure may be beneficial with respect to driving immune activation prior to the establishment of an infection in an infant exposed to HIV-1, however may have some detrimental consequences in the case of existing infection acquired *in utero*. Understanding the synergistic interaction between HIV-1 infection and NVP in substantially enhancing immune activation in some individuals but not in others will provide important insights that could lead to a means to identify mothers who present with this phenomenon, as these are the mothers who are likely to transmit HIV-1 during pregnancy.

CHAPTER FIVE

Consequences of HIV-1 exposure/infection and presence/absence of single-dose NVP exposure on cytokine and chemokine levels of newborn infants

5.1 Introduction

Alterations in T-lymphocytes (Clerici *et al*., 2000), impaired interleukin production (Chougnet *et al*., 2000), a greater susceptibility to apoptosis (Economides *et al*., 1998) and impaired thymic development (Nielsen *et al*., 2001) are some of the consequences associated with an infant being born to an HIV-1 seropositive mother. In Chapter Four we demonstrated that exposure of an infant *in utero* to HIV-1, regardless of single-dose NVP exposure, resulted in significant increases in immune activation markers in cord blood plasma. We therefore questioned whether immune activation in combination with short-course NVP could be associated with changes in levels of other innate immune factors, particularly specific cytokines and chemokines, which play important roles both in response to HIV-1 infection and in the development of a functional immune system, in early life.

The biological functions of cytokines and chemokines and their receptors are numerous and involve: cell recruitment (Rossi and Zlotnik, 2000); Th1/Th2 development or Th1/Th2 polarization of $CD4^+$ and $CD8^+$ T-cell cytokine-secretion patterns (Lusso 2002); immunomodulation, organogenesis and haematopoiesis (Zou *et al*., 1998); proliferation, maturation and function of myeloid progenitor cells (Gasson 1991); regulation of the development, proliferation and homeostatic maintenance of naïve (Tan *et al*., 2001) and antigen-stimulated mature T-cells, both through peripheral expansion and through thymus-dependent mechanisms (Hofmeister *et al*., 1999). In adults, chronic immune activation induced by HIV-1 infection results in dysregulation of the cytokine network (Cohen *et al*., 1997) which contributes to HIV-1 pathogenesis by stimulating viral replication or suppressing the immune system to mount a strong antiviral response and even inducing cytokine-mediated cytopathic effects (Cohen *et al*., 1997). The cytokine/chemokine interaction with HIV-1 is complex (Chatt *et al*., 2002) and is recognised to influence viral dynamics, replication and disease progression.

Little research to date has documented the effects of HIV-1 exposure and/or infection in the absence and presence of single-dose NVP on the factors that regulate haematopoiesis, lymphocyte homing and angiogenesis, least of all in the developing immune system, of newborns. In this study we focus on the consequences of these exposures on levels of the cytokines IL-7, IL-10, GM-CSF, the CXC chemokine CXCL12, as well as the CC chemokines CCL3, CCL4 and CCL5 measured in cord blood plasma of newborn infants.

5.2 Results

5.2.1 Clinical characteristics of HIV-1 seropositive mothers and their infants

Clinical characteristics of the mother-infant participants who were included in this nested case-control study have been presented in Chapter Four (Table 4.1).

5.2.2 NVP-unexposed infants exhibit similar levels of IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4 and CCL5 to control infants

In order to establish if exposure to HIV-1 in the absence of single-dose NVP influences levels of IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4 or CCL5 as evidenced by increased or decreased levels of these factors in cord blood plasma, EU infants born to HIV-1 infected mothers were compared to control infants born to HIV-1 uninfected mothers. Statistically, there were no significant differences between EU PEP infants for any of these factors when compared to control infants (Table 5.1).

5.2.3 Single-dose NVP exposure has no appreciable effect on cytokine and chemokine levels in EU infants

As no consequences of *in utero* exposure were demonstrated on the levels of IL-7, IL-10, GM-CSF, CXCL12, CCL3, CCL4 or CCL5 we questioned whether single-dose NVP exposure would influence levels of these factors. EU DART infants demonstrated a trend towards elevated levels of the factors IL-10, CXCL12, GM-CSF, CCL4 and CCL5 (Table 5.1) with a reduction in levels in CCL3 relative to EU PEP infants. However, overall statistically, there were no significant differences between levels of any of the factors of EU PEP and EU DART infants. NVP therefore had no appreciable effect on levels of any of the factors measured.

5.2.4 Levels of factors do not differ significantly between HIV-1 exposed infants and those that become infected IU or IP in the presence or absence of single-dose NVP

There is a general consensus that HIV-1 infection and disease progression are directly related to immune activation and subsequent immune dysregulation. In the absence of significant differences between levels of IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4

or CCL5 of drug-exposed and drug-unexposed infants we were curious as to whether infants infected *in utero* or that became infected just prior to or during labour (IP) might exhibit levels of these factors which could account for transmission. In the absence of single-dose NVP, no significant differences were demonstrated between levels of IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4 or CCL5 of exposed infants (EU PEP) and infants infected IP or IU (IP PEP, IU PEP) (Table 5.1). With the exception of IL-7, which in the IU DART infants was significantly reduced relative to EU DART infants, all other immune factors were similar between EU DART versus IP or IU DART infants (Table 5.1).

Table 5.1 Median and interquartile ranges of levels of IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4 and CCL5 of infants stratified based on infection outcome

	Control	**PEP cohort**			**DART cohort**		
Infection outcome	**Uninfected**	**EU**	**IP**	**IU**	**EU**	**IP**	**IU**
N	30	63	13	4	54	3	4
Median (IQR)							
IL-7 (pg/ml)	2.3 (1.4-3.6)	2.3 (1.4-3.9)	2.4 (1-3.9)	2.4 (1.9-3.5)	2.4 (1.6-3.9)	3.1 (1.4-3.7)	0.7[1;2] (0.4-2.2)
IL-10 (pg/ml)	17.4 (15.6-27.2) *12/30	19.8 (17.5-31.5) *56/63	19.2 (16.8-30.3) *9/13	38.9[3] (23.5-70.8) *3/4	26.7[4] (17.5-43.1) *32/54	15.1 (13.8-28.8)	ND
CXCL12 (pg/ml)	311 (233.2-502.9)	323.8 (253.2-420.1)	330.2 (277.8-432.9)	387.7 (294.9-442.7)	371 (260.9-485)	240.4 (234-362.3)	491.5[5] (395.4-701.2)
GM-CSF (pg/ml)	6.4 (4.3-8.4)	4.7 (1.9-9.2)	7 (3.7-9.1)	6.9 (1.7-8.3)	7.7 (1.2-9.9)	1.6[6] (0.2-3)	7.3 (3-11.6)
CCL3 (pg/ml)	32.9 (23.7-37.2) *20/30	23.0 (18.2-60.7) *40/63	28 (19-136.7)	28 (11.7-41.3)	18.1 (10.7-50.2) *19/54	25.5 (18.1-255.2)	18.1 (18.1) *1/4
CCL4 (pg/ml)	87.6 (70.3-120) *20/30	97.8 (71.1-158) *40/63	100.84 (77.2-230.1)	84.8 (63.5-109.3)	170.2[7] (97.5-318.8) *19/54	85.1 (58.7-2025)	ND
CCL5 (ng/ml)	48.8 (40-58.1) *20/30	41.9 (25.3-55) *40/63	48.8 (33.4-54.1)	47.6 (11.6-61)	57.6 (38.1-78.9) *19/54	68.9 (20.9-71.5)	2.1 (2.1) *1/4

IQR - Interquartile range i.e. 25th and 75th percentiles
Significant differences within groups: [1] control and IU DART (p=0.033); [2] EU DART and IU DART (p=0.027); [3] control and IU PEP (p=0.030); [4] control and EU DART (p=0.041); [5] IP DART and IU DART (p=0.034); [6] control and IP DART (p=0.008); [7] control and EU DART (p=0.017)
Significant differences between PEP and DART groups: GM-CSF - IP PEP (n=13) and IP DART (n=3) (p=0.043). All other comparisons between levels of the factors of PEP and DART infant groups were not significant (p>0.05)
ND - not determined as samples too few for statistical significance or due to insufficient sample for analysis
* = n/N: proportions tested

5.2.5 Maternal status may influence immunomodulatory factors such as IL-10 in the infant

Convincing evidence indicates that anti-inflammatory cytokines such as IL-10 play an essential role in regulating Th1 immune reactions. We therefore questioned how maternal IL-10 levels might influence infant levels. It was interesting that mothers with high viral load (PEP) had plasma levels of IL-10 that correlated with infant levels (EU PEP) (r=0.622, p=0.031). However, when adjusting for maternal viral load this did not account for the IL-10 levels.

5.2.6 Haematopoietic growth factors are modulated independently of those in the mother

It was important to determine whether *in utero* exposure to HIV-1 could impact on the haematopoietic growth factors of newborn infants. Within the control group there were no significant correlations between mothers' and infants' levels of IL-7 (r=0.714, p=0.111), CXCL12 (r=0.232, p=0.658) or GM-CSF (r=0.364, p=0.087) and infants had significantly raised levels of GM-CSF (p<0.001), significantly reduced levels of CXCL12 (p=0.028) and elevated levels of IL-7 (2.6 pg/ml versus 1.5 pg/ml) compared to their mothers, suggesting that these factors develop independently from those of the mother (Table 5.2).

5.2.7 Correlations exist between haematopoietic growth factors in the newborn infant (control infants)

The neonatal immune system is considered to be one that is developing and in the context of HIV-1 exposure it has become increasingly important to distinguish between changes associated with developmental factors versus defects induced by HIV-1 exposure. It was therefore interesting to determine at birth whether control infants demonstrated a relationship between the haematopoietic growth factors IL-7, CXCL12, GM-CSF, CCL3, CCL4 and CCL5. IL-7 levels were strongly correlated with levels of CXCL12 (r=0.650, p=0.000) but negatively correlated with levels of GM-CSF (r=-0.423, p=0.020). CCL3 strongly correlated with CXCL12 (r=0.606, p=0.005) as well as with CCL4 (r=0.588, p=0.006). Furthermore, increased levels of CCL4 strongly correlated with increased levels of CCL5 (r=0.529, p=0.016).

5.2.7.1 Correlations exist between IL-7 and CXC- and CC-chemokines in EU infants

Having demonstrated significant correlations between haematopoietic growth factors in control infants we questioned how HIV-1 exposure (EU infants) would influence these relationships. In the absence of single-dose NVP (EU PEP infants) weak but significant correlations were demonstrated between IL-7 and CXCL12, CCL3 and CCL4 (r=0.350, *p*=0.005; r=0.361, *p*=0.022; r=0.415, *p*=0.008 respectively), with a stronger correlation with CCL5 being demonstrated (r=0.731, *p*=0.000). The correlations between IL-7 and CXCL12 (r=0.402, *p*=0.003) and IL-7 and CCL5 (r=0.778, *p*=0.000) remained in the presence of NVP (EU DART). However, this group of infants also demonstrated a weak but significant negative correlation between IL-10 and IL-7 (r=-0.365, *p*=0.040).

5.2.8 CBMC of NVP-unexposed and NVP-exposed infants produce similar levels of IL-7, CXCL12 and GM-CSF when primed with HIV-1 Env peptides or PHA

Having demonstrated that *in utero* HIV-1 exposure does not significantly impact on cord blood plasma levels of IL-7, CXCL12 or GM-CSF, we sought to ascertain whether the ability of CBMC to produce these factors might be altered by *in utero* HIV-1 exposure or infection, or by single-dose NVP. The production of these haematopoietic growth factors, either due to spontaneous release (medium) or in response to stimulation with both HIV-1 Env peptides and PHA was measured in a limited number of infants from the nested case-control study. In the absence of both infection and single-dose NVP (EU PEP, IP PEP) (Figure 5.1A) (i) CBMC of all infants had similar abilities to produce IL-7 (Figure 5.1Ai-iii) and CXCL12 (Figure 5.1Aiv-vi) as control infants, although there was a trend towards decreased IL-7 in infants born to HIV-1 positive mothers, (ii) exposed infants had a significantly greater ability to produce GM-CSF in response to HIV-1 envelope peptide and PHA stimulation than control infants (Figure 5.1Aviii,ix) and (iii) CBMC of infants with pre-existing infection (IU infants) produced significantly less IL-7 spontaneously or in response to HIV-1 peptides (Figure 5.1Ai, ii) but significantly higher levels of GM-CSF in response to HIV-1 Env peptides and PHA compared to control infants (Figure 5.1Aviii,ix). In the presence of single-dose NVP but in the absence of infection (EU DART, IP DART) (Figure 5.1B) however, CBMC of infants produced similar levels of IL-7 and GM-CSF as the control infants in response to HIV-1 Env peptides (Figure 5.1Bii,viii) but significantly higher levels of CXCL12 compared to control infants (Figure

5.1Bv). However, CBMC from EU PEP and EU DART infants did not differ significantly (p>0.05) in their ability to produce IL-7, CXCL12 and GM-CSF when primed with HIV-1 Env peptides and PHA. EU PEP infants were able to spontaneously produce significantly more GM-CSF (p=0.031) than their EU DART counterparts while the spontaneous release of IL-7 and CXCL12 by these groups was similar (p>0.05).

Table 5.2 IL-7, IL-10, CXCL12 and GM-CSF levels of mother and infant pairs

Factor		Mother		Infant		
IL-7 (pg/ml)	**n**	**Median**	**IQR**	**Median**	**IQR**	***P****
Control	6	1.5	1-2.8	2.6	1.2-3.7	NS
PEP						
EU	10	1.7	1.4-2.7	3.1	0.9-5.0	NS
IP	9	1.7	0.8-3.2	2.6	1.1-4.1	NS
IU	3	2.4	0.9-2.9	2.5	2.2-3.9	NS
DART						
EU	6	2.2	1.2-7.5	2.8	1.8-4.1	NS
IP	1	0.7	0.7	1.4	1.4	ND
IU	ND	ND	ND	ND	ND-	ND
IL-10 (pg/ml)						
Control	4	28.3	19.2-33.3	29.8	22.6-34.1	NS
PEP						
EU	12	40.7	29.4-84.2	35.8	26.1-39.5	NS
IP	2	52.7	41.7-63.7	33.2	21.6-44.7	NS
IU	3	31.6	23.6-56.9	33.9	23.5-70.8	NS
DART						
EU	17	47.6	23.4-86	43	30.2-98.3	NS
IP	3	23	20.1-31.1	15.1	13.8-28.8	NS
IU	ND	ND	ND	ND	ND	ND
CXCL12 (pg/ml)						
Control	6	976.1	891-1088.5	311	228-351.1	0.028
PEP						
EU	10	1066	749.2-1420.3	313	259-407.1	0.005
IP	8	1083.2	852.1-1339.5	332.7	292.8-445.9	0.008
IU	4	856.4	693.6-1069.6	387.7	294.9-442.7	NS
DART						
EU	6	996.9	796.6-1286.6	416.9	283.7-521.2	0.046
IP	1	595.5	595.5	240.4	240.4	ND
IU	ND	ND	ND	ND	ND	ND
GM-CSF (pg/ml)						
Control	23	0.6	0.5-0.7	6.1	4.4-8.6	<0.001
PEP						
EU	35	0.4	0.3-0.5	2.1	1.3-7.5	<0.001
IP	5	0.3	0.3-0.5	8.4	3.7-10.3	NS
IU	4	0.3	0.2-0.6	6.9	1.7-8.3	NS
DART						
EU	17	0.3	0.2-0.6	6.2	1.0-9.6	0.001
IP	2	0.3	0.1-0.5	0.9	0.2-1.6	NS
IU	ND	ND	ND	ND	ND	ND

IQR - Interquartile range i.e. 25^{th} and 75^{th} percentiles

*P** Wilcoxon signed rank test for paired (mother-infant) data sets. Statistical tests were two-tailed and significant at $p<0.05$

ND - not determined as samples too few for statistical significance or due to insufficient sample for analysis

NS – no significant differences between infant and mother pairs

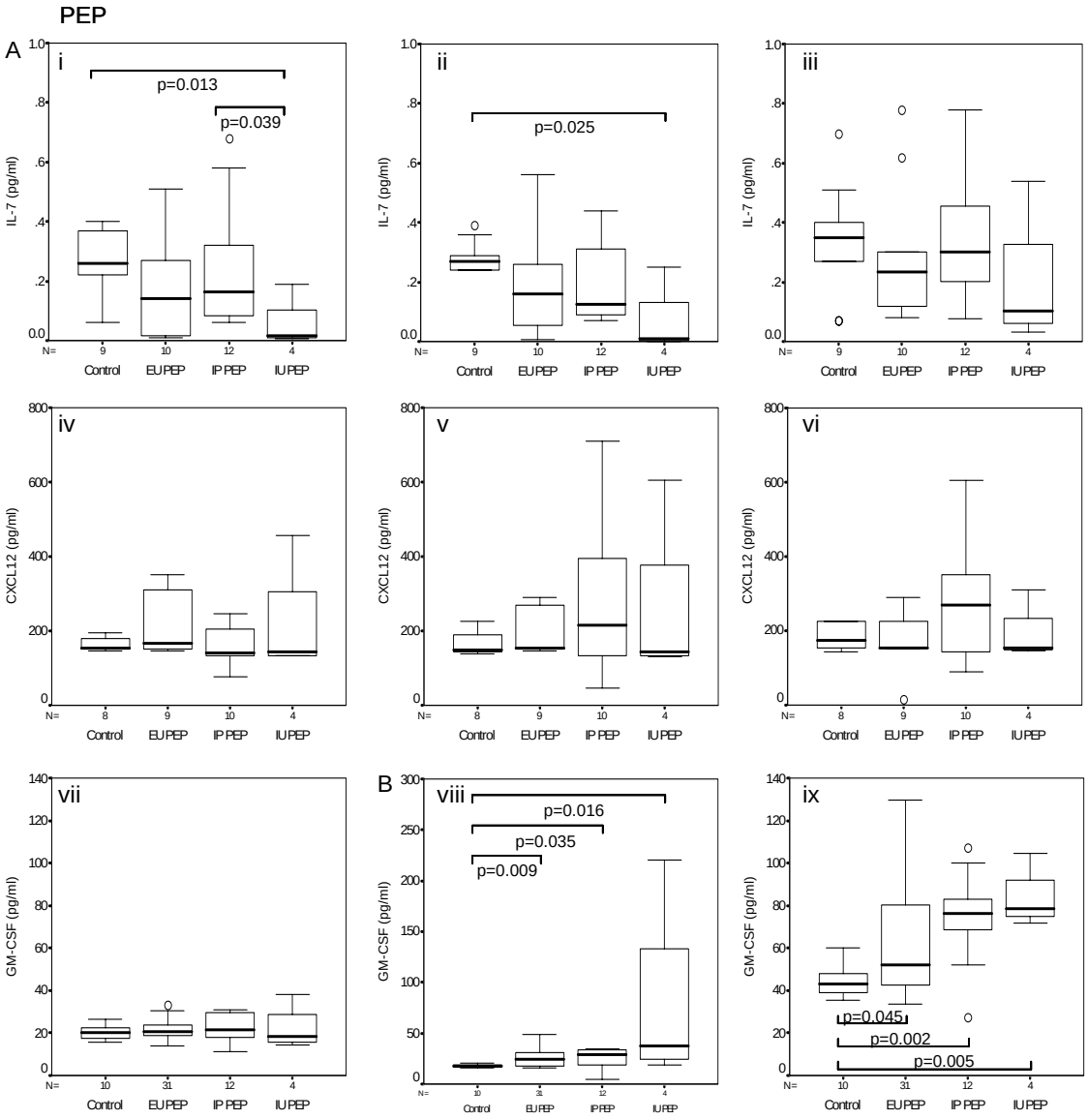


Figure 5.1A Ability of CBMC isolated from infants given PEP within 72 hours of birth to produce IL-7 (pg/ml), CXCL12 (pg/ml) and GM-CSF (pg/ml) spontaneously (i,iv,vii), in response to HIV-1 envelope peptides (ii;v,viii) and PHA (iii;vi,ix). Infant groups are stratified on the basis of infection outcome: exposed uninfected (EU), infected intrapartum (IP) or infected *in utero* (IU). An unexposed uninfected (control) group is included. The data are presented using box plots with medians being reflected by the horizontal bars, the 25th and 75th percentiles by the boxes and the 10th and 90th percentiles by the bars. Significant differences between groups and the number of samples tested per group are indicated.

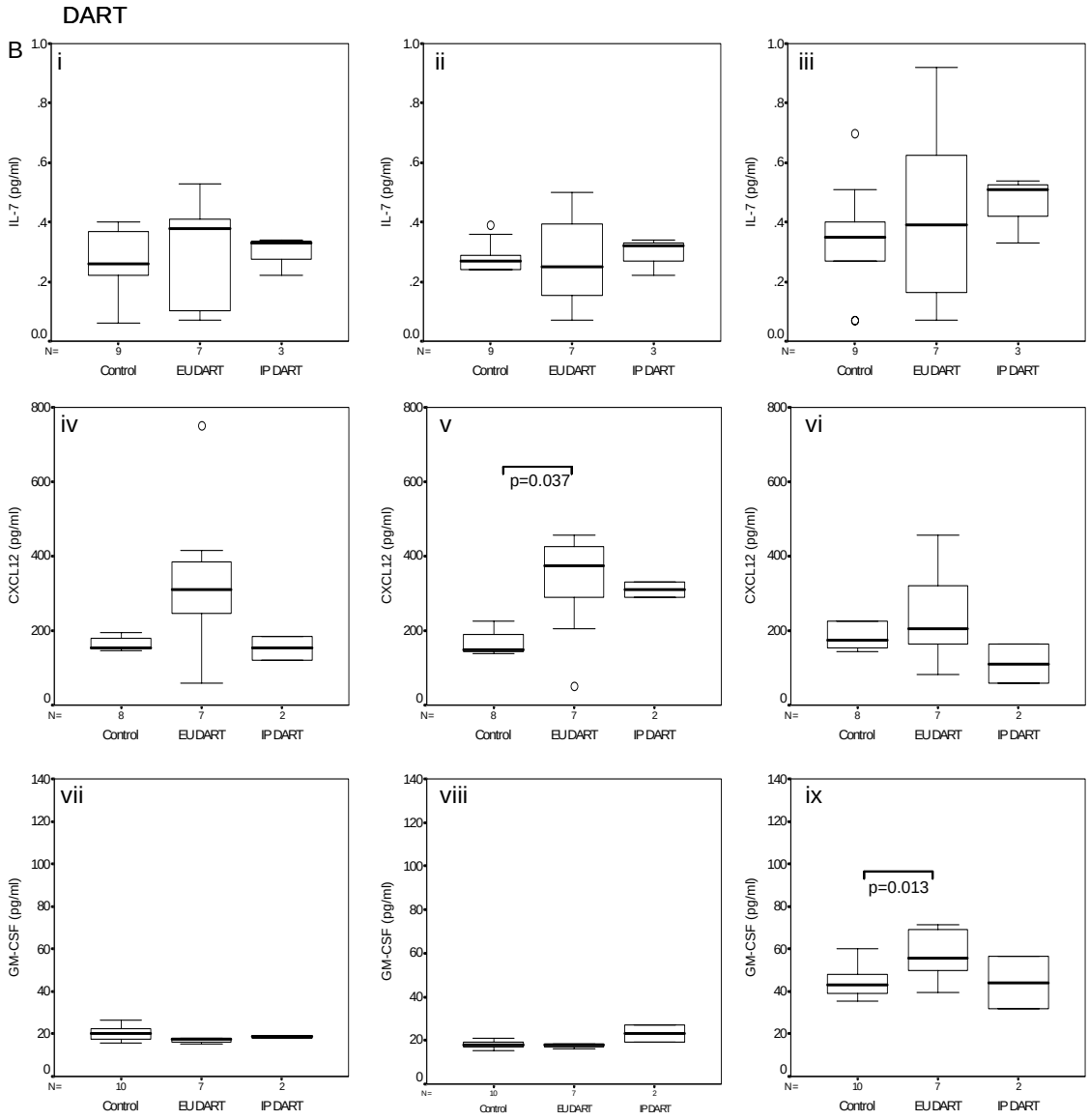


Figure 5.1B Ability of CBMC isolated from infants exposed to a single-dose of NVP to produce IL-7 (pg/ml), CXCL12 (pg/ml) and GM-CSF (pg/ml) spontaneously (i,iv,vii), in response to HIV-1 envelope peptides (ii,v,viii) and PHA (iii,vi,ix). Infant groups are stratified on the basis of infection outcome: exposed uninfected (EU) or infected intrapartum (IP). An unexposed uninfected (control) group is included. The data are presented using box plots with medians being reflected by the horizontal bars, the 25th and 75th percentiles by the boxes and the 10th and 90th percentiles by the bars. Significant differences between groups and the number of samples tested per group are indicated.

5.3 Discussion

GM-CSF, IL-7 and CXCL12 play important roles in regulating haematopoiesis, lymphocyte homing, B-lineage cell growth and angiogenesis. IL-10 has been described as an immunosuppressive and anti-inflammatory cytokine that functions to control type-1-mediated inflammation. Chemokines (CCL3, CCL4 and CCL5) are also recognized for their involvement in a variety of immune and inflammatory responses. Interestingly, these cytokines/chemokines have been demonstrated to play a role in individuals that are HIV-1 infected, that is, levels of CCL3, CCL4 and CCL5 produced in response to stimulation with HIV-1 proteins are associated with reduced viral replication *in vivo* (Ferbas *et al*., 2000). In light of the involvement of these cytokines/chemokines in immune responses we aimed to assess levels of IL-7, IL-10, GM-CSF, CXCL12, CCL3, CCL4 and CCL5 in cord blood plasma of infants born to HIV-1 infected mothers and to determine whether NVP exposure or HIV-1 infection influences the production of these factors.

Our results have shown that (i) in the absence of single-dose NVP cord blood plasma levels of IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4 and CCL5 from EU infants were not significantly different from those of uninfected (control) infants, (ii) exposure to single-dose NVP has no significant effect on the levels of these factors, (iii) regardless of single-dose NVP exposure there were no significant differences between levels of IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4 and CCL5 of EU infants, IP or IU infants, (iv) levels of infant haematopoietic factors (IL-7, CXCL12 and GM-CSF) were correlated and developed independently from those of their mothers, (v) maternal immunomodulatory cytokines such as IL-10 may influence levels in infants and, (vi) single-dose NVP exposure appears to influence the ability of cells to spontaneously produce GM-CSF.

The plasma levels of the factors in newborns are measures of immunomodulatory events that have occurred prior to labour. Our data suggests that *in utero* HIV-1 exposure does not significantly affect the cytokines and chemokines tested that are involved in immunomodulation and haematopoiesis. However, pre-existing infection may affect cytokine/chemokine levels significantly e.g. IL-10. The observation that CCL3, CCL4 and CCL5 levels of drug-unexposed infants born to HIV-1 positive mothers are similar to those of uninfected infants would be in agreement with the fact that naïve or resting cells do not produce chemokines (Moser *et al*., 2004) and suggests that *in utero* HIV-1

exposure is not sufficient to prime these cytokines. Importantly our data would indicate that whilst CBMC may have the potential to produce these chemokines they are not readily detected in cord blood plasma of newborn infants. Significant amounts of CCL3 and CCL4 are released spontaneously and following PHA induction by CBMC of infants given PEP compared to infants exposed to single-dose NVP (Meddows-Taylor, 2003). No difference in the levels of spontaneous and PHA-induced CCL5 between EU PEP and EU DART infants has been demonstrated (Meddows-Taylor, 2003). CC-chemokine production may represent an important cellular response for regulating extent of viral replication and recruitment of immune cells (Fantuzzi *et al*., 2001). However these chemokines also play a significant role in blood cell regulation (Broxmeyer *et al*., 1993) with the CC-chemokine CCL3 being reported to suppress colony formation of myeloid progenitor cells that are stimulated by GM-CSF, in a dose-dependent manner (Broxmeyer *et al*., 1993). This might suggest that the strict immunomodulatory/regulatory control of CC-chemokines may be of particular relevance to infants and their developing immune systems.

It is interesting that no statistically significant differences were demonstrated between IL-10 levels of control infants and their mothers, but that a strong correlation between maternal and infant levels was demonstrated. This would suggest that IL-10 levels could influence the developing infant immune system since IL-10 can downregulate T-cell functions. Whilst the haematopoietic growth factors IL-7, GM-CSF and CXCL12 are modulated independently in infants, levels of IL-10 might play a more important role than previously recognised. It has been postulated that in infants, an immunoregulatory circuit may exist in which IL-10 has a central role in the modulation of T-cell production of inflammatory cytokines (Wegmann *et al*., 1993). This could have far reaching consequences for infants born to HIV-1 infected mothers particularly since significant negative correlations were demonstrated between IL-10, which can downregulate Th1 type functions and IL-7, which regulates naïve and mature T-cells, in drug-exposed infants (EU DART infants). Interestingly, significant positive correlations were demonstrated between IL-7, CCL3, CCL4 and CCL5 in drug-unexposed infants (EU PEP). A dose-dependent production of CCL3, CCL4 and CCL5 induced by IL-7 has been demonstrated in PBMC of HIV-1 uninfected and HIV-1 infected individuals, suggesting that IL-7 may regulate CC-chemokine production *in vivo* (Llano *et al*., 2003). We further demonstrated that in the absence of single-dose NVP, CBMC spontaneously produced

more GM-CSF. In the case of infants born to HIV-1 seropositive mothers, immunosuppressive cytokines such as IL-10 may play a significant role in suppressing immune and inflammatory responses and reducing antigen-specific proliferation and cytokine production of T-cells. This may have implications for the developing immune system of infants and could therefore influence the course of HIV-1 infection and AIDS in infants.

In conclusion, HIV-1 exposure does not influence levels of IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4 and CCL5 significantly. Single-dose NVP exposure does not significantly affect levels of any of these factors but may influence trends in the levels of cytokines/chemokines. Regardless of single-dose NVP exposure, levels of these factors do not change between HIV-1 exposed and infected infants and therefore do not predict infection status. Haematopoietic growth factors independently modulate the infants developing immune system but maternal factors such as IL-10 and exposure to single-dose NVP, which reduces stimulation of CBMC, could impact on the infant. This data suggests that while the direct antiviral effects of single-dose NVP are not disputed, indirect consequences of NVP exposure include an altered ability of CBMC to produce cytokines such as GM-CSF.

CHAPTER SIX

Immune factor(s) of infants born to HIV-1 seropositive mothers that develop HIV-1 envelope specific cellular immune responses and those that do not

6.1 Introduction

The vast majority of T-lymphocytes in infants are naïve cells reflecting their relatively short exposure to external stimuli (Ibegbu *et al*., 1994). Low dose exposure to HIV-1 can selectively activate the cellular arm of the immune system resulting in the presence of strong HIV-1 specific T-cell immunity (Shearer and Clerici, 1996). Studies demonstrating specific T-helper and cytotoxic T-cell responses in cord blood from HIV-1 uninfected newborns of HIV-1 seropositive mothers (Clerici *et al*., 1993a; Kuhn *et al*., 2002, Wasik *et al*., 1999) point to the ability of the neonatal immune system to respond *in utero* to HIV-1 or its soluble products. A decrease in this T-cell function is reported to occur in subsequent months following termination of the exposure to HIV-1 from the mother (Shearer and Clerici, 1996). T-helper cell responses to HIV-1 specific antigens in newborns have been implicated in protection from HIV-1 infection, with a study conducted in a breastfeeding population in South Africa finding no HIV-1 transmission to infants that had a T-helper cell response to HIV-1 envelope peptides in cord blood compared to 17% transmission among infants with no T-helper cell responses (Kuhn *et al*., 2001a).

It has become increasingly important to establish the functional maturity of the immune system of infants, particularly with respect to infants born to HIV-1 seropositive mothers. Although studies have shown that a proportion of HIV-1 exposed infants have been primed to HIV-1 *in utero* and remain uninfected, when this priming event takes place is unclear. While it is particularly interesting that protective T-helper cell responses can be demonstrated in newborns in the absence of antiretroviral treatment these responses are attenuated in neonates receiving short-course antiretroviral treatment to prevent MTCT (Kuhn *et al*., 2001c). We attempted to determine whether levels of certain immune activation markers, immunomodulatory/immunoregulatory and haematopoietic cytokines, could predict transmission and whether quantitating these factors might further distinguish infants that develop HIV-1 specific cellular immune responses (Env^+) from

those that lack such responses (Env^-). Identifying and characterizing markers of protective immunity will contribute to understanding immune responsiveness in newborns and will provide important information for the design and evaluation of HIV-1 vaccines.

6.2 Results

6.2.1 Clinical characteristics of HIV-1 seropositive mothers and their infants

Clinical characteristics of the mother-infant participants who were included in this nested case-control study are presented in Table 6.1. Maternal viral loads and $CD4^+$ T-lymphocyte counts were similar between mothers of infants that remained uninfected or became infected IP or IU (Table 6.1).

6.2.2 Levels of immune activation markers, immunomodulatory/immunoregulatory and haematopoietic cytokines do not predict maternal-infant HIV-1 transmission

T-helper cell responses to HIV-1 Env peptides detected in uninfected infants at birth have been associated with lack of subsequent IP infection (Kuhn *et al*., 2001a). T-helper cell responses have also been detected in cord blood of some infants infected *in utero* (Kuhn *et al*., 2001a). It was therefore interesting to question whether levels of neopterin, β_2-m, sL-selectin, IL-7, CXCL12 and IL-10 measured in cord blood plasma of infants could predict IP infection (infants defined with a negative HIV-1 DNA PCR within 24 hours of birth). Infants infected IU (infants defined with a positive HIV-1 DNA PCR within 24 hours of birth) were included to assess these same immune factors with the knowledge that existing infection was likely to affect these measurements. No statistically significant differences were demonstrated between neopterin, β_2-m, sL-selectin, IL-7, CXCL12 or IL-10 levels of EU or IP infants (Table 6.1) indicating that at birth these factors do not predict the likelihood of IP transmission. However, significantly elevated levels of sL-selectin and IL-10 were detected in infants with pre-existing infection (IU) relative to EU infants implying that there is significant immunological priming associated with infection. As cord blood measures in IU infected infants demonstrated consequences of existing infection they were excluded from all subsequent analyses.

6.2.3 Presence of T-helper envelope responses predict the lack of IP transmission

Corroborating earlier findings (Kuhn *et al*., 2001a) a significantly higher proportion of EU infants elicited cellular immune responses to HIV-1 Env peptides, while IP infants were unresponsive to Env (Fisher's exact test, p=0.029; Table 6.2). These T-helper Env

responses which are detectable at birth in EU infants therefore serve as a marker of protective immunity. In an attempt to further characterize these responses we wished to establish whether differences exist in other immune parameters (e.g. neopterin, β_2-m, sL-selectin, IL-7, GM-CSF, CXCL12 and IL-10) between those infants that elicit such responses and those who do not. Furthermore, we questioned if differences in these immune parameters could distinguish between IP infants (Env^-) and EU infants (Env^-).

Table 6.1 Maternal viral loads, maternal $CD4^+$ T-lymphocyte counts and infant levels of immune factors

	PEP cohort with known HIV-1 envelope response outcome			
Infection outcome	**Control**	**Exposed uninfected**	**Infected intrapartum**	**Infected *in utero***
N	20	60	13	3
Mean (standard deviation)				
Maternal $CD4^+$ T-lymphocyte count[1]	ND	483.2 (263.3)	430 (188.7)	504 (466.7)
Median (IQR)				
Maternal HIV-1 RNA (log_{10})[2]	-	4.7 (4.0-5.1)	4.8 (4.5-5.3)	5.4 (3.6-5.5)
Neopterin (ng/ml)[3]	2.7 (2.2-3.6)	3.7 (2.9-4.8)	4.3 (3.3-6.7)	5.2 (3.3-5.6)
β_2-microglobulin (μg/ml)[4]	1.6 (1.3-2.0)	2.2 (1.7-3.0)	2.2 (1.6-2.8)	2.8 (2.6-5.4)
sL-selectin (ng/ml)[5]	554.5 (484.0-613.5)	666.6 (565.5-794.2)	703.1 (581.9-790.4)	1046.6 (734.3-1629.1)
GM-CSF (pg/ml)	5.3 (4.2-6.9)	4.1 (1.7-9)	7 (3.7-9.2)	7.7 (6.1-8.5)
CXCL12 (pg/ml)	311 (253.2-510.0)	323 (248.4-418.4)	330.2 (277.8-432.9)	393.8 (381.6-459)
IL-7 (pg/ml)	2.8 (1.8-4)	2.2 (1.4-3.5)	2.4 (1-3.9)	2.2 (1.9-2.5)
IL-10 (pg/ml)[6]	17.4 (15.6-20.3) *(11/20)	19.8 (17.5-27.2)	19.2 (16.9-30.3) *(9/13)	54.8 (38.9-70.8) *(2/3)

IQR - Interquartile range i.e. 25th and 75th percentiles
[1] Mothers $CD4^+$ T-lymphocyte counts are not significantly different
[2] Maternal viral loads are not significantly different
Significant differences between: [3] control and EU PEP (p=0.001), control and IP PEP (p=0.001), control and IU PEP (p=0.028); [4] control and EU PEP (p=0.006), control and IP PEP (p=0.036), control and IU PEP (p=0.022); [5] control and EU PEP (p=0.001), control and IP PEP (p=0.015), control and IU PEP (p=0.011), EU and IU PEP (p=0.026); [6] control and EU PEP (p=0.025), control and IU PEP (p=0.029)
* = n/N: proportions tested

6.2.4 Mothers of infants with HIV-1 specific cellular immune responses have lower viral loads

HIV-1 exposed uninfected individuals from different risk groups do not produce detectable antibodies against HIV-1 and show no signs of infection, yet they exhibit HIV-1 specific cell-mediated immunity (Ranki *et al*., 1989; Clerici *et al*., 1992; Cheynier *et al*., 1992) suggesting that exposure to defective viruses or to sub-infectious doses of HIV-1 may play a role in the development of protective cell-mediated immune responses. To test

the latter hypothesis, we compared viral loads of non-transmitting mothers (EU infants: Env^- and Env^+) and of IP transmitting mothers (IP infants: Env^-). Mothers whose infants elicited IL-2 responses to HIV-1 Env peptides had lower median viral loads than those whose infants did not elicit these responses (this being true for infants who became infected IP and those who did not)(Table 6.2). Since mothers of EU and IP Env^- infants had higher viral loads compared to mothers of Env^+ infants ($log_{10}4.8$ and $log_{10}4.1$, respectively), we might have expected the former groups to demonstrate some associated immunological differences. However, adjusting for maternal viral load, viral load did not account for variations in levels of neopterin, β_2-m, sL-selectin, IL-7, IL-10, CXCL12 or GM-CSF between the infant groups.

6.2.5 Infants with HIV-1 specific cellular immune responses exhibit different immune capabilities compared to those without responses

The measurement of plasma activation factors is indicative of events that have occurred prior to birth. Also, the presence of cellular responses to HIV-1 Env responses is indicative of prior HIV-1 exposure, an event that is expected to be associated with immune activation.

All infants regardless of their ability to elicit HIV-1 specific responses exhibited significantly elevated neopterin levels compared to control infants (Table 6.2; Figure 6.1A). In contrast to what might be expected, β_2-m and sL-selectin were not different in EU Env^+ infants compared to control infants (Fig 6.1B;C), however levels were increased in EU infants without Env responses. There was no apparent difference in immune activation (neopterin, β_2-m and sL-selectin) between the EU and IP Env^- groups.

Table 6.2 IL-2 dependent cellular immune responses of exposed uninfected and intrapartum infected infants as well as levels of the immunomodulatory and immunoregulatory cytokines of infants. Maternal viral load and $CD4^+$ T-lymphocyte counts are also presented

	No response to HIV-1 Env peptides			**IL-2 response to HIV-1 Env peptides**
Infection outcome	**Control**	**Uninfected**	**Infected intrapartum**	**Uninfected**
N	20	42	13	18
Mean SI (standard deviation and n/N)				
PHA response	182.7 (145.9) (20/20)	237 (218.9) (42/42)	262 (303.6) (13/13)	199 (154.4) (18/18)
Env response	1.0 (0.5) (20/20)	1.1 (0.5) (42/42)	0.9 (0.4) (13/13)	4.5 (1.4) (18/18)
Maternal $CD4^+$ T-lymphocyte count[1]	-	482 (259) (41/42)	430 (188.7) (11/13)	488 (288) (13/18)
Median (IQR)				
Maternal HIV-1 RNA (log_{10})	-	4.8 (4.3-5.2) [2]	4.8 (4.4-5.3)[3]	4.1 (3.5-4.9)
Neopterin (ng/ml)[4]	2.74 (2.2-3.6)	3.7 (2.8-4.9)	4.3 (3.3-6.7)	3.3 (2.9-4.3)
β_2-microglobulin (μg/ml)[5]	1.6 (1.3-2.0)	2.2 (1.9-3.4)	2.2 (1.6-2.8)	1.9 (1.5-2.4)
sL-selectin (ng/ml)[6]	554.5 (484-613.5)	695.4 (568.8-842.8)	703.1 (581.9-790.4)	593.6 (509.1-667.1)
IL-7 (pg/ml)	2.8 (1.8-4.0)	2.3 (1.2-3.3)	2.4 (1.0-3.9)	2.1 (1.4-4.7)
CXCL12 (pg/ml)	311 (245.2-520)	325.4 (259-408,8)	332.7 (277.3-445.9) (12/13*)	298.2 (214.7-452.2)
IL-10 (pg/ml)[7]	17.4 (15.6-20.3) (11/20*)	20.2 (18.1-35.6)	19.2 (16.8-30.3) (9/13*)	19 (17.1-21.7)

IQR - Interquartile range i.e. 25^{th} and 75^{th} percentiles

[1]Mothers' $CD4^+$ T-lymphocyte counts are not significantly different

[2]Significant difference (p=0.024) between maternal viral loads of exposed uninfected infants that elicit HIV-1 specific cellular responses and those that do not

[3]Maternal viral load higher than that of mothers whose infants elicit specific cellular responses; not significant (p=0.055);

Significant differences between: [4] control and EU Env^- (p=0.001); control and IP Env^-(p=0.001); control and EU Env^+(p=0.016); [5] control and EU Env^- (p=0.001); control and IP Env^-(p=0.036); EU Env^- and EU Env^+(p=0.018); [6] control and EU Env^- (p<0.001); control and IP Env^-(p=0.015); EU Env^- and EU Env^+(p=0.013); [7] control and EU Env^- (p=0.012)

* = n/N: proportions tested

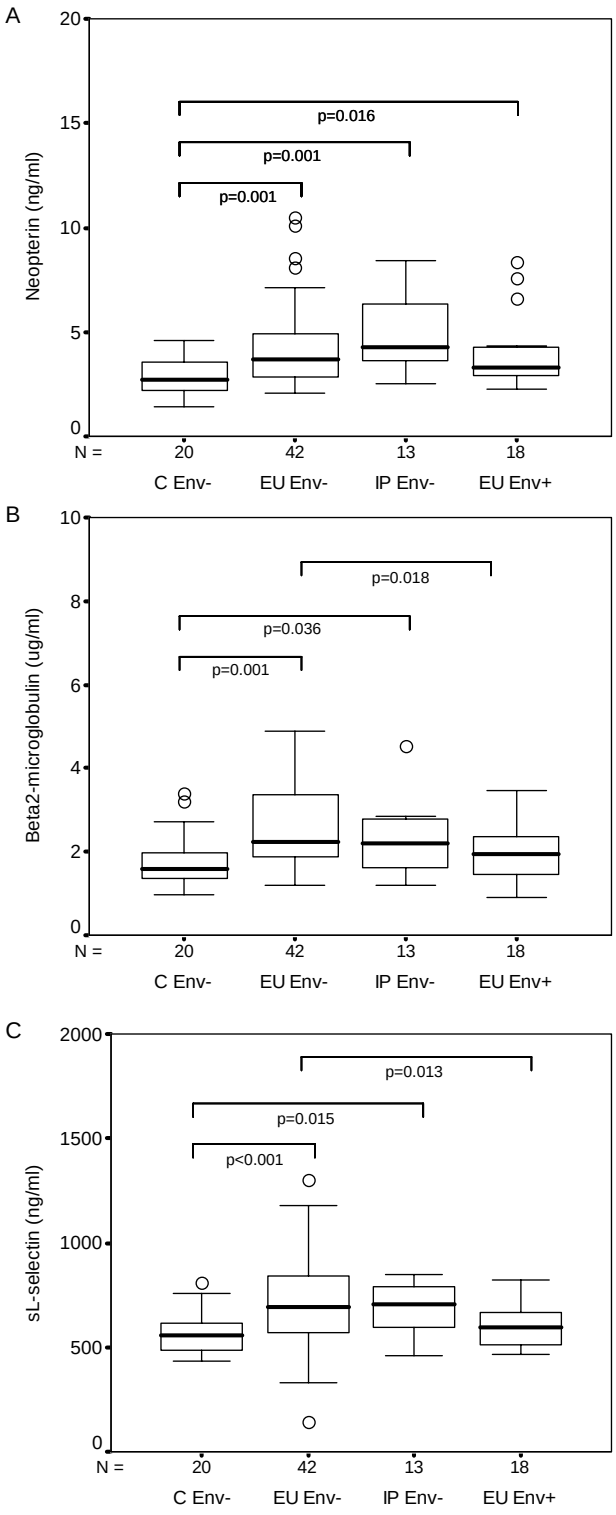


Figure 6.1 Levels of neopterin (ng/ml) (A), β_2-m (μg/ml) (B) and sL-selectin (ng/ml) (C), of infants born to HIV-1 seropositive mothers in the absence of single-dose NVP (PEP) and stratified on the basis of infection outcome and cellular immune responses to HIV-1 envelope peptides (Env^- and Env^+ infants). Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes) and 10th and 90th percentiles (bars). Significant differences between groups and sample numbers per group are indicated.

6.2.6 Infants with HIV-1 specific responses have reduced plasma GM-CSF levels

As the activation of helper T-cells results in the co-ordinate expression of a number of cytokines involved in the differentiation, proliferation and activation of the haematopoietic system, we questioned whether elevated levels of cytokines involved in immunomodulation and haematopoiesis would be associated with Env^+ infants. IL-7 and CXCL12 (Table 6.2) levels were similar regardless of whether or not infants had been exposed to HIV-1 *in utero* or elicited Env responses. Interestingly, Env^+ infants had significantly reduced levels of GM-CSF compared to Env^- infants (Figure 6.2A). Given these findings we were curious as to whether maternal GM-CSF levels might influence those of their infants however, GM-CSF levels of HIV-1 infected mothers were substantially lower than those of their infants and all tended to be decreased relative to those of uninfected mothers (Figure 6.2B). Adult plasma GM-CSF levels were characteristically low compared to those measured in cord blood plasma's of the different groups. This difference was maintained even amongst EU Env^+ infants and their mothers (p=0.002).

6.2.7 CBMC of Env^+ infants exposed to HIV-1 demonstrated a reduced ability to produce GM-CSF in response to stimulation with PHA

As Env^+ infants demonstrated reduced GM-CSF plasma levels we questioned whether this was associated with an inability of CBMC to produce GM-CSF. Env^+ infants demonstrated no altered ability to spontaneously release GM-CSF (Figure 6.3A). In response to stimulation with HIV-1 Env peptides (Figure 6.3B) all groups (Env^- and Env^+ infants) were able to release significantly higher amounts of GM-CSF than the control group. In response to stimulation with PHA however, CBMC of Env^+ infants produced significantly less GM-CSF than EU Env^- infants. If we compare the proportions of infants that had GM-CSF levels greater than the control group median for each stimulus then 44% EU Env^-, 50% IP Env^- and 62% EU Env^+ infants produced more GM-CSF spontaneously, 72% EU Env^-, 75% IP Env^- and 85% EU Env^+ infants produced more GM-CSF in response to HIV-1 Env peptides and 89% EU Env^-, 92% IP Env^- and only 46% EU Env^+ infants produced more GM-CSF in response to stimulation with PHA (Figure 6.3a-c).

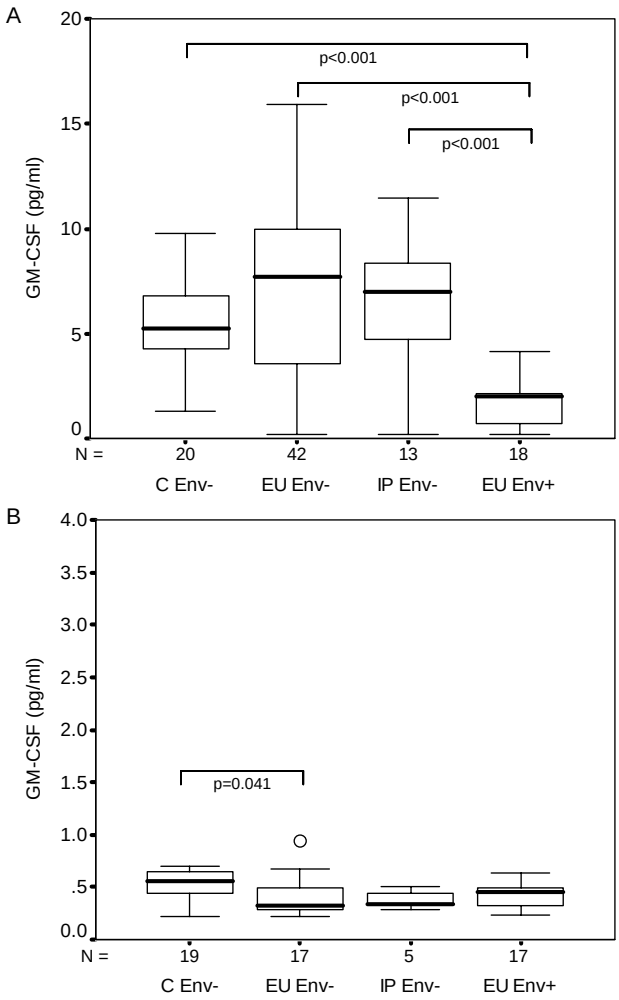


Figure 6.2 Infant (A) and maternal (B) plasma GM-CSF levels. Infants and their mothers were stratified on the basis of infection outcome and HIV-1 specific cellular immune responses to Env peptides (Env^- and Env^+). Data are presented as medians (horizontal bar), 25^{th} and 75^{th} percentiles (boxes) and 10^{th} and 90^{th} percentiles (bars). Significant differences between groups and sample numbers per group are indicated.

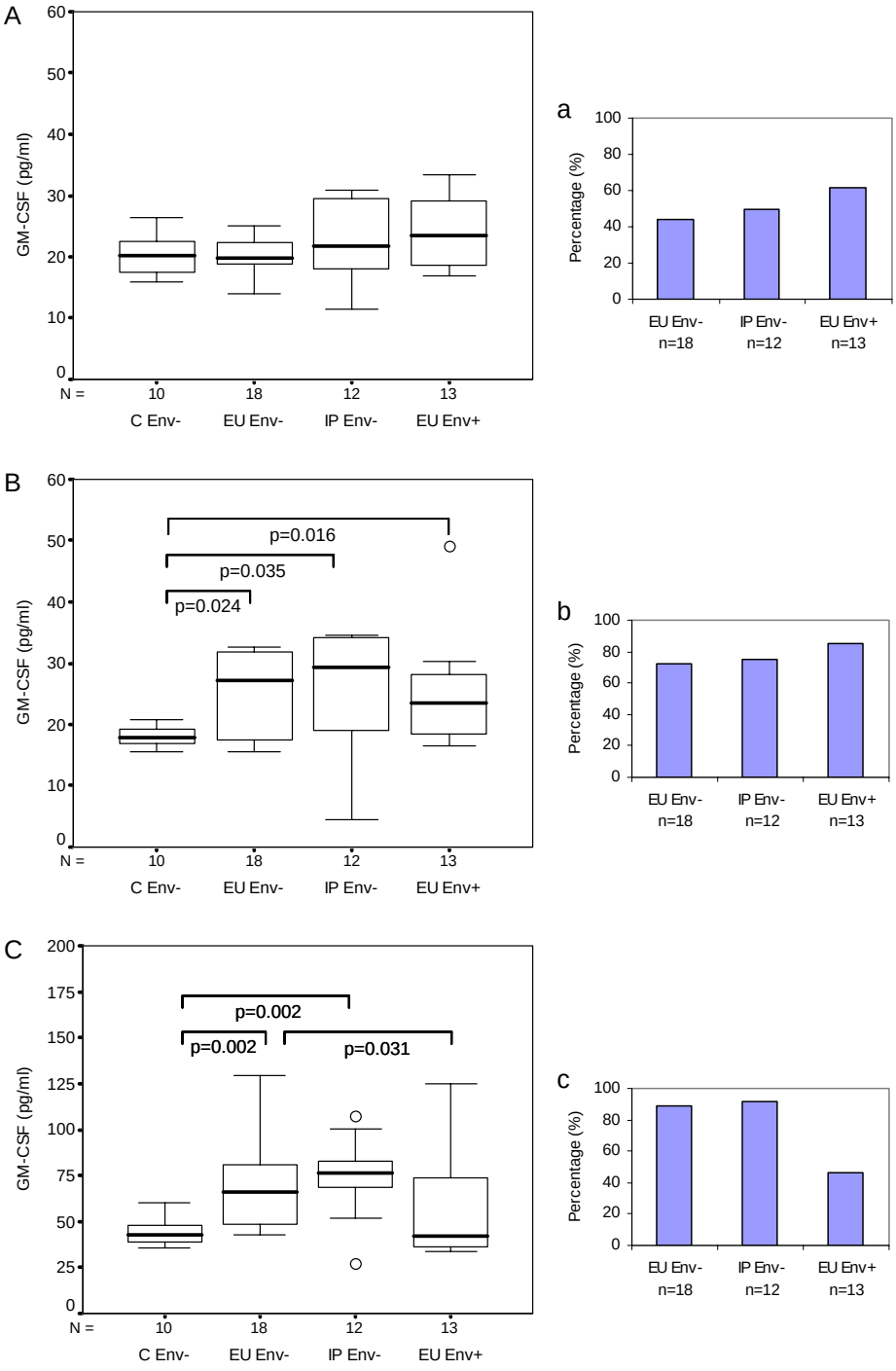


Figure 6.3 Ability of CBMC to produce GM-CSF (pg/ml) spontaneously (A), in response to HIV-1 envelope peptides (B) and PHA (C). Results are stratified on the basis of infection outcome and HIV-1 specific cellular immune responses to Env peptides (Env^- and Env^+). Data are presented as medians (horizontal bar), 25^{th} and 75^{th} percentiles (boxes) and 10^{th} and 90^{th} percentiles (bars). Significant differences between GM-CSF levels (pg/ml) of the groups and sample numbers per group are indicated. Inserts a, b and c represent the proportion of infants that had GM-CSF levels greater than the control group median for each stimulus.

6.2.8 Exposed-uninfected infants without HIV-1 specific cellular responses have significantly elevated plasma IL-10 levels

IL-10 is recognised for its ability to downregulate several major functions of T-helper type-2 cells and macrophages as well as controlling T-helper type-2 mediated inflammatory processes. It was therefore interesting to observe that Env non-responder infants (EU group) had significantly higher levels of IL-10 than control infants (Figure 6.4; Table 6.2) but that levels were not significantly different compared to EU Env^+ infants. Proportions of infants with IL-10 levels greater than the control group median where higher in the EU Env^- group compared to the IP Env^- and EU Env^+ groups (Figure 6.4a). Adjusting for maternal viral load did not account for the significantly elevated IL-10 levels observed among EU Env^- infants (p=0.214).

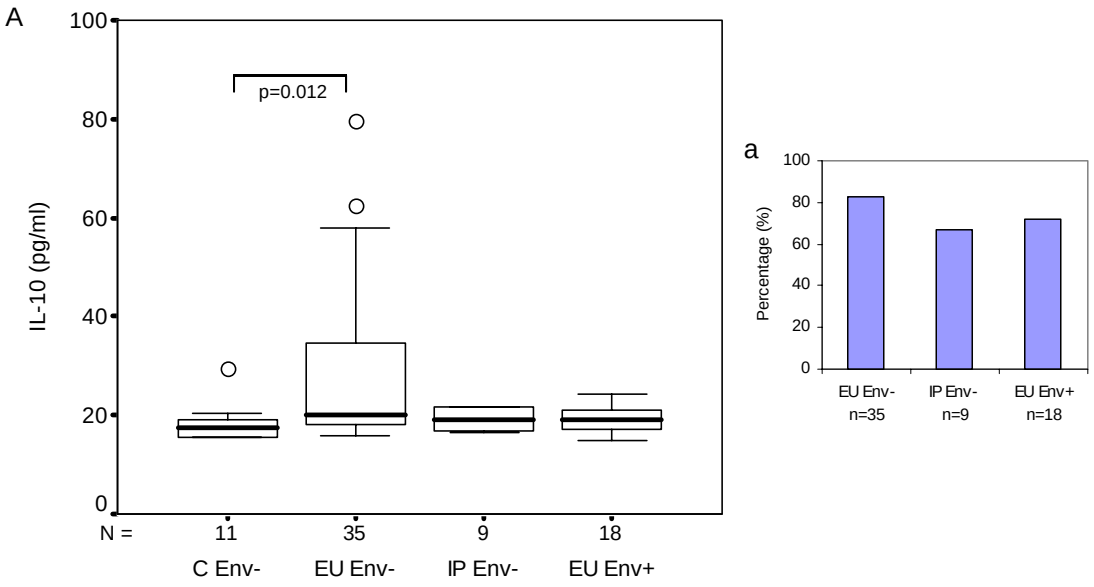


Figure 6.4 Levels of IL-10 (pg/ml) in plasma (A) of infants born to HIV-1 seropositive mothers in the absence of single-dose NVP (PEP) and stratified on the basis of infection outcome and cellular immune responses to HIV-1 Env peptides(Env^- and Env^+ infants). Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes) and 10th and 90th percentiles (bars). Significant differences between groups and sample numbers per group are indicated. Insert a represents the proportion of infants with IL-10 levels greater than he control group median.

6.3 Discussion

T-helper cell responses have been considered critical for controlling HIV-1 infection (Rosenberg *et al*., 1997). It is interesting therefore that detection of T-helper cell responses to HIV-1 envelope peptides in cord blood have been predictive of absence of IP and breast-feeding HIV-1 transmission. The study specifically demonstrating this point observed a 17% transmission rate amongst infants with no T-helper cell responses in cord blood (Kuhn *et al*., 2001a) but found that the proportion of infants born to HIV-1 infected mothers that elicited specific cellular immune responses were associated with lack of infection, even after prolonged re-exposure through breast-feeding (Kuhn *et al*., 2002). In this study we questioned firstly, whether levels of the immune activation markers neopterin, β_2-m, sL-selectin, the immunomodulatory and haematopoietic factors IL-7, CXCL12, GM-CSF and the immunoregulatory cytokine IL-10 were significantly different between infants exposed to HIV-1, or that became infected during labour, or that were infected *in utero* given that they received NVP as PEP within 72 hours of birth. Secondly, since a proportion of the exposed uninfected infants elicited HIV-1 specific cellular responses to envelope peptides (Env^+), whether infants in the EU group (Env^+ and Env^-) were different from each other or from infants that acquired infection IP (birth PCR negative, 6 week PCR positive) particularly with reference to the factors tested.

While all exposed uninfected infants had significantly elevated levels of neopterin, relative to the control group, indicating early T-cell responses, levels of IL-7 and CXCL12 were similar. Interestingly, EU Env^- infants had significantly elevated levels of β_2-m (indicating increased lymphocyte turnover) and sL-selectin (released following T-cell activation), relative to EU Env^+ and control infants, with elevated IL-10 (a Th2 cytokine) levels relative to control infants only, whilst Env^+ infants had significantly reduced cord blood levels of GM-CSF relative to EU Env^- and control infants. Our data suggests that levels of the immune factors tested in this study are not predictive of HIV-1 transmission, but confirm that infants with HIV-1 specific cellular responses to envelope peptides in cord blood are associated with lack of subsequent infection. Furthermore, we demonstrated that Env^+ infants exhibit some unique features which might suggest that these infants have different immune capabilities. Our results point to some interesting observations in Env^- infants: (i) these infants demonstrate significant immune activation yet exhibit T-cell unresponsiveness which may merely reflect the antigenically 'naïve'

immune status, (ii) the lack of response may be a consequence of ongoing exposure to HIV-1, resulting in chronic stimulation leading to immunodeficiency which would result in infants having altered immune responsiveness (iii) elevated IL-10 levels (EU Env^- infants) may suggest a bias towards Th2 immune responses - this might indicate that the Th2 cytokine milieu prevailing during pregnancy and at the materno-foetal interface (Lin *et al*., 1993) could contribute to shaping immune responses for a period of time after birth (Martinez *et al*., 1997).

Since a large majority of HIV-1 exposed infants remain uninfected, this raises the question of whether deficient immunological priming or the immature nature of a newborn's immune system influences maternal HIV-1 transmission. High neopterin concentrations are predominantly associated with T-cell anergy (Widner *et al*., 2000), but this compound has been reported to stimulate haematopoietic cell proliferation and differentiation *in vitro* by activating stromal cell function (Aizawa *et al*., 1998). Since IL-10 can prevent Th1 effector functions by inducing long-lasting T-cell unresponsiveness (Groux *et al*., 1996), neopterin and IL-10 levels measured in the Env^- infants may reflect an inability of these infants to respond to specific antigen, factors that could point to an antigenically 'naïve' yet developing immune system. Factors that support this are the elevated levels of all the immune activation markers and of GM-CSF.

It is interesting to note that HIV-1 mutants which are deleted in the integrase/endonuclease gene are unable to integrate but can competently produce HIV-1 core and envelope antigen (Stevenson *et al*., 1990) which could potentially prime immune responses in exposed-uninfected infants. Although the structural integrity of term human placenta is such that it can effectively impede the transplacental passage of cell-free HIV-1, it has been shown that p24 antigen is able to permeate through this barrier (Bawdon *et al*., 1994). Infants exposed to increased levels of p24 antigen, defective virus particles or HIV-1 from aborted infections (which could account for the HIV-1 specific responses to envelope peptides) that result in immune stimulation can therefore be associated with antigen-induced lymphokine production and cellular proliferation. It is interesting that the levels of the immune activation markers were higher in Env^- infants and that the mothers of this group of infants also had significantly higher viral loads than their Env^+ counterparts, indicating that these infants tend to be in a higher state of immune activation. Whether the elevated state of immune activation in Env^- infants might promote

HIV-1 infection on subsequent exposure in some infants (e.g. through mothers breast-milk) is not clear.

It is important to emphasize that despite the association of HIV-1 specific immune responses with lack of IP infection, a large proportion of infants (up to two-thirds) do not elicit responses to HIV-1 antigens, yet remain uninfected. The inheritance of certain class I alleles has been associated with either a functional immunological advantage or disadvantage in controlling HIV-1 infection (Tang and Kaslow, 2003), and in the case of the EU Env$^-$ infants recognition of other peptide motifs (from non-envelope genomic regions), which may also be associated with protection, might account for the lack of infection. In an adult study, Env-specific T-helper responses were found to be induced early <30 days p.i. but responses were more Gag than Env specific (16% versus 5%) (Malhotra *et al*., 2003). The inability to detect these responses over time was suggested to reflect low frequencies or impaired proliferative capacity (Malhotra *et al*., 2003). It is important to note that the selection of HIV-1 envelope peptides used to induce T-helper responses was based on previous studies which had identified these peptides to be broadly immunogenic across MHC haplotypes (Cease *et al*., 1987; Hale *et al*., 1989; Berzofsky *et al*., 1991), and which have documented T-helper cell responses to these peptides in several, independent populations of HIV-1 exposed, uninfected individuals (Clerici *et al*., 1991; Clerici *et al*., 1992; Clerici *et al*., 1994a; Mazzoli *et al*., 1997; Kuhn *et al*., 2001a). The failure observed amongst Env$^-$ infants to recognise these peptides would result either in unresponsiveness or suppression/diminished responses. Furthermore, failure to respond to an otherwise immunogenic peptide could be due to amino acid substitutions which would influence the anchoring of peptides on MHC molecules or due to MHC restriction.

It is interesting that infants without specific cellular immune responses were born to mothers with higher viral loads, yet infants exposed to lower viral loads elicited HIV-1 specific responses, a phenomenon documented to occur amongst different risk groups exposed to HIV-1 who fail to produce antibodies to HIV-1 but exhibit strong HIV-1 specific cell mediated immunity (Ranki *et al*., 1989; Clerici *et al*., 1992; Cheynier *et al*., 1992; Kelker *et al*., 1992; Rowland-Jones *et al*., 1993; Clerici *et al*., 1993c; Clerici *et al*., 1994a). Murine studies have demonstrated that (i) DNA immunization within the first week of life can induce adult-like Th1 or mixed Th1/Th2 and CTL responses, although, Th2-biased responses induced after early priming cannot be reverted by Th1-driving

DNA vaccines (Martinez *et al*., 1997); (ii) the inability to develop a CTL response to high doses of virus (Cas-Br-M murine leukaemia virus) correlated with the induction of a non-protective type-2 cytokine response and was not the result of immunological immaturity (Sarzotti *et* al., 1996). The higher IL-10 levels and lack of HIV-1 specific cellular immune responses in Env^- infants suggest a Th2 cytokine polarization in this group of infants in our study and would lend support to the murine studies which suggest that the initial antigen dose may be critical to the development of protective immunity in newborns. In further support of this, using a non-human primate model (macaques), it was found that sub-infectious doses of SIV led to SIV-specific T-cell responses that appeared to confer long-term protection against subsequent virus challenge (Clerici *et al*., 1994b). Similar to the primate study, our study suggests that the maternal viral load dosage could lead to effective immunological T-helper cell priming that is also protective against subsequent virus challenge.

One of the most interesting findings from this study was the significantly lower cord blood plasma GM-CSF levels in EU Env^+ infants compared to the controls. That high GM-CSF levels can be measured in cord blood of infants is supported by another study that measured levels in cord blood of normal full-term neonates and proposed that the role of the GM-CSF was to maintain adequate neutrophil production (Laver *et al*., 1990). The reduced ability of CBMC from Env^+ infants to produce GM-CSF following stimulation with PHA suggests that GM-CSF is poorly stimulated or not inducible. Besides GM-CSF being recognised for its regulatory role of granulocyte and monocyte cell lineage maturation, it influences phagocytosis, cytokine secretion and antigen presentation capability. Furthermore, GM-CSF is not only produced by activated (but not resting) T-cells of both Th1 and Th2 phenotype (Chan *et al*., 1986; Shannon *et al*., 1997), but by a variety of cell types in response to specific activating signals (Gasson, 1991). Since GM-CSF is known to augment immune responses to antigens *in vivo* and *in vitro* (Somani *et al*., 2002), and due to its numerous immunomodulatory properties, it has been administered as a vaccine adjuvant to enhance cellular or humoral responses to specific antigen. Recently, studies however propose GM-CSF to be less effective as an immune adjuvant than earlier reports suggest (Somani *et al*., 2002). It is intriguing that as a vaccine adjuvant the effects of GM-CSF are related to type-1 and type-2 DCs, with DC1 priming naïve T-cells towards a cellular (Th1) response and DC2 promoting differentiation to humoral immune responses (Th2). DC ratios shift towards a

predominance of DC1 when peripheral blood stem cells are mobilised with GM-CSF and granulocyte colony stimulating factor (G-CSF) (Somani *et al*., 2002). This would suggest that haematopoietic growth factors shift T-cell responses.

In an attempt to explain the reduced GM-CSF levels measured in EU Env^+ infants we propose that (i) reduced levels are indicative of a priming event (T-helper cell response) or (ii) GM-CSF levels reflect the extent of immune maturation i.e. high neopterin levels are indicative of extensive immune activation, which can be associated with T-cell anergy and apoptosis however, neopterin can stimulate haematopoietic cell proliferation (Aizawa *et al*., 1998) and GM-CSF can prevent apoptosis in target cells (Thatte and Dahanukar, 1997), both of these factors are reduced relative to those of Env^- infants. Furthermore, this might suggest that a newborn's immune system is not as immature (naïve) as is speculated and that associated with cells acquiring a memory phenotype, GM-CSF levels are approaching levels measured in adults or (iii) plasma levels of GM-CSF may be indicative of both immune responses (whether Th1 or Th2) and might importantly reflect predominant cell types and expression from specific cells. Expression of GM-CSF in T-cells, fibroblasts and endothelial cells is controlled by changes in the rate of transcription (Shannon *et* al., 1997) or (iv) since GM-CSF can inhibit the growth of T-cells (Janeway *et* al., 2001) this event may be dose-dependent i.e. the influence that GM-CSF has on the immune response could be determined by the GM-CSF levels or (v) GM-CSF levels are indicative of pathological events/encounters since GM-CSF protein expression has been associated with certain pathological processes (Gasson, 1991; Shannon *et* al., 1997) or (vi) defects in normal signalling pathways for GM-CSF gene activation might account for the reduced GM-CSF plasma levels and the reduced ability of CBMC to produce GM-CSF in response to potent stimuli such as PHA.

In conclusion, our data are consistent with a macaque study which reports that exposure to sub-infectious doses of SIV (either due to abortive infection/with a virus of low replication competency) may prime the immune system sufficiently to confer protection (Clerici *et al*., 1994b). Neopterin, β_2-m, sL-selectin, CXCL12, IL-7 or IL-10 cannot predict transmission, although, HIV-1 specific cellular immune responses are of immunological significance and in support of studies that have demonstrated these responses to be correlates of protection, can predict lack of infection. That differences in the immune characteristics between Env^- and Env^+ infants exist are highlighted. Clearly

strategies that optimize the cellular components of the immune system (the activation and expansion of virus-specific $CD4^+$ and $CD8^+$ T-cell responses) as an approach for a prophylactic AIDS vaccine (Salk *et al*., 1993) are important and our data may provide some additional clues to how protective responses might develop. In neonates, induction of the Th1 responses in early life could require a prolonged exposure to low doses of antigen (Martinez *et* al.,1997) so as to drive $CD4^+$ T-cells along the Th1 differentiation pathway (Szabo *et al*., 1997; Rogge *et al*., 1997). It remains important however to identify other markers of protective immunity that are not attenuated by antiretroviral treatment.

CHAPTER SEVEN

Thymic function in newborn infants of drug naïve HIV-1 infected mothers

7.1 Introduction

T-cells are produced either through thymopoiesis which generates diversity of the pool or proliferative expansion of post-thymic T-cells which results in clonal expansion of the T-cells. In neonates, T-cell production occurs by proliferative expansion which is facilitated by increased cytokine responsiveness and thymopoiesis (Schönland *et* al., 2003). Peripheral blood naïve T-cells predominate in healthy newborns and decline with age whilst memory T-cell numbers seem less dependent on age (Hazenberg *et al*., 2004).

During their passage through the thymus, T-cell precursors rearrange their T-cell receptor (TCR) genes, a process known as V(D)J T-cell receptor rearrangement. This requires excision of segments of DNA, the ends of which are subsequently ligated to form small circles of episomal DNA known as T-cell receptor rearrangement excision circles (TRECs) (Douek *et al*., 1998). TRECs contain the signal joint sequences from the *TCRAD* locus δRec to ψJα recombination event, which is common to approximately 70% of thymocytes destined to become mature TCRαβ T-cells (Okamoto *et al*., 2002). TRECs therefore indirectly measure thymopoeisis and have the added advantage of being stable (Livak and Schatz, 1996) and not being replicated during cell division and diluted out (Takeshita *et al*., 1989). In peripheral blood, increased TREC levels relate to thymic output whilst decreased TREC levels are associated with peripheral expansion (Douek *et al*., 1998).

Numerous studies have suggested that HIV-1 may be involved in the direct killing of thymocytes (Kourtis *et al*., 1996), destroy dendritic cells required for normal thymocyte development (Valentin *et al.,* 1994), damage thymic epithelial cells required for thymopoiesis (Stanley *et al*., 1993) or inhibit thymocyte signalling (Haynes et al., 2000). That morphological changes occur in the thymus in response to HIV-1 is supported by the observed clinical decrease in thymocyte numbers and enhanced involution, (Rozenweig *et al*., 1993; Papiernik *et al*., 1992) as well as decreases in naïve T-cells and TREC

concentrations in paediatric and adult patients infected with HIV-1 (Douek *et al*., 1998). Furthermore, thymic dysfunction and early thymic involution have been associated with rapid disease progression in a subset of infants infected perinatally with HIV-1 (Kourtis *et al*., 1996; Nahmias *et al*., 1998). Direct evidence of HIV-1 induced thymic damage comes from studies demonstrating thymic lesions, located predominantly in the thymic cortex, in foetuses aborted from HIV-1 infected mothers (Papiernik *et al*., 1992). That the thymus itself may be a source of HIV-1 infection (Al-Harthi and Landay, 2002), albeit latent infection, (Brooks *et al*., 2001) comes from analyses of human thymic tissue from HIV-1 infected infants, children and adults which have demonstrated the presence of HIV-1 RNA and proteins (Tanaka *et al*., 1992; Rozenzweig *et al*., 1993). Studies from explant to animal models demonstrate that HIV-1 infection of thymus/liver results in severe and preferential depletion of immature ($CD4^+CD8^+$) human thymocytes (Autran *et al*., 1996), defects in the thymic microenvironment (Autran *et al*., 1996) and defective thymopoiesis (Bonyhadi *et al*., 1993).

In Chapter Four we showed newborn infants to have substantial immune activation in response to *in utero* exposure to HIV-1, or as a consequence of having an HIV-1 infected mother, and as a result of *in utero* HIV-1 infection. In Chapter Six we confirmed that infants with HIV-1 specific cellular responses to envelope peptides detected in cord blood are associated with lack of subsequent infection, but we also demonstrated these infants to have lower GM-CSF levels suggesting that immunologically they are different from those infants without such responses. Based on these findings we had good reason to question whether TREC levels in infants born to HIV-1 infected mothers might be altered. Also, since studies have clearly demonstrated the involvement of HIV-1 infection in thymic damage and in alterations in the thymic microenvironment we questioned, using newborn infants whose mothers were not administered antiretrovirals at any stage prior to labour or intrapartum to prevent maternal-infant transmission of HIV-1 whether: (i) increased immune activation that occurs in infants born to HIV-1 seropositive mothers would be associated with decreased TREC levels, (ii) a decrease in TREC concentrations occurs in newborn infants that elicit HIV-1 specific immune responses to Env peptides (a specific marker of HIV-1 exposure) and (iii) raised levels of IL-7 could be demonstrated with HIV-1 exposure or infection which would imply a direct effect on naïve T-cell output.

7.2 Results

7.2.1 Characteristics of infants

In order to determine whether *in utero* HIV-1 exposure or infection could show early alterations in thymic output we studied infants born to HIV-1 seronegative and seropositive mothers collected anonymously when the PETRA trial placebo group (Kuhn *et al*., 2001c) was discontinued for ethical reasons. These mothers therefore had not received any antiretrovirals and were unaware of their HIV-1 status at the time of delivery. The viral load, TREC determinations and immunological characteristics of the infant participants included in this study are presented in Table 7.1. The study included samples from a total of 76 infants, 47 who were born to HIV-1 seronegative mothers (controls) and 29 to HIV-1 seropositive mothers. As mothers and infants were collected anonymously, infants were not followed prospectively, and so for the purpose of this particular study, infection outcomes at birth were based on infant viral loads, that is, those with <400 RNA copies/ml in cord blood plasma were grouped as EU and those with >400 RNA copies/ml as IU infections.

7.2.2 Infants exposed to HIV-1 demonstrate significant immune activation but not significant reductions in TREC concentrations

We have previously demonstrated that there is significant immune activation either as a consequence of being born to a HIV-1 seropositive mother or as a result of *in utero* HIV-1 exposure, and that existing infection at birth (IU) is marked by the most elevated levels of plasma immune activation markers (neopterin, β_2-m and sL-selectin). Neopterin showed the most profound differences between the PEP study groups (that is, cord blood at birth not exposed to antiretrovirals) and was therefore selected as the marker for comparison in this study. Infants were stratified on the basis of their viral loads, namely infants having viral loads <400 copies/ml (EU) and infants with detectable viral loads (>400 copies/ml) (IU) being grouped together and compared to control infants of HIV-1 uninfected mothers. In order to establish whether HIV-1 exposure or infection leads to early alterations in thymic output in newborn infants of HIV-1 seropositive mothers, TREC levels were determined from CBMC isolated from cord blood collected at the time of birth. Irrespective of the infant viral load, HIV-1 exposure or infection did not

significantly impact on thympoiesis (Figure 7.1C). Furthermore, the results from this study provide further support to our earlier observations for neopterin, although, increased immune activation was not associated with reductions in TREC levels (Figure 7.1A and 7.1C).

Amongst the infected infants, it is interesting to observe that viral load did not affect TREC levels measured (Table 7.1). In fact, there was no correlation between viral load and TREC levels in infected infants (r=-0.2, p=0.8).

Table 7.1 Viral load, TREC determinations and immunological characteristics of infants

	N	Infants' viral load (log_{10})	Neopterin (ng/ml)	IL-7 (pg/ml)	TREC (log_{10}) (copies/µg DNA)
		Median (IQR)			
Uninfected (control)	**47**	-	4.82 (3.92-5.95)[3]	4.06 (2.98-5.58)	3.81 (3.4-4.18)
$Env^{+/-}$	ND				
Exposed uninfected*	**25**	2.6 (2.6)[1]	5.95 (4.73-7.77)[3]	2.98 (2.06-5.42)	3.66 (3.34-3.97)
$Env^{+\#}$	5	2.6 (2.6)[1]	6.27 (3.75-11.90)	4.03 (2.08-5.03)	3.69 (3.02-4.44)
Env^{-}	13	2.6 (2.6)[1]	5.95 (4.81-7.77)	4.10 (2.16-6.71)	3.54 (2.97-3.93)
$Env^{+/-}$ ND	7	2.6 (2.6)[1]	5.79 (4.65-6.08)	2.38 (4.65-6.08)	3.75 (3.66-3.90)
Infected*	**4**	4.33 (3.15-5.57)[2]	8.44 (6.46-10.90)[3]	4.10 (3.43-8.76)	3.74 (3.65-3.79)
		Individual values from infected infants			
$Env^{+\#}$		4.65 5.88	6.27 11.24	3.37 3.61	3.72 3.77
Env^{-}		2.86 4.01	9.85 7.03	4.60 10.14	3.80 3.63

IQR - Interquartile range i.e. 25th and 75th percentiles

Env^{+} - Infants that elicited HIV-1 specific cellular immune responses in the EU/IU groups

Stimulation indices (median (IQR)) in response to:

HIV-1 Env peptides: EU Env^{+} infants (n=5) 4.69 (3.85-6.46);
individual values for IU Env^{+} infants: 6.33 and 5.45, respectively

PHA: EU infants tested for T-helper responses (n=28) 22.40 (19.72-27.50);
IU infants tested for T-helper responses (n=4) 29.48 (17.13-45)

Env^{-} - Infants that did not elicit HIV-1 specific cellular immune responses in the EU/IU groups

[1] EU infants with viral loads <400 copies/ml (<log_{10}2.6)

[2] IU infants with viral loads >400 copies/ml (>log_{10}2.6)

[3] Neopterin levels of EU an IU infants are significantly different from control infants (p=0.030 and p=0.012, respectively)

* Infants' neopterin, IL-7 and TREC (log_{10}) levels are not statistically different between EU (n=25) or IU (n=4) infants

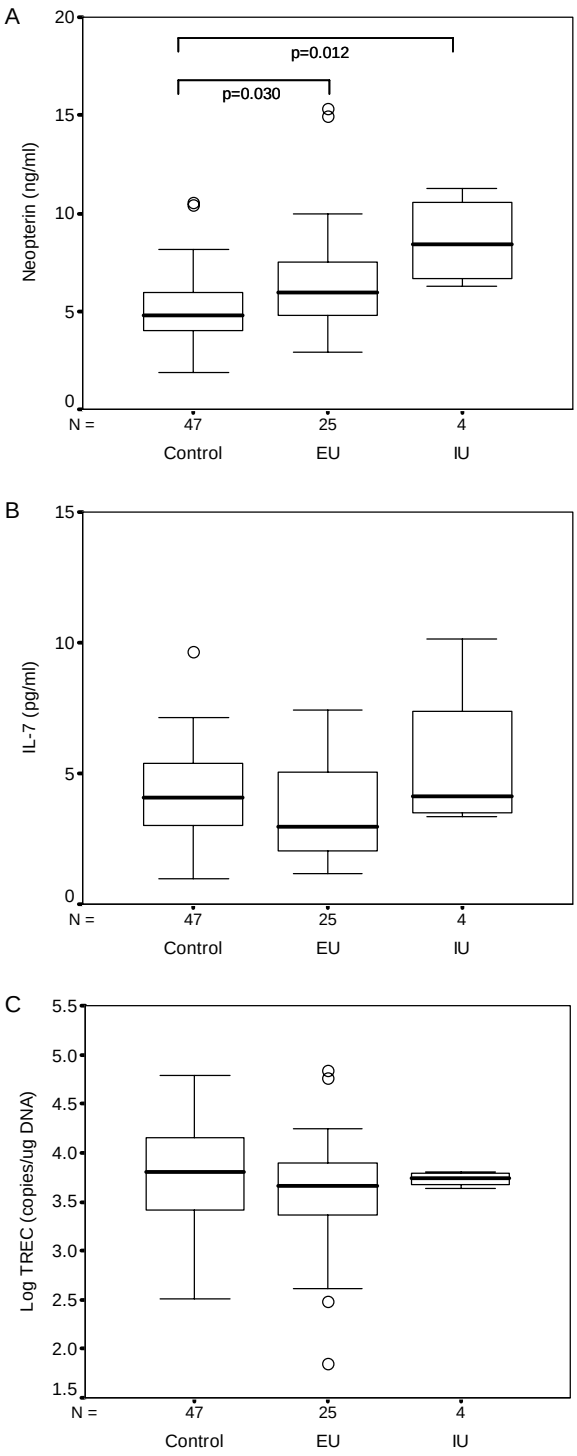


Figure 7.1 Cord blood plasma levels of neopterin (A) and IL-7 (B) and thymic function (C) measured by analysis of T-cell receptor excision circles (Log_{10} TREC copies/µg DNA) for uninfected (control) infants and infants born to HIV-1 infected mothers stratified on the basis of their viral loads. Infants with viral loads <400 copies/ml were considered to be HIV-1 exposed but uninfected (EU) whilst those with viral loads >400 copies/ml infected *in utero* (IU). The data are presented as medians (horizontal bar), 25^{th} and 75^{th} percentiles (boxes) and 10^{th} and 90^{th} percentiles (bars). Significant differences and sample numbers per group are indicated.

7.2.3 Cord blood plasma IL-7 levels do not reflect diminished thymic output

Since IL-7 is involved in thymopoiesis and in the survival of developing thymocytes (Kees and Ford, 1999) and from direct evidence of HIV-1 induced thymic damage (Papiernik, 1992), and that increased IL-7 levels are associated with impaired T-cell homeostasis (Clerici *et al*., 2000), we hypothesized that HIV-1 exposure or infection could induce thymic changes that might be reflected by the IL-7 levels measured in cord blood plasma. However, contrary to what might be expected EU and IU infants demonstrated no significant differences in IL-7 levels compared to unexposed (control) infants (Table 7.1). However, it is interesting to note that IL-7 levels tended to be higher amongst the HIV-1 infected infants compared to the EU and control infants (Table 7.1).

7.2.4 The presence of HIV-1 specific immune responses in cord blood do not influence thymic output measured in infants

Having shown that HIV-1 exposure influences neopterin levels demonstrably without affecting TREC or IL-7 levels in newborn infants, we questioned whether the presence of HIV-1 specific cellular immune responses (determined as previously described in Chapter Two section 2.2.8 and which are indicative of memory responses to HIV-1 Env), would be accompanied by a decrease in TREC levels. Based on a stratification using only those infants that were tested for HIV-1 specific cellular responses, no significant differences were demonstrated between EU Env^+ (n=5) and EU Env^- (n=13) infants, although TREC levels tended to be higher amongst the EU Env^+ group.

If one looks at the infected infants individually, then TREC levels, irrespective of the presence of HIV-1 specific immune responses, do not differ substantially (Table 7.1). The magnitude of T-helper cell responses to HIV-1 Env peptides and PHA are expressed as stimulation indices (SI's) with SI's >3 representing a positive response. While only two infected infants and five EU infants demonstrated T-helper cell responses to HIV-1 Env peptides (SI's shown as a footnote to Table 7.1) all infants (EU Env^+ and IU Env^+) responded strongly to stimulation with PHA stimulation (SI's shown as a footnote to Table 7.1). No correlation between TREC levels and SI's in response to HIV-1 Env peptides or PHA was demonstrated in either the EU or IU group of infants.

7.3 Discussion

This study was a unique opportunity to assess the effect of *in utero* HIV-1 exposure and infection on thymic ouput in the absence of antiretroviral therapy. Furthermore, based on earlier work demonstrating that HIV-1 exposure and infection result in extensive immune activation and that a small proportion of infants elicit T-helper Env responses (a specific marker of exposure and an indication that post-thymic memory T-cell expansion has taken place), we questioned how these immune responses could influence TREC levels of newborns. Based on studies which have demonstrated lower naïve $CD4^+$ counts and thymic output in HIV-1 seronegative infants of HIV-1 seropositive mothers (Nielsen *et al*., 2001) and augmented memory T-cells but diminished naïve T-lymphocytes in HIV-1 uninfected newborns of HIV-1 infected mothers (Clerici *et al*., 2000), we hypothesized that *in utero* exposure/infection would affect the thymic output (TREC levels) reflected both by the extent of immune activation and by circulating levels of IL-7. In contrast to what we hypothesized, TREC levels were not significantly different between uninfected (control) infants and those born to HIV-1 seropositive mothers regardless of stratification on the basis of viral load (infection outcome at the time of birth), demonstration of substantial immune activation or the presence of HIV-1 specific immune responses. Furthermore, exposure did not significantly affect the IL-7 levels measured from cord blood plasma.

HIV-1 infection has been suggested to influence the TREC content of naïve T-cells either by (i) directly infecting the thymus thereby affecting thymic production, (ii) causing an increased loss of naïve T-cells either in response to HIV-1 mediated killing of naïve cells, (iii) an increased rate of priming into memory cells, (iv) increasing T-cell division in response to direct activation of naïve T-cells or (iv) as a result of virus-induced depletion of naïve T-cells inducing a homeostatic response (Hazenburg *et al*., 2000a). Our results did not demonstrate either an increased loss of naïve T-cells or an increased rate of memory T-cell priming associated with decreases in TREC levels. Importantly our study suggests that impaired production of T-cells, mediated by HIV-1 exposure or infection, cannot be readily demonstrated using TREC measures on early infant (cord blood) samples. However a study which enriched for $CD4^+$ and $CD8^+$cells for TREC determinations has demonstrated that the frequency of $CD4^+$ cells with TRECs was significantly lower in HIV-1 seronegative infants than control infants (gestational age

~37.7 weeks; gestational age was defined by these authors as the age of the infant determined as the number of weeks after the first day in the last menstruation) (Nielsen *et al*., 2001). Importantly, the cohort studied by Nielsen *et al*. (2001) received antiretrovirals (predominantly AZT) antepartum, intrapartum and postpartum, maternal $CD4^+$ counts ranged from 80 to 621 cells/µl and maternal viral loads ranged from <20 to 34 000 RNA copies/ml. While a weakness of our study is the lack of maternal clinical data, the infants in our study were not exposed to any antiretrovirals. Interestingly, in a cohort of healthy infants (born 26 to 41 weeks gestation), TREC concentrations varied but were stable over the time range examined which suggested that the production of $TREC^+$ T-cells by thymic production and the generation of $TREC^-$ T-cells by proliferative expansion was constant (Schönland *et al*., 2003). These authors demonstrated a high proliferative turnover of the peripheral T-cell pool in newborns as well as maximal thymopoiesis and high proliferative expansion of the mature naïve T-cells. The data from our study suggests that impaired production or function of T-cells attributed to HIV-1 infection of thymocytes or thymic epithelial cells may only become evident when the infant is a few weeks/months old. Rapid production of new naïve T-cells in an attempt to counter homeostatic changes in response to HIV-1 mediated effects may account for the TREC levels we have measured. This may be supported by the fact that in neonates thymic T-cell release and post-thymic expansion are interlinked events, leading to stability of clonal size in the T-cell compartment (Schönland *et al*., 2003). Studies in children using HAART have also shown that high rates of recovery of naïve, memory and total $CD4^+$ T-cells occurs in children younger than 3 years of age (Cohen Stuart *et al*., 1998). Furthermore, the ability of the infant thymus to contribute to *de novo* T-cell synthesis in early life is demonstrated in the setting of complete DiGeorge syndrome (a congenital malformation of the third and fourth pharyngeal pouches in which the thymus is absent and patients are completely devoid of T-cells and function) where transplantation with postnatal thymus (0-3 months) leads *in vivo* to the regeneration of a new T-cell repertoire (Markert *et al*., 1997; Markert *et al*., 1999).

Although evidence of early immune stimulation due to HIV-1 exposure or as a consequence of being born to a HIV-1 infected mother is again demonstrated in this study, immune activation measured by plasma neopterin levels was not associated with reductions in TREC levels which would imply that at birth HIV-1 induced depletion of naïve T-cells may be offset by the induction of a homeostatic response. Chronic immune

activation in children infected with HIV-1 leads to the loss of the naïve T-cell pool (Hazenburg *et al*., 2004). As it is not known how T-cell activation levels are affected in HIV-1 infection in children, it is not clear to what extent immune hyperactivation may play a role in paediatric HIV-1 pathogenesis (Hazenburg *et al*., 2004).

While HIV-1 exposure or infection did not significantly affect the IL-7 levels measured from cord blood plasma in this study, the IL-7 levels tended to be increased in the IU group of infants with high viral loads, particularly those that did not have HIV-1 specific immune responses to HIV-1 Env peptides. One can only infer that the higher IL-7 levels may reflect an increased need to enhance thymopoiesis and survival of developing thymocytes. IL-7 plays an important role in TCR rearrangement in the thymus (Al-Harthi and Landay, 2002) and has been suggested to directly enhance TREC generation (Okamoto *et al*., 2002). Increased plasma levels of IL-7 have been reported in uninfected infants (mean age 30 days) and older children (mean age 7.2 years) of HIV-1 infected mothers suggesting impaired T-cell homeostasis (Clerici *et al*., 2000).

Since decreased TREC levels resulting from increased T-cell division are expected in response to direct activation of naïve T-cells, one could reason that infants demonstrating HIV-1 specific immune responses, (which reflects specific peripheral T-cell expansion of memory cells), would exhibit lowered TREC levels compared to exposed uninfected infants without these cell responses. It needs to be emphasized however that HIV-1 specific immune responses in infants are weak, this being the case more so in EU than IU infants. Furthermore, although only a proportion of infants demonstrated HIV-1 specific Env responses, infants may well demonstrate responses to other regions of the HIV-1 genome. This is supported by an adult study which has found the probability of detecting HIV-1 specific lymphoproliferative responses to be low and when present to be more Gag than Env specific (Malhotra *et al*., 2003). If all responses to HIV-1 are weak then it is possible that T-cell expansion of these memory cells may not be reflected by TREC levels. That TREC concentrations remained stable between infants with and without responses might well suggest a balance between thymopoiesis and expansion of selected T-cells in the newborn infant. This would infer that there is little difference in the naïve T-cells produced by infants exposed to HIV-1 and control infants. One study has suggested that the neonatal system aims for a stable clonal size for each specific T-cell rather than proliferative expansion functioning to compensate for insufficient thymic T-

cell release (Schönland *et al*., 2003). Two possibilities have been proposed to explain this (i) recent thymic emigrants have a pre-determined probability to enter the cell cycle which is dependent on the age of the T-cell, (ii) 'new' T-cells leaving the thymus home preferentially to anatomically defined microenvironments where they encounter IL-7 (Schönland *et al*., 2003).

Our results beg the question of how antiviral prophylaxis affects thymic output of infants? One infant study attributes low $CD4^+$ counts and reduced thymic output in HIV-1 uninfected infants of HIV-1 infected mothers to impaired progenitor function (Nielsen *et al*., 2001). It is interesting that the mothers and infants of this particular study were administered antiretrovirals antepartum, intrapartum and postpartum. Based on our findings we can only infer that the reduction in TREC levels demonstrated by Nielsen *et al*. (2001), may be a consequence of the antiretrovirals, with TREC levels not being significantly affected in the absence of antiretroviral regimens. Importantly, whether TREC levels in the infant are affected by maternal HIV-1 status ($CD4^+$ and viral load) needs to be elucidated. What effect exposure to HIV-1 antigen or other immune factors of the mother may have on (i) the thymus of newborn infants or (ii) how this impacts on the development of HIV-1 specific T-cells and/or (iii) whether exposure to HIV-1 results in clonal deletion of antigen specific T-cells within the thymus, is unknown. What is evident is that studies that have demonstrated impaired thymic development (Nielsen *et al*., 2001) and altered T-cell development due to HIV-1 exposure have also shown that these changes tend to persist during childhood (Clerici *et al*., 2000).

In conclusion, while it is recognised in adults that HIV-1 infection results in T-cell depletion and that regeneration of T-cells via the thymus is reduced and therefore largely dependent on peripheral T-cell expansion, our study suggests that newborn infants exposed to or infected with HIV-1 appear to exhibit no differences in their thymic output at birth suggesting that either (i) at birth HIV-1 influences cannot be demonstrated when using TREC to assess *de novo* T-cell synthesis or (ii) newborns have an ability to balance thymic ouput as a result of virus-induced depletion of naïve T-cells inducing a homeostatic response or (iii) the timing/duration of HIV-1 exposure or infection influences the TREC levels. What is thought-provoking is whether (i) at any stage during human thymocyte development, prior to positive selection, HIV-1 exposure influences T-cells to such an extent that they are unable to recognise HIV-1 or (ii) whether as a

consequence of extensive immune activation, limited HIV-1 exposure (dose and duration) renders T-cells anergic such that even when normal adequate costimulatory signals are provided by the APC, T-cells are unable to respond or (iii) thymocytes from EU infants become latently infected serving as HIV-1 reservoirs until activation postpartum.

CHAPTER EIGHT

Concluding remarks

Clinical trials have shown that in developing countries short-course antiretroviral therapy can substantially reduce MTCT of HIV-1 (Connor *et al*., 1994, Guay *et al*., 1999; Taylor *et al*., 2000). Furthermore, short-course intervention is a highly attractive option due to its affordability, effectiveness and easy implementation. Although there are significant benefits to these short-course interventions the mechanisms responsible for reduced maternal transmission of HIV-1 are not well established in fact, drug-induced suppression of maternal viral load has been found to account for only a small proportion of the reduction in transmission (Sperling *et al*., 1996). The effectiveness of PEP in preventing MTCT is based on the premise that antiviral prophylaxis, soon after exposure, will suppress viral replication enabling the hosts' immune system to clear the inoculum (Shih *et al*., 1991). This has been supported by studies of health care workers that have a decreased risk of HIV-1 seroconversion following AZT administration as PEP (Cardo *et al*., 1997; Gerberding, 2003; Hardie and Yeats, 2003). Two non-experimental studies also reported reduced rates of maternal HIV-1 transmission following adminstration of ART to infants shortly after birth but whose HIV-1 infected mothers had not received ART (Wade *et al*., 1998a, Bulterys *et al*., 1999), a finding not confirmed in another study (Fiscus *et al*., 1999). A randomized control study conducted at Chris Hani Baragwanath, South Africa to assess the efficacy of PEP in reducing MTCT of HIV-1 has demonstrated that single-dose NVP given to an infant within 24 hours of delivery offers protection against HIV-1 infection (Gray *et al*., 2005) and although not tested directly in the same population, reduces transmission rates to similar figures reported for the HIVNET 012 protocol (where NVP is given to the mother and to the newborn).

In this thesis we have demonstrated that while there are substantial benefits to such therapy, which are generally assumed to be associated only with the direct interaction of NVP with HIV-1, there are immunomodulatory consequences of *in utero* exposure to HIV-1 and single-dose NVP given to the mother at the onset of labour. While the effects of antiviral drugs on immune function have not been well defined it is these effects which indirectly, may also contribute to the prevention of the establishment of an infection in infants.

Reports documenting immunological changes associated with ART of HIV-1 exposed uninfected patients and studies which suggest that antiretroviral agents modulate immunological responses independently of antiviral activity have been discussed in Chapter One section 1.14.

Using an *in vitro* system we attempted to examine the viral infection outcome of a primary HIV-1 isolate, M502L, using primary cell cultures (normal donor PBMC) and adding either NVP or AZT at different times either prior or subsequent to infection. Notably, the effectiveness of the drugs and the ability to be infected with HIV-1 and sustain the replication (permissiveness) varies between primary human cells from different donors. These variations most likely represent the differences that occur in patient populations. Furthermore, in certain blood donors elevated levels of p24-Ag (indicative of virus replication) suggested that HIV-1 replication was in fact enhanced in the presence of NVP or AZT. The lack of inhibition of HIV-1 replication by NVP or AZT in certain donors might well suggest that resistant mutations were rapidly selected, however, this seems highly unlikely since selection for NRTI or NNRTI resistance in culture appears to depend on the repeated passage of primary isolates in the presence of increasing concentrations of drug (Loemba *et al*., 2002; Reid *et al*., 2005). While the experimental approach was challenging due to the use of primary cells, findings emphasized the complexity of the virus-host interactions. Importantly, a better understanding of these interactions will enable therapeutic strategies to be developed which can more effectively control HIV-1 in infected individuals.

As the *in vitro* model highlighted host variability (most likely genetically encoded and influencing factors and receptors important in HIV-1 infection) as a major factor affecting permissiveness of infection or drug reaction it was therefore difficult to establish by extrapolation, how timing of drug administration might affect viral infection outcome. We therefore chose to study levels of numerous peripheral blood immune factors that are indicative of immunological events that have occurred *in vivo*. Changes in the levels of such factors might provide evidence that antivirals influence cellular events and therefore may indirectly affect HIV-1 replication potential in the infant (aside from their direct antiviral effects).

Table 8.1 summarizes our findings with respect to the impact of HIV-1 exposure *in utero* (or other consequences of being born to a HIV-1 seropositive mother), of HIV-1 infection and the effects of HIV-1 and single-dose NVP exposure on the infant's immune system. Our studies have shown that:

(i) Existing infection (IU) is marked by substantially elevated levels of all immune activation markers (neopterin, β_2-m and sL-selectin). Furthermore, there is evidence of further increased immune activation in cord blood of HIV-1 infected infants exposed to NVP,

(ii) Levels of the haematopoietic, immunoregulatory or immunosuppressive cytokines and chemokines such as IL-7, IL-10, CXCL12, GM-CSF, CCL3, CCL4 and CCL5 measured from the cord blood of EU infants did not demonstrate consequences of HIV-1 or NVP exposure,

(iii) Levels of the immune activation markers, haematopoietic, immunoregulatory or immunosuppressive cytokines and chemokines tested did not predict infection,

(iv) HIV-1 specific cellular immune responses are of immunological significance and as correlates of protection their detection at birth can predict lack of subsequent infection. Furthermore, low GM-CSF levels are associated with these HIV-1 specific responses,

(v) Control infants demonstrated significantly elevated levels of peripheral blood immune activation markers (neopterin, β_2-m and sL-selectin), significantly raised peripheral levels of the haematopietic factor GM-CSF, significantly reduced peripheral levels of the immune modulator CXCL12 and elevated IL-7 levels (regulator of naïve and mature T-cells) compared to their mothers, indicating that the immune system of the infant develops independently of the mother,

(vi) Exposure to single-dose NVP (DART infants) reduces the ability of CBMC to spontaneously produce cytokines such as GM-CSF,

(vii) Maternal viral load did not influence levels of peripheral blood immune activation markers (neopterin, β_2-m and sL-selectin), GM-CSF, CXCL12 or IL-7. However, our data demonstrated an association between HIV-1 specific cellular immune responses in cord blood, lower maternal viral load and lack of infection,

(viii) Maternal/placental immunomodulatory cytokines such as IL-10 may influence those of their infants. These Th2 maternal cytokines may play a role in suppressing immune and inflammatory responses and reducing antigen-specific proliferation and cytokine production of T-cells in infants,

(ix) Thymic output of HIV-1 exposed or infected newborn infants is not significantly different from that of uninfected control infants. Furthermore, we were unable to demonstrate an association between decreased TREC levels and HIV-1 specific priming and immune activation (both indicative of peripheral T-cell expansion), in newborn infants.

Table 8.1 Infant plasma levels of immune factors associated with HIV-1 exposure/infection in the presence and absence of single-dose NVP and their association with IP transmission. Infant plasma levels relative to those of their mothers are also presented

Immune factor	Association with IP transmission	HIV-1 exposure (EU PEP)[1]	HIV-1 infection (IU PEP)[1]	NVP effect *in vivo* (EU DART)[1]	Level relative to mothers level (Control group)	EU PEP vs EU DART[2]
Activation markers						
Neopterin	No	↑	↑	↑	↑	↑ in EU DART infants
β_2-m	No	↑ (NS)	↑	↑	↑	
sL-selectin	No	↑ (NS)	↑	↑	↑	↑ in EU DART infants
Haematopoietic						
GM-CSF	No	No	No	↑ (NS)	↑	NS
IL-7	No	No	↓	No	Similar	NS
CXCL12	No	No	↑ (NS)	↑ (NS)	↓	NS
Immunoregulatory						
CCL3	No	No	No	↓ (NS)	ND	NS
CCL4	No	No	No	↑	ND	NS
CCL5	No	No	No	↑ (NS)	ND	NS
Immunosuppressive						
IL-10	No	No	↑	↑	Similar	NS
Memory response						
T-helper (to HIV-1 envelope peptides)	Yes (Th Env^+ responses in infants are associated with lack of infection and with ↓ GM-CSF levels)	Yes	Yes	No	ND	EU PEP Env^- vs EU PEP Env^+ infants ↑ β_2-m ↑ sL-selectin

[1] ↑ or ↓ ($p<0.05$) - increases or decreases significantly **relative to control infants**
[2] Statistically significant increase (↑ $p<0.05$) – EU PEP infant group compared to EU DART infant group
NS – differences in levels not statistically significant
↑ (NS) or ↓ (NS) - levels increase or decrease, but difference is not statistically significant (NS), that is, an increased or decreased trend in levels
ND - not determined

In Figure 8.1 we illustrate the possible HIV-1 infection outcomes of infants that are born to HIV-1 seropositive mothers and that are exposed to single-dose NVP either as a result of a dose being given to the mother at the start of labour or due to a dose being given to the infant within 72 hours of birth. HIV-1 exposure or the immune consequences of having an HIV-1 infected mother as well as single-dose NVP influences the extent of immune activation which affects the type of immune response observed in the newborn infant. We propose that the resultant consequences of these exposures are either (i) T-cell anergy or immunodeficiency, (ii) protective immunity (Th1 responses) or (iii) immune activation (either Th1 or Th2 but which is determined by the cyokine/chemokine milieu). In scenario (iii) however, some infants do become infected. This would suggest that either (i) infection in the infant is determined by the cytokine patterns that are linked to a polarization of cell-mediated (Th1) or humoral immune responses (Th2) or (ii) host genetic factors account for susceptibility to infection of infants that become infected despite favourable immune responses/activation (not determined in this study). Although our study has made specific reference to cells of the adaptive immune system we note that DC and macrophages play an important role in HIV-1 disease pathogenesis and have thus included them in Figure 8.1.

Figure 8.1 (page 163) Possible outcomes of *in utero* viral exposure in infants born to HIV-1 seropositive mothers

(A) R5 viruses from a viral quasi-species of R5 and X4, which use the CCR5 and CXCR4 co-receptors, respectively, to infect cells are predominantly transmitted across the placental barrier (Wolinsky *et al*., 1992; Scarlatti *et al*., 1993). Alternatively, p24 antigen which diffuses (Bawdon *et al*., 1994) or defective virus particles cross the placental barrier.

(B) Transplacental passage can occur due to placental damage (pre-existing inflammatory conditions such as sexually transmitted diseases (Fawzi *et al*., 2001; Nair *et al*., 1993), active genital ulcers, chorioamnioitis, inflammatory cytokines (Fawzi *et al*., 2001; Nair *et al*., 1993; St Louis *et al*., 1993; Wabire-Mangen *et al*., 1999, Raghupathy, 1997), transcytosis (by a vesicular pathway) (Bomsel, 1997) or infection of placental cells (Maury *et al*., 1989; Sprecher *et al*., 1986; Zachar *et al*., 1994)).

(C) After R5 virus crosses the placental barrier it infects resting and activated $CD4^+$ T-cells, DC and macrophages. The interaction of complex host and viral factors will determine the risk for initial HIV-1 acquisition and the progression to disease.

(D Part a) Infants infected IU (presence or absence of NVP): demonstrate elevated immune activation (increased neopterin, β_2-m and sL-selectin) as a consequence of being born to a HIV-1 seropositive mother. Infants that become infected *in utero,* demonstrate

excessive immune activation, the presence of single-dose NVP augments the already activated immune system. The consequence of excessive immune activation may be T-cell anergy or immunodeficiency. In this group of infants virus replicates, that is, there is systemic dissemination of virus and infected cells resulting in the establishment of a lymphatic tissue reservoir of HIV-1 that spreads infection to other organs and peripheral tissues. In this group of infants single-dose NVP, given to the mother at the onset of labour or to the infant as PEP is unlikely to have a significant impact on the extent of the viral replication.

(D Part b) Infants that develop T-helper responses in the absence of single-dose NVP: immune activation in some infants results in the development of Th1-mediated responses (HIV-1 specific $CD4^+$ T-helper responses). Detection of these responses implies that encounter with HIV-1 occurred *in utero*. In these infants prolonged exposure to low doses of HIV-1 (lower maternal viral load) primes the immune system to induce Th1 responses. These infants are protected from infection upon subsequent re-exposure to HIV-1.

(D Part c) Exposure to HIV-1 either in the presence or absence of NVP – infants remain uninfected (EU) or become infected IP: short-course NVP intervention has been shown to lower the rate of transmission of HIV-1 to between 10.6% and 13% (Ayouba *et al*., 2003), that is, although transmission is reduced some infants will still become infected during delivery or postpartum. This group of infants cannot be distinguished from uninfected infants at birth (as the PCR is negative at this time). Exposed uninfected infants demonstrate elevated immune activation. In these infants immune activation and host factors (antigen-induced lymphokine production and cellular proliferation) may result in (i) abortive HIV-1 infection or (ii) virus is harboured in quiescent cells (in this case virus may be activated even after further activation of the immune system (IP infants)), alternatively (iii) these infants do not directly encounter HIV-1. Infants that become infected IP may also encounter virus during labour or postpartum. In this case, virus may replicate due to the cytokine milieu e.g. a bias to a more Th2-type such as IL-10 which would downregulate host T-cell function and reduce antigen-specific proliferation. Alternatively, these infants are genetically more predisposed to HIV-1 infection. NVP might act by binding directly to the HIV-1 reverse transcriptase or by making cells anergic thereby preventing efficient replication of HIV-1.

(D Part d) Naïve T-cell output in newborn infants does not appear to be affected by HIV-1 exposure, HIV-1 infection, immune activation or post-thymic memory T-cell expansion (development of HIV-1 specific $CD4^+$ T-helper responses).

While some interesting observations were made from the *in vitro* study, such studies may not adequately reflect the *in vivo* situation particularly if one considers that physiologic processes associated with labour impact on NVP absorption, distribution and elimination (Mirochnick *et al*., 1998b). These processes will most probably also impact on the biovailability of AZT and will affect the rate and extent of AZT phosphorylation, as well as the dose and rate of AZT elimination. Furthermore, the antiviral activity of both NVP and AZT as tested *in vitro* using PBMC from healthy donors may not adequately reflect the sensitivity of maternally transmitted HIV-1 to single-dose prophylaxis, and the influence of viral characteristics such as dose and tropism, as measured *in vivo*, is not readily comparable to these measures *in vitro*.

Based on the data from this study the following will be important:

1. To establish whether other drugs or drug combinations used to prevent maternal HIV-1 transmission similarly enhance immune activation.
2. To determine what direct effect(s) NVP and other drugs or drug combinations have on different cell types which might influence, amongst others, characteristics such as antigen-presentation and chemotaxis.
3. To determine, using microarray analysis, how different antiviral drugs alone, and in combination with HIV-1, influence gene expression regulating immune activation, cell trafficking and stimulatory or inhibitory cytokines and chemokines.
4. To identify other markers of protective immunity that are not attenuated by antiretroviral treatment, and to determine whether reduced GM-CSF levels are commonly associated with infants demonstrating HIV-1 specific cellular immune responses to viral proteins in addition to Env.
5. To determine which cells of infants are responsible for the increased release of neopterin and whether the neopterin levels measured in plasma directly correlate with γ-interferon (IFN-γ) release from antigenic stimulation of monocytes or T-helper cell subsets. This would address the question of IFN-γ production in newborn infants since studies have reported a reduced ability of cord blood T-lymphocytes to produce Th1 derived cytokines IFN-γ and IL-2 upon stimulation with specific mitogens or alloantigen (Paloczi, 1999; D'Arena *et al*., 1999) and a significantly decreased ability of CBMC to produce IFN-γ (Roncarolo *et al*., 1994). In a recent study, an increased IFN-γ production to mycobacterial antigens, has been demonstrated in a proportion of HIV-1 exposed newborns, indicating that *in utero*

HIV-1 exposure primes T-cells (Van Rie *et al*., In press). Furthermore, IFN-γ is known to activate tryptophan degradation which appears to result in the reduced ability of intracellular pathogens to survive and limits the proliferation of cells including T-lymphocytes (Widner *et al*., 2000). Decreased T-cell responsiveness and development of immunodeficiency may be the result of increased neopterin formation and degradation of tryptophan (Widner *et al*., 2000).

6. To determine the extent of the influence of the Th2 bias prevailing during pregnancy and at the materno-foetal interface on the shaping of infant immune responses specifically in the case of infants born to HIV-1 seropositive mothers.
7. To determine whether exposure of the thymus to HIV-1, either as a consequence of aborted infections or from defective virus particles, influences specific precursor T-cells and subsequent antiviral responses.
8. To conduct a longitudinal study to determine how immune factors (such as those examined in this study) change and whether thymic disturbances and the extent of the dysfunction are reflected over time between HIV-1 exposed, HIV-1 infected and control infants.
9. To establish whether maternal virus phenotype influences the substantially increased immune activation noted in the presence of single-dose NVP exposure and whether viral phenotype accounts for differences in infection outcome in infants (EU, IP and IU infants).

In conclusion, the outcome of HIV-1 infection of infants born to HIV-1 seropositive mothers reflects a complex host-virus interaction. The risk for an infant of acquiring maternally transmitted HIV-1 is determined in part by a critical balance between the level of immune activation, extent of the priming events and the stimulatory/inhibitory cytokines and chemokines. The ability of the infant to remain uninfected may be dependent on the time of exposure and may be compromised by the dose and phenotype of the virus. While single-dose NVP may act by directly binding to HIV-1 reverse transcriptase and slowing the rate of DNA synthesis our study suggests that NVP can influence immune activity – possibly by affecting the 'anergic' state of the cells, the extent of which may determine the replicative potential for HIV-1.

Unfortunately, many of the resource-poor countries such as Sub-Saharan Africa, the Caribbean and parts of Asia are still affected by the HIV-1 pandemic. Notable progress

has been made in many areas of the developing world in reducing the transmission of HIV-1, however these countries are still the ones most affected by HIV-1 infection. Cellular and humoral immune responses play an important role in controlling HIV-1, however, the true correlates of immune protection and immune failure need to be better defined (Letvin and Walker, 2003). By broadening our understanding of the role of cellular and humoral immune responses in controlling HIV-1 this should provide greater insight into defining goals for immuno-therapeutic intervention and for vaccine strategies – a task made more difficult by viral heterogeneity and the diverse HLA backgrounds of infected individuals (Letvin and Walker, 2003).

AIDS started as a highly fatal infection but has become a treatable, chronic infectious disease in the developed world as a result of the development of antiretroviral therapy. Despite HIV-1 resistance to current therapy, metabolic complications, adverse side effect and adherence difficulties, the improvements made in the treatment regimens have prolonged the survival of HIV-1 infected patients (Pomerantz and Horn, 2003). The biggest challenge to date is to implement affordable and appropriate approaches in the developing world. For scientists the greatest task is to develop an effective HIV-1 vaccine. However, the AIDS pandemic threatens the success of existing vaccine programs and may accelerate the emergence of new infectious diseases (Weiss, 2003). The need therefore to develop novel approaches to combat the HIV-1 infection are evident. Maternal-infant transmission provides an important model for the continued study of protective processes that will provide important insights towards the development of novel strategies for the prevention and treatment of HIV-1 infection in both infants and adults.

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APPENDIX A: Abbreviations

ACTG	AIDS Clinical Trial Group
AIDS	acquired immune deficiency syndrome
ALLO	allogeneic peripheral blood mononuclear cells
AMT	3'-amino-3'-deoxythymidine
APC	antigen presenting cell
ARC	AIDS related complex
ART	antiretrovitral therapy
AZT	zidovudine
AZT-DP	dideoxynucleoside diphosphate
AZT-MP	dideoxynucleoside monophosphate
AZT-TP	dideoxynucleoside triphosphate
bd	twice daily
BFU-E	erythroid burst-forming units
BH_4	tetrahydrobiopterin
β_2-m	β_2-microglobulin / beta2-microglobulin
CBL	cord blood leukocytes
CBMC	cord blood mononuclear cells
CCL3	macrophage inflammatory protein-1α (MIP-1α)
CCL4	macrophage inflammatory protein-1β (MIP-1β)
CCL5	regulated upon activation, normal T-cell expressed and secreted (RANTES)
cDNA	complimentary deoxyribonucleic acid
°C	degree Celsius
CFU-GM	granulocyte macrophage colony-forming units
C_{max}	peak plasma concentration
CSF-1	colony stimulating factor 1
CYP	cytochrome
Da	Dalton
DC(s)	dendritic cell(s)
D gene segments	diversity gene segments
dGTP	guanosine 5'-triphosphate
ddNTP	di-deoxynucleoside triphosphate

dNTP	deoxynucleoside triphosphate
DITRAME	Diminution de la Transmission Mère-Enfant
DNA	deoxyribonucleic acid
DTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Env	envelope
EU	exposed uninfected
FCS	foetal calf serum
GalC	galactosylceramide
GAZT	glucuronyl zidovudine
GM-CSF	granulocyte macrophage colony stimulating factor
gp	glycoprotein
GPCRs	G-protein-coupled receptors
GTP	guanosine triphosphate
HAART	highly active antiretroviral therapy
HLA	human leukocyte antigen
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HIVNET	HIV Network for Prevention Trials
IC_{50}	50% inhibitory concentration
IFN-α	interferon-alpha
IFN-β	interferon beta
IFN-γ	interferon gamma
IgG	immunglobulin G (characterized by δ heavy chains)
IL	interleukin
IL-2R	interleukin-2 receptor
IP	infected intrapartum
IU	infected *in utero*
J gene segment	joining gene segment
k_{cat}	enzymatic catalysis
K_d	Dissociation constant
k_s	enzymatic substrate
kDa	kiloDalton

$KHCO_3$	potassium carbonate
L-selectin	L-selectin (also called CD62L)
l	litre(s)
LTR	long terminal repeat
µ	micro
M	molar
MAb	monoclonal antibody
M-CSF	macrophage colony stimulating factor
mg	milligram(s)
MHC	major histocompatability complex
ml	millilitre(s)
mmol	millimole
MLRs	mixed lymphocyte reactions
moi	multiplicity of infection
mtDNA	mitochondrial DNA
MTCT	mother-to-child transmission
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDP	nucleoside diphosphate
ng	nanogram
NH_4Cl	ammonium chloride
NK	natural killer
NNRTI	non-nucleoside reverse transcriptase inhibitor
nm	nanometre
NRTI	nucleoside reverse transcriptase inhibitor
NVP	nevirapine
p24-Ag	p24 antigen (a major capsid antigen of HIV)
PACTG	Pediatric AIDS Clinical Trial Group
PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-alpha derived growth factor
PEP	post-exposure prophylaxis
PETRA	Perinatal Transmission Study

PHA	phytohemagglutinin
PI	protease inhibitor
p.i.	post infection
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
SABTS	South African Blood Transfusion Service
SAINT	South African Intrapartum Nevirapine Trial
SD	standard deviation
SDF	stromal-cell derived factor
SI	syncytium-inducing
SI's	Stimulation indices
SIV	simian immunodeficiency virus
sjTREC	single-joint T-cell receptor excision circles
sL-selectin	soluble L-selectin
$t_{½}$	elimination half-life
3TC	lamivudine
$TCID_{50}$	50% tissue culture infectivity dose
TCR	T-cell receptor
tds	three times daily
TdT	terminal deoxynucleotidyl transferase
Th	T-helper
Th1	T-helper type 1
Th2	T-helper type 2
TIBO	tetrahydrobenzodiazepine
TMP	thymidine monophosphate
TREC(s)	T-cell receptor excision circle(s)
TTP	thymidine triphosphate
TNF-α	tumour necrosis factor alpha
UNAIDS	United Nations Programme on HIV/AIDS
USA	United States of America
UTR	untranslated region
µg	microgram
µl	microlitre

μM	micromolar
V region	variable region
WHO	World Health Organisation

APPENDIX B: Antiretroviral agents in clinical use and under clinical development for adults and children

Table 1 Antiretroviral agents available to adults – their properties and clinical status

Compound (Abbreviation) (Trade name)	T½ (hours)	Primary route(s) of metabolism and elimination	T_{max} (hours)	Bio-availability (%)	Dosage forms	Targets Mechanisms Clinical status
Nucleoside analogues[1, 2, 3, 5, 11]						
Zidovudine (AZT/ZDV) (Retrovir)	1-1.1	Renal excretion following hepatic metabolism of the parent drug and glucuronide	0.4-1.5	60-70	Capsule, tablet, syrup, injection	Substrate (dNTP) binding site of RT; Chain termination; Approved for HIV-1 treatment others under clinical development
Didanosine (ddI) (Videx)	0.97-1.6	Renal excretion	0.25-2	25-43	Tablet, solution	
Zalcitabine (ddC) (HIVID)	1-3 adults 0.8 children	Metabolism by liver NS Renal excretion	0.8 fasting 1.6 food	> 80	Tablet	
Stavudine(d4T) (Zerit)	0.9-1.1	Renal excretion	0.5-1.5	78-86	Capsule, powder	
Lamivudine (3TC) (Epivir)	8.5	Renal excretion	0.9	86-88	Tablet, solution	
Abacavir sulphate (ABC) (Ziagen)	1.5	Renal excretion		83	Tablet, solution	
Emtricitabine(FTC) (Emtriva)	10	Renal excretion	1-2	93	Capsules	FDA approved July 2003; Substrate (dNTP) binding site of RT; Chain termination
Approved nucleoside combinations [11]						
AZT and 3TC (Combivir)						
AZT+3TC+abacavir (Trizivir)						
Protease inhibitors[1, 2, 3, 5, 11]						
Saquinavir (SQV) (Fortovase Invirase)	13.2	Hepatic metabolism by CYP3A4 Faecal elimination	>1 fasting; 3 food	4-13	Capsule	Inhibits HIV-1 aspartic protease; Release of immature non-infectious HIV-1 particles; Approved for HIV-1 treatment others under clinical development
Ritonavir(RTV) (Norvir)	3-5	Hepatic metabolism by CYP3A4 and CYP2D6 Faecal elimination	2-4	60	Capsule, solution	
Indinavir sulphate (IDV) (Crixivan)	1.8 adults 1.1 children	Hepatic metabolism by CYP3A4 Faecal elimination	0.8 fasting		Capsule	
Nelfinavir mesylate (NFV) (Viracept)	3.5-5 13yrs+	Hepatic metabolism by CYP3A4 Faecal elimination	2-4	17-47 (animal data)	Tablet, powder	
Amprenavir (APV) (Agenerase)	7.1-10.6	Hepatic metabolism by CYP3A4 Faecal elimination	1.9 adults 1.1 children	Low	Capsule, solution	
Lopinavir (Aluviran)						
Lopinavir-ritonavir (latter pharmaco-enhancer of former) (Kaletra)	5-6	Lopinavir metabolism by CYP3A4 ritonavir induces own metabolism and inhibits CYP3A4 therby increasing .plasma levels of lopinavir Faecal elimination	4		Capsule, solution	
Atazanavir sulphate (ATV) (formerly BMS-232632) (Reyataz)		Metabolised through monoxygenation and dioxygenation Faecal elimination	2.5		Capsule	FDA approved June 2003 in combination with other ART; Inhibits processing of Gag and Gag-Pol pp; Release of immature non-infectious HIV-1 particles
Fosamprenavir (FPV) (Lexiva)	7.7	Hepatic metabolism by CYP3A4 Minimal faecal, urine elimination	1.5-4	Not established	Tablets	
Non-nucleoside reverse transcriptase inhibitors[1, 2, 3, 5, 11]						
Nevirapine (NVP) (Viramune)	>22	Hepatic metabolism by CYP3A4 and CYP2B6 Renal excretion	1.5-4	90-93	Tablet, oral suspension	Nonsubstrate binding site of RT; Inhibition of RT through interaction with allosteric pocket site; Approved for HIV-1 treatment others under clinical development
Delavirdine mesylate (DLV) (Rescriptor)	2.4	Hepatic metabolism by CYP3A4 and CYP2D6	1-1.2	85	Tablet	
Efavirenz (EFV) (Sustiva/Stocrin)	52-76	Hepatic metabolism by CYP3A4 and CYP2D6 Faecal elimation	3-5		Capsules, tablet	
Nucleotide analogue (acyclic nucleoside phosphonates)[3, 5, 6]						
Tenofovir disoproxil fumarate[2;3] (TDF) (Viread)		Not metabolized by liver enzymes Renal excretion	1	25 fasting 65 food	Tablet	HIV-1 RT; Phase 3
Receptor antagonists[4, 5, 8, 11]						
Membrane fusion inhibitors						
Enfuvirtide[4; 5; 8] (T-20) (Fuzeon)	3.8-6	To be elucidated	4-8	84.3	Subcutaneous injection	Interferes with gp41-mediated fusion; Phase 3; FDA approved

Table 2 Antiretroviral agents under clinical development

Compound	Company producing the ART	Proposed mechanism of action	Clinical status
New generation nucleoside reverse transcriptase inhibitor [11]			
Amdoxovir (DADP)	Emory University Inc	Inhibit HIV-1 RT	Studied for use in cases of virologic failure; Phase 1
Alovudine (MIV-310)	Boehringer Ingelheim	Inhibit HIV-1 RT	
D-D4FC (DPC 817)	Pharmasset Inc	Inhibit HIV-1 RT	Safety, tolerability and pharmacokinetics being determined
Elvucitabine (ACH-126,443)	Achillion	Inhibit HIV-1 RT	
New generation non-nucleoside reverse transcriptase inhibitor [7, 9, 11]			
TMC 125	Tibotec-Virco	Inhibition of RT through interaction with allosteric pocket site	Phase 2
Calanolide A	Sarawak MediChem Pharmaceuticals Inc	Inhibition of RT through interaction with specific sites of RT enzyme	Phase 1 trials
Capravirine	Pfizer / Agouron Pharmaceuticals Inc	Inhibition of RT	Phase 1-2 trials
New generation non-peptidic protease inhibitors [9; 10, 11]			
Tipranavir (TPV)	Boehringer Ingelheim	Binds to active site of HIV-1 PI; Suppress viral replication	Phase 2-3
TMC114	Tibotec	Inhibits HIV aspartic protease	Phase 2 trials
Receptor antagonists [4, 5, 8, 11]			
Entry and fusion inhibitors			
T-1249[5;8]	Hoffmann- La Roche	Interferes with gp41-mediated fusion	Salvage therapy for patients failing T-20; Phase 1-2
'Five-helix'[5]		Binds to gp41, inhibits fusion	Preclinical
TNX-355[5]	Tanox	IgG4 anti-CD4 domain 2 mAb	Phase 1a safety study
AMD11070	AnorMED	CXCR4 inhibitor	Safety, tolerability and pharmacokinetics being determined in clinical phase 1-2 trials
Re-combinant soluble CD4[5]		Competitively binds to gp120 receptor	Limited activity phase 1-2 trials
PRO 542[5]		Tetrameric CD4 incorporated into γ-globulin	Phase 1-2
BMS-805[7]		Binds to gp120 to block CD4 binding	Preclinical
Chemokine receptor inhibitors			
SCH-C[5]		RANTES antagonist, competes for CCR5	Phase 1-2
PRO-140[5]		MAb to CCR5	Preclinical
AMD3100[5]		Bicyclam inhibitor of CXCR4	Phase 2
Other attachment inhibitors			
Dextran sulphate[5]		Binds to gp120 and inhibits CXCR4	Phase 1-2 Toxic
PRO 2000[5]		Binds to CD4 and interferes with gp120 binding	Phase 2
Cyanovirin-N[5]		Binds to gp120, interferes with CD4 and CXCR4	Preclinical
Integrase inhibitors			
S-1360[7]			Clinical
Anionic polymers[3]			
Polysulphonates		Virus absorption; Interaction with V3 loop of gp120	Under consideration as vaginal microbicides
Polysulphonates			
Polycarboxylates			
Chicoric acid			
Zintevir			
Cosalane derivitives			
Other microbicides [11]			
Carrageenan (Carraguard)	FMC Biopolymer	Topical microbicide to prevent sexual transmission of HIV-1	Phase 1 trials as a vaginal microbicide
Carbopol 974P (Buffer gel)	ReProtect LLC		Phase 1 trials as a vaginal microbicide
C31G	Biosyn Inc		Phase 1 trials as a vaginal microbicide, oral rinse and dermal application
Hydroxyethyl cellulose (Cellulosize)	Union carbide		Safety currently being determined
UC-781	Biosyn Inc	Inhibit HIV-1 RT	Studied in animal models; will soon enter phase 1 trials as a NNRTI vaginal microbicide

References for Tables 1 and 2, Appendix B

[1] Li and Chan 1999; [2] Hammer 2002; [3] De Clerq 2001; [4] Fletcher 2003; [5] Kilby and Eron 2003; [6] McColl and Miller 2003; [7] McCarthy M 2002; [8] Moyle 2003; [9] Vella 2003; [10] Yeni 2003; [11] AIDS information http://aidsinfo.nih.gov/drugs/htmldrug_tech.asp

Table 3: Antiretroviral agents recommended in children under 3 months of age (South Africa) (Cohen *et al*., 2002)

Category	ART	Formulation	Dosage
I (NRTI-thymidine base)	Stavudine	Suspension 1 mg/ml	<29 days: 0.5 mg/kg/dose twice daily (bd) >30 days: 1 mg/kg/dose bd
	Zidovudine	Suspension 10 mg/ml	4 mg/kg/dose three times daily (tds) until 29 days then 160 mg/m^2/dose tds
II (NRTI-other)	Didanosine	Suspension 10 mg/ml; tablets 25 mg	50 mg/m^2/dose bd
	Lamivudine	Suspension 10 mg/ml	<1 month: 2 mg/kg/dose bd >1 month: 4 mg/kg/dose bd
III (NRTI)	Nevirapine	Suspension 10 mg/ml	5 mg/kg/day x 14 days then 120 mg/m^2/dose bd x 14 days then 200 mg/m^2/dose bd
IV (PI)	Ritonavir	Suspension 80 mg/ml	> 1 month: 450 mg/m^2/dose bd
	Nelfinavir	Powder 50 mg/g; tablets 250 mg	55 – 65 mg/kg/dose bd
V (NRTI-new)	Abacavir	Suspension 20 mg/ml	8 mg/kg/dose bd

Babies < 3 months should not be on treatment unless they fulfil specific indications for starting ART (based on clinical category or CD4%<20%). In cases where ART is suggested the regimens used include: 3 NRTIs (one from category I, one from category II, category V) plus 1 PI (category IV) or 3 NRTIs (one from category I, one from category II, category V) plus NVP or 2 NRTIs plus 1 PI plus 1 NNRTI (this last regimen leaves few alternatives available for future use and should only be considered in special circumstances).

APPENDIX C: Clinical trials undertaken to investigate the efficacy of different antretroviral regimens in reducing perinatal HIV-1 transmission

Table 1: Phase 3 antiretroviral prophylaxis clinical trial regimes and their efficacy in reducing perinatal HIV-1 transmission

Drug regimen	Study and site	Breast fed	Maternal antepartum	Maternal intrapartum	Maternal post partum	Infant postpartum	Transmission
AZT	PACTG 076 [(1)]; USA	No	100 mg orally 5x/day starting 14-34 weeks gestation	Intravenous 2.0 mg/kg over 1 hour then continuous infusion of 1 mg/kg/hr	-	2 mg/kg orally 4x/day for 6 weeks	68% reduction at 18 months:
AZT	PACTG 185 [(2)]; USA	No	Non-study maternal AZT treatment	Intravenous 2.0 mg/kg over 1 hour then continuous infusion of 1 mg/kg/hr	-	2 mg/kg orally 4 times daily for 6 weeks	5% at 18 months
AZT	Bangkok trial [(3)]; Thailand	No	300 mg orally 2x/day starting 36 weeks gestation	300 mg orally every 3 hours	-	-	50% reduction at 6 months
AZT	Cote d'Ivoire trial [(4)]	Yes	300 mg orally 2x/day starting 36 weeks gestation	300 mg orally every 3 hours	-	-	37% reduction at 3 months
AZT	DITRAME ANRS 049 [(5)] Cote d'Ivoire Burkino Faso	Yes	300 mg orally 2x/day starting 36-38 weeks gestation	600 mg orally at onset of labour	300 mg orally 2x/day for 1 week	-	38% reduction at 6 months
AZT/3TC	PETRA [(6)] South Africa, Uganda, Tanzania (3- vs 2- vs 1-part regimen)	Mixed	300 mg oral AZT and 150 mg oral 3TC 2x/day starting 36 weeks gestation	300 mg oral AZT every 3 hours and 150 mg oral 3TC every 12 hours	300 mg oral AZT and 150 mg oral 3TC twice daily for 1 week	4 mg/kg oral AZT and 2 mg/kg oral 3TC 2x/day for 1 week	At 6 weeks 63% reduction (3-part) vs 42% reduction (2-part) vs NS reduction (1-part)
NVP	HIVNET 012 [(7)] Uganda	Yes	-	Single 200 mg oral NVP dose at onset of labour	-	Single 2 mg/kg oral NVP dose at age 48-72 hours	47% reduction at 14-16 weeks:
AZT/3TC vs NVP	SAINT [(8)] South Africa	Yes (40%)	-	300 mg oral AZT every 3 hours and 150 mg oral 3TC every 12 hours vs single 200 mg oral dose at onset of labour	300 mg oral AZT and 150 mg oral 3TC 2x/day for 1 week vs single 200 mg oral dose at 48-72 hours postpartum	4 mg/kg oral AZT and 2 mg/kg oral 3TC 2x/day for 1 week vs single 2mg/kg oral dose at age 48-72 hours after birth	Transmission at 8 weeks 10.2% with AZT/3TC vs 13.3% with NVP

AZT, zidovudine; 3TC, lamivudine; NVP, nevirapine; NS, not significant

[1] Connor *et al*., 1994; [2] Stiehm *et al*., 1999; [3] Shaffer *et al*., 1999a; 4 Wiktor *et al*., 1999; [5] Dabis *et al*., 1999; [6] The PETRA Study Team 2002; [7] Guay *et al*., 1999; [8] Moodley *et al*., 2003

APPENDIX D: Synthetic HIV-1 envelope peptides used to induce T-helper cell responses

Table 1. HIV-1 subtype B envelope peptides (Berzofsky *et al*., 1991; Hale *et al*., 1989; Cease *et al*., 1987) used to stimulate IL-2 production

HIV-1 gp120 peptides	Peptide sequences	Amino acid residue
Constant regions		
T2	HEDIISLWDQSLK	112-124
T1	KQIINMWQEVGKAMYA	428-443
TH4.1	DRVIEVVQGAYRAIR	834-848
Hypervariable loop		
P18 MN	RIHIGPGRAFYTTKN	315-329
P18 IIIB	RIQRGPGRAFVTIGK	315-329

APPENDIX E: Composition of buffers and media

1. Phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free

Use de-ionized water

i. Dulbecco 10x concentrate Mg^{2+} free:

NaCl	800g
KCl	20g
Na_2HPO_4	115g
K_2HPO_4	20g

Dissolve the chlorides in 5 L of de-ionized water. Use de-ionized water (~100°C) to dissolve the Na_2HPO_4. Add the dissolved K_2HPO_4. Make up to 10 L. Check pH. Filter sterilize with positive pressure and store at room temperature.

ii. PBS Ca^{2+} and Mg^{2+} free:

Dilute the Dulbecco Mg^{2+} free 10-fold with de-ionized water. Check the pH. Filter sterilize with positive pressure. Store at 4°C.

2. DMSO/FCS freezing solution for PBMC

i. Mix together:

FCS	90%
DMSO	10%

ii. Resuspension of cells:

Working on ice, resuspend cells at a concentration of 1-5x10^6 cells/ml in the above mixture

iii. Freezing conditions:

To freeze cells at the recommended controlled cooling rate of -1°C/minute, vials containing sample (cells) are placed into a Nalgene Cryo 1°C freezing container and placed at -70°C for a minimum of 4 hours. Frozen samples are then removed and stored at -170°C. Cells may be kept stored at -70°C however, the success rate of reconstituting cells which have been frozen for periods greater than three months is increased with storage at -170°C.

APPENDIX F: List of suppliers

Item	Manufacturer
Plasticware	
Blue Max™ polypropylene conical tubes (15 ml / 50 ml)	Becton Dickinson Labware, Franklin Lakes, NJ, USA
Costar 48-well cell culture cluster (flat-bottomed)	Corning, Inc., NY, USA
Costar 96-well cell culture cluster (U-bottomed)	Corning, Inc., NY, USA
Costar tissue culture flasks (25 cm^2 / 75 cm^2)	Corning, Corp.,Cambridge, USA
Nunc cryotube™ vials	Nalge, Nunc Int. Denmark
Nunclon™ 96-well microtitre plate (flat-bottomed)	Nalge, Nunc Int. Denmark
Disposable plastic pipettes (2ml / 5 ml / 10 ml)	Sterilin Ltd. Staffordshire, UK
Polystyrene reagent reservoir (50 ml)	Corning, Inc. NY, USA
Safe lock micro test tubes (0.5 ml / 1.5 ml / 2 ml)	Eppendorf, Hamburg, Germany
Sarstedt tubes (1.5 ml / 2 ml)	Nümbrecht, Germany
Nalgene Cryo 1°C freezing container	Nalge Nunc International, Rochester, NY, USA
Cell culture media	
RPMI 1640	Gibco, Invitragen Life Technologies NY, USA
Foetal calf serum	Gibco, Invitragen Life Technologies NY, USA
Penicillin G	Sigma, Steinheim, Germany
Streptomycin	Sigma, Steinheim, Germany

L-glutamine	Sigma, Steinheim, Germany

Reagents/items used for cell culture

EDTA vacutainer tubes	Becton Dickinson, USA
Ficoll-Paque™ Plus	Amersham Biosciences, Uppsala, Sweden
Dimethyl sulfoxide	Sigma, Steinheim, Germany
Interleukin-2 (human, recombinant)	Roche Diagnostics Corporation, Indianapolis, USA
Trypan blue	Sigma, Steinheim, Germany
Phytohaemagglutinin	Sigma, Steinheim, Germany
Celltiter 96™ non-radioactive cell proliferation assay	Promega, Madison, USA

Reagents/items for HIV-1 studies

AZT	AIDS Research and reference reagent programme (Division of AIDS, NIAID, NIH, USA)
FACSCount System	Becton Dickinson, San Jose, CA
HIV-1 antibody	Abbott Laboratories, Abbott Park, Illinois, USA
NVP	AIDS Research and reference reagent programme (Division of AIDS, NIAID, NIH
p24-Antigen ELISA	Coulter Corp., Hileah, FL, USA
Roche DNA monitor assay	Roche Diagnostics Systems, Inc., New Jersey, USA
Roche Amplicor RNA monitor assay	Roche Diagnostics Systems, Inc., New Jersey, USA

Enzyme linked immunosorbent assay kits

Biotrak high sensitivity (h)GM-CSF	Amersham Biosciences UK Ltd.
Biotrak high sensitivity (h)IL-10	Amersham Biosciences UK Ltd.

Immunotech neopterin kit	Beckman Coulter, France
Quantikine $Beta_2$-microglobulin	R&D Systems, Inc. Minneapolis, MN, USA
Quantikine high sensitivity SDF-1α	R&D Systems, Inc. Minneapolis, MN, USA
Quantikine sL-selectin	R&D Systems, Inc. Minneapolis, MN, USA
Quantikine high sensitivity IL-7	R&D Systems, Inc. Minneapolis, MN, USA
Quantikine MIP-1α	R&D Systems, Inc. Minneapolis, MN, USA
Quantikine MIP-1β	R&D Systems, Inc. Minneapolis, MN, USA
Quantikine RANTES	R&D Systems, Inc. Minneapolis, MN, USA
Quantiglo human IL-2	R&D Systems, Inc. Minneapolis, MN, USA

Real-time PCR

AmpErase^R UNG	SYBR Green PCR Core Reagents, PE Biosystems, Foster City, CA, USA
AmpliTag Gold^R DNA polymerase	SYBR Green PCR Core Reagents, PE Biosystems, Foster City, CA, USA
QIAmp^R DNA Mini kit	Qiagen GmbH, Hilden, Germany

General chemicals

Ammonium chloride	Sigma, South Africa
Ethylenediamine tetraacetic acid	Roche, South Africa
Ethanol (absolute)	Merck, South Africa
	SkyChem, South Africa
Potassium carbonate	Merck, South Africa

APPENDIX G: Statistical methods

SPSS version 11.0 (SPSS inc, Chicago, USA) was used to perform all statistical tests.

Paired-samples *t*-test

This test establishes using the *t*-statistic whether two means collected from the same sample (or related observations) differ significantly. In the *in vitro* study, this test was used to compare differences between untreated (control) samples and those treated pre- or post infection with either NVP or AZT.

Mann-Whitney U-test (for unpaired data set comparisons)

Is a nonparametric equivalent to the t-test and tests whether two independent samples are from the same population. It uses the ranks of cases and requires an ordinal level of measurement. U is the number of times a value in the first group precedes a value in the second group, when values are sorted in ascending order. In our case, the Mann-Whitney U-test was used to compare data between groups (EU-, IP- and IU-PEP; EU-, IP- and IU-DART).

Spearman's rank correlation coefficient

A nonparametric measure of the correlation between two ordinal variables. Rank correlation coefficients were used to compare data within groups e.g to determine the relationship (correlation) between immune factors within the PEP and DART groups. The correlation coefficient is a measure of the interdependence between two variables. It is a measure that takes on values between –1 (indicating a perfect negative association) and +1 (indicating a perfect positive association) with the intermediate value 0 indicating the absence of association.

Wilcoxon signed rank test (for paired data sets)

A nonparametric procedure used with two related variables to test the hypothesis that the two variables have the same distribution. It makes no assumptions about the shapes of the distributions of the two variables. This test takes into account information about the magnitude of the differences within pairs. The test statistic is based on the ranks of the absolute values of the differences between the two variables. This test was used to compare immune factors from mother-infant PEP and DART pairs.

Fisher's exact test

A test to determine differences in proportions between groups. Thus, this test was used to determine whether the proportion of infants with HIV-1 specific immune responses and those without differs between the EU and IP infected groups.

APPENDIX H: Ethical clearance

This study was approved by the University of the Witwatersrand, Johannesburg Committee for Research on Human Subjects (Medical), protocol number M02-04-25. Patients were recruited after informed consent was obtained and the confidentiality of all records ensured.