HIV-1 genotypes and phenotypes associated with mother to child transmission

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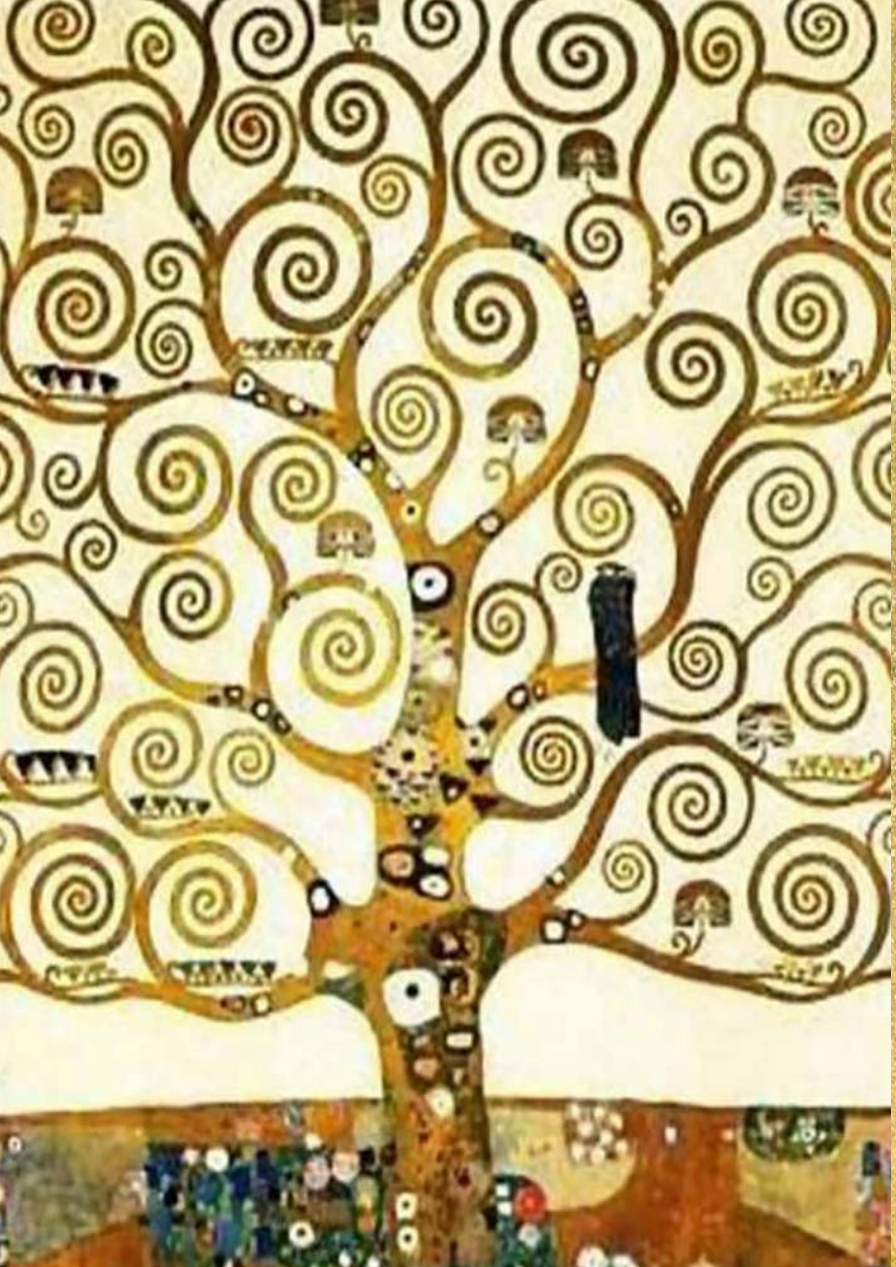
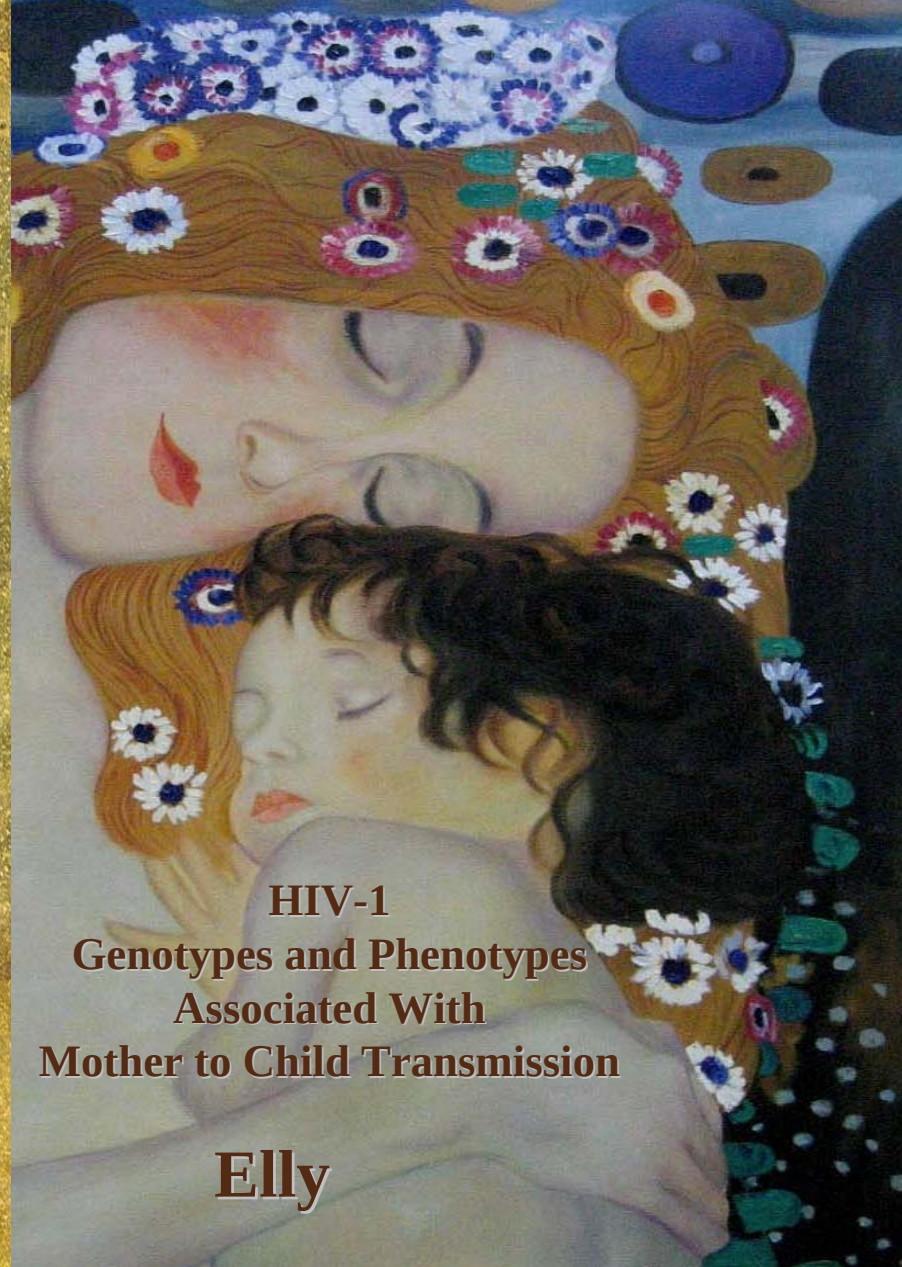


HIV-1 Genotypes and Phenotypes Associated With Mother to Child Transmission Elly



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HIV-1 Genotypes and Phenotypes Associated with Mother to Child Transmission

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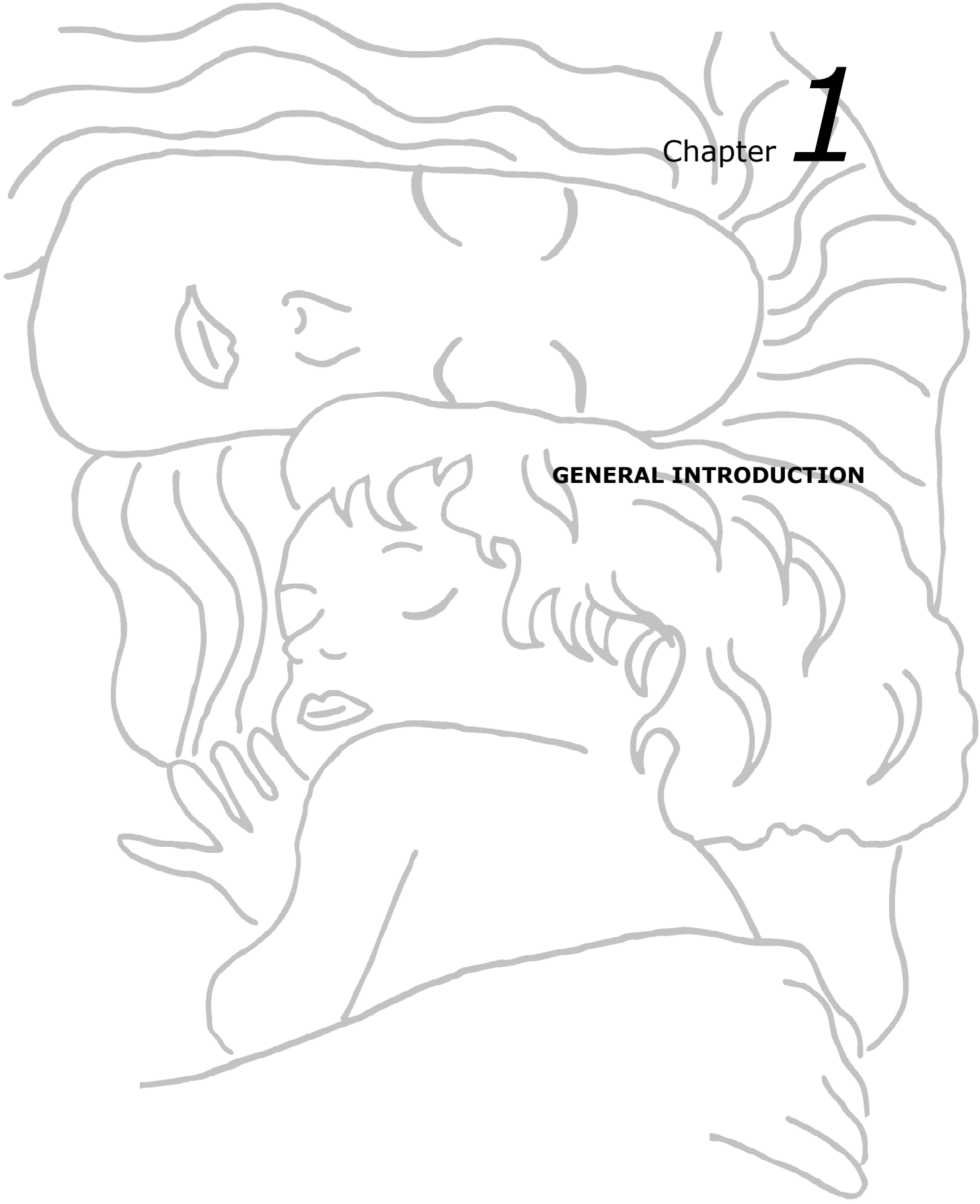

Chapter 1

GENERAL INTRODUCTION

1. Emergence of the AIDS epidemic

In 1981 a number of reports documented the occurrence of unusual diseases indicative of severe immune dysfunction in homosexual men [1-4], subsequently referred to as acquired immunodeficiency syndrome (AIDS). It soon became apparent that homosexual men were not the only group at risk of developing AIDS. The disease was found to spread in several ways, by intimate homosexual as well as heterosexual contact [5], by needle sharing during intravenous drug use [6], by administration of blood or blood products [7] and via mother to child transmission (MTCT) [8,9]. All the data pointed towards an infectious agent causing the selective depletion of $CD4^+$ T-cells and the search began for the pathogen responsible. In 1983 Barre-Sinoussi et al reported on the isolation of a retrovirus from T-lymphocytes obtained from a patient with lymphadenopathy [10]. The evidence suggested this to be the causative agent of AIDS: the virus first named lymphadenopathy associated virus (LAV) later became known as human immunodeficiency virus (HIV).

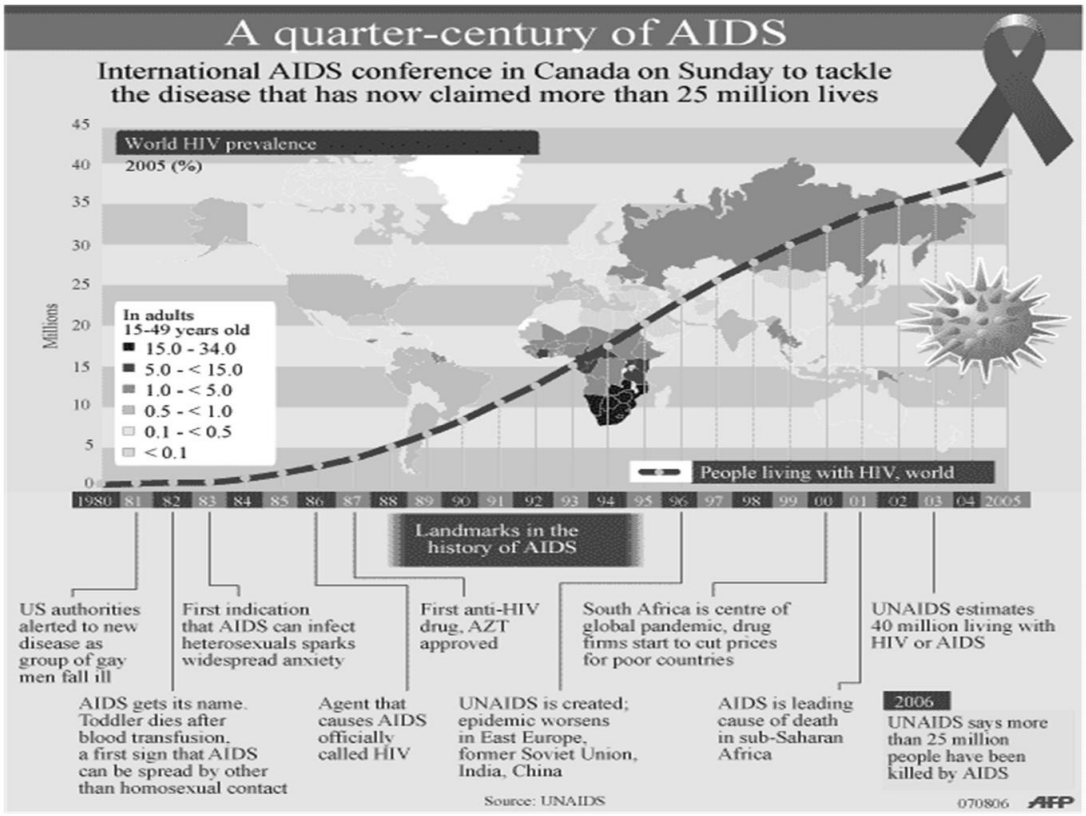

Figure 1. History of the HIV-1 epidemic

From the recognition of the first AIDS cases the pandemic has rapidly spread and by the end of 2011 approximately 34.0 million people were reported living with HIV (UNAIDS report 2012), with the history of the epidemic depicted in Figure 1. HIV belongs to the genus Lentiviruses, a group of retroviruses that can infect a broad range of animals, including simians (simian immunodeficiency virus, SIV), cats (feline immunodeficiency virus, FIV), sheep (Visna/Maedi virus) and horses (equine infectious aneamia virus, EIAV) [11]. Two types of HIV have been identified, HIV-1 and HIV-2, with the vast majority being infected with type 1. Phylogenetically HIV-1 can be divided into three groups, group M (major), group O (outlier) and group N

(non-M and non-O) which were introduced into the human population from chimpanzees and through separate cross-over events. Group M viruses can be divided into variant subtypes; A, B, C, D, F, G, H, J and K (Figure 2). Many recom-

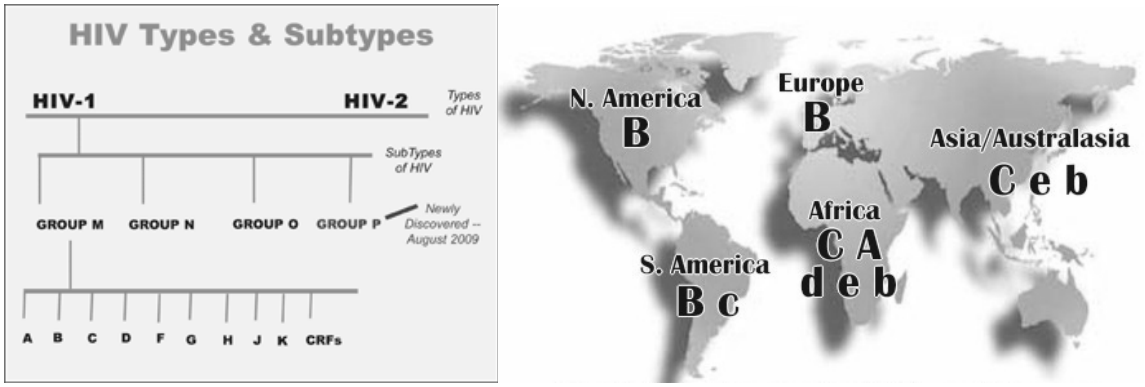

Figure 2. HIV subtypes and global distribution. Major subtypes in capitals

binant forms of HIV-1 have been described with AE being considered the most important. Subtype B is the most dominant subtype circulating in the Western World, whilst subtypes A and C circulate predominantly in Africa and South East Asia with the recombinant AE form being the main subtype present in Thailand (Fig. 2).

2. Virus composition and replication cycle

The genome of HIV-1 consists of two identical positive strand RNA molecules of approximately 10 kb. The genome encodes nine proteins, including the three major proteins *gag, pol and env* (as shown in Figure 3) [12-14]. The *gag* gene encodes

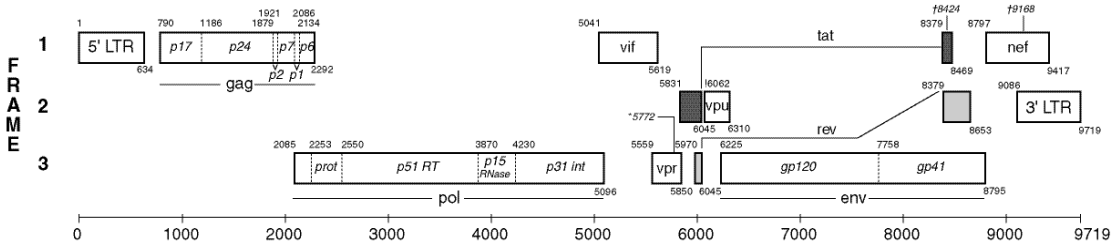

Figure 3 HIV-1 gene-map

four structural proteins: the capsid (CA-p24), the matrix (MA-p17) and the nucleocapsid (NC –p7 and NC-p6) [15-18]. The *pol* gene encodes three enzymes: reverse transcriptase (RT), integrase (IN) and protease (PR) [15-20]. The e*nv* gene encodes for the viral envelope glycoprotein gp160 (Env), which after cleavage generates the gp120 and the gp41 proteins that associate to form the envelope glycoprotein (gp120/gp41 Env) [21]. In addition several accessory and regulatory proteins are encoded by HIV-1 (Vpr, Vif, Vpu, Tat, Rev and Nef) which possess various functions in the viral life-cycle and which classically distinguishes lentiviruses from more simple retroviruses [16,22-24].

The viral RNA molecules are coated with the NC proteins and reside within the inner core together with the enzymes RT, IN and PR [19,20,25,26]. The structural proteins make up the inner core (CA) and shell (MA) of the virus [15-18]. The outer surface of the viral particle is composed of the cell membrane from the producer cell with the gp41 protein anchored in the membrane and non-covalently linked to the gp120 molecule [21]. The gp120/gp41 complex (envelope complex, Env) is expressed as a trimer on the surface of the viral particle and is involved in the recognition of the next target cell, followed by interaction with required receptors before fusion of the viral and cellular membranes [27]. Upon fusion the viral core is released into the cytoplasm of the cell where it is uncoated and a pre-integration complex is formed between host factors and the content of the viral core (Figure 4).

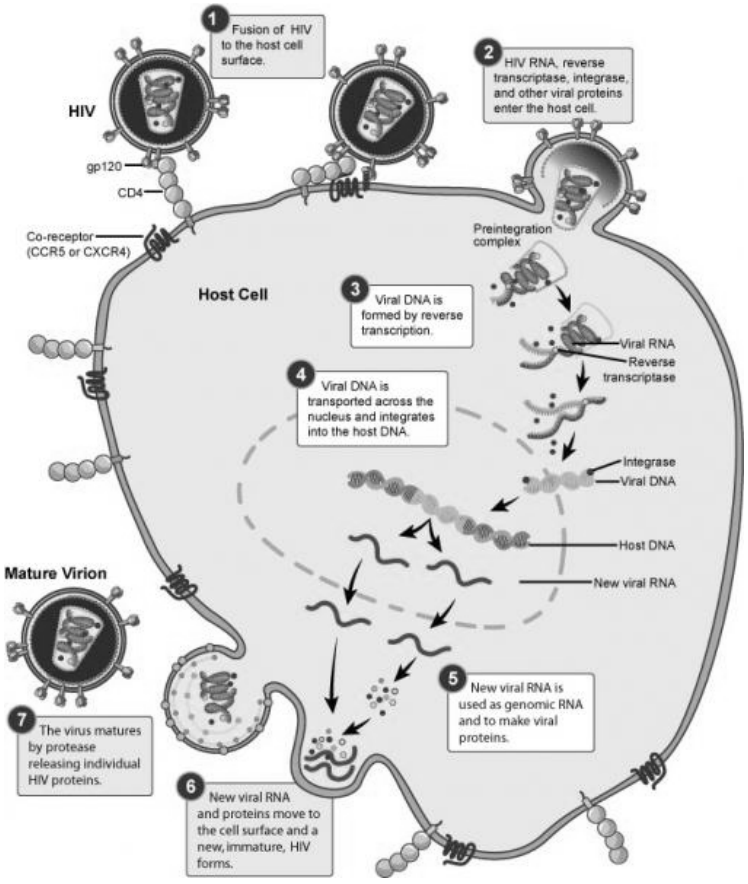

Figure 4. HIV-1 repication-cycle

This complex enters the cell nucleus and the viral RNA is reverse transcribed by the viral RT into DNA followed by integration into the host genome [26]. RNA synthesis and protein translation by the host cell machinery results in the production of new infectious viral particles. HIV RT lacks proofreading activity to correct errors made when it converts the RNA into DNA**.** The new viral particles are released from the cell by a budding process. The life-cycle of HIV can be short (1.5 days) from time of viral entry, through replication, assembly, and release of virus particles and the subsequent infection of new cells [28]. The short life-cycle period, large progeny size and high polymerase error rate all provide for a virus with a high degree of genetic diversity. Most of the mutations either are inferior to the parent virus or convey no advantage, but some provide for a selective advantage which can allow

the virus to circumvent antiretroviral drugs or evade the human immune system. In 1996 David Ho presented viral dynamics data showing that the average untreated HIV-1 infected individual produces 10 billion virions per day [29].

3. HIV-1 envelope glycoprotein and receptor usage

The HIV-1 Env spikes are composed of non-covalently linked gp120 and gp41 molecules anchored in the cell membrane which form functional trimers [30,31]. Both gp120 and gp41 are involved in the interaction, recognition and fusion of viral with cellular membranes and which determine viral tropism [32]. The gp120 molecule is composed of five constant regions (C1-C5) alternated with five highly variable regions (V1-V5) as depicted in Figure 5. The conserved regions form the central core and the variable regions form the surface-exposed loops of the molecule. The envelope proteins are the most immunogenic components of HIV-1 particles since they are the only viral proteins expressed on the viral membrane.

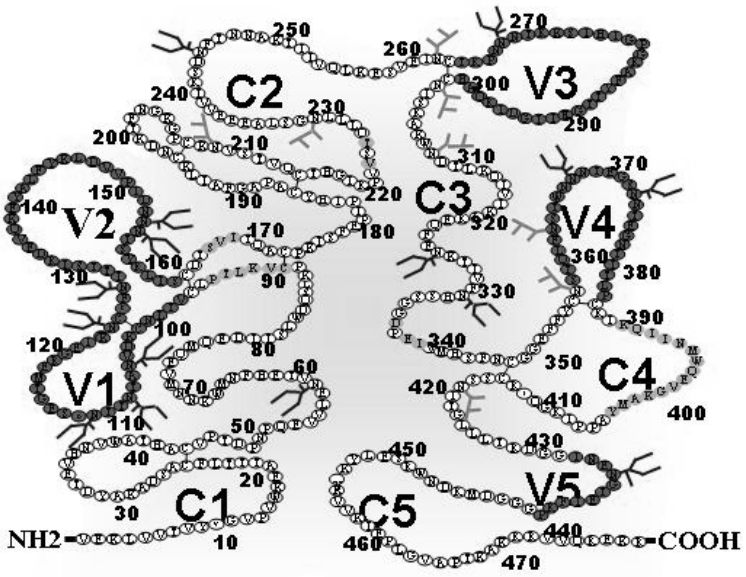

Figure 5. HIV-1 gp120 envelope protein

The envelope glycoprotein of HIV-1 gp160 is synthesized in the endoplasmic reticulum and subjected to extensive N-linked glycosylation, resulting in high-mannose chains attached to N residues at either the N-X-S or the N-X-T glycosylation sites. N-linked glycans comprise approximately 50% of the mass of the gp160 protein [33]. These sugar moieties are involved in various activities such as metabolism, transport, structural maintenance of the cell, protein folding, recognition of particular cell types and adhesion to other cells. The N-linked glycosylation pattern of the Env protein forms a "glycan shield" which is one of the major mechanisms described to block or minimize the effects of neutralizing antibodies (NAbs) and as shown in Figure 6 [34].

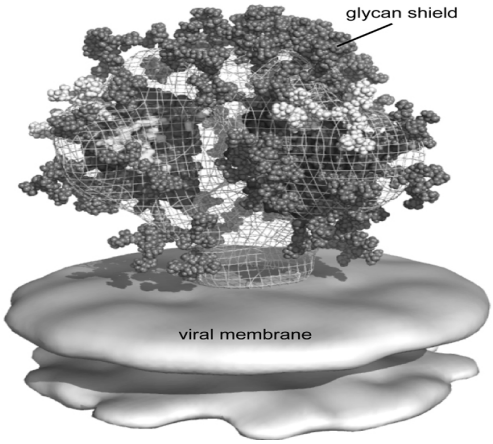

Figure 6. HIV-1 gp160 glycan shield

The cell-types infected with HIV-1 are numerous and include $CD4^+$ T-lymphocytes, thymocytes, macrophages, monocytes, dendritic cells (DCs) and microglial cells [35-41]. The CD4 molecule on these cells serves as the primary receptor for virus binding [42]. Subsequent to CD4 binding the gp120/gp41 structure undergoes a conformational change that enables the molecule to interact with a coreceptor that is essential for membrane fusion and infection. Several CC- and CXC-chemokine receptors have been shown to function as coreceptors for HIV-1 entry with CCR5 and CXCR4 being the two most prominent and significant (Figure 7) [43].

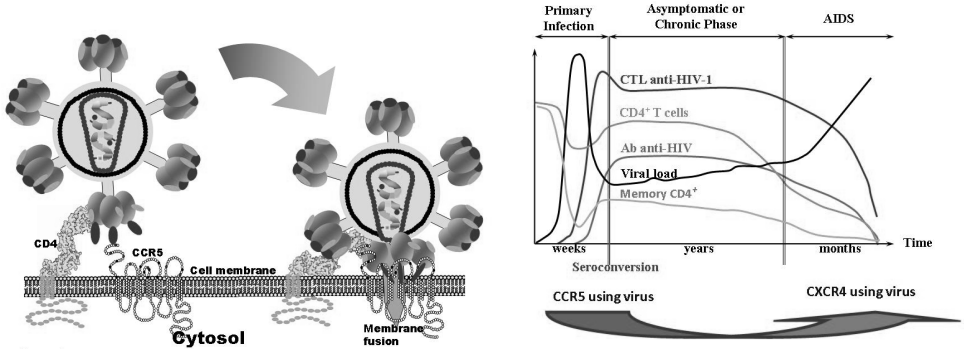

Figure 7. HIV-1 co-receptor usage and disease

Viruses can utilize either CCR5 (R5) or CXCR4 (X4) with dual-tropic viruses (R5X4) being able to utilize both receptors. In approximately half of HIV-1 infected individuals the virus population switches coreceptor phenotype from being R5 to R5X4 or X4 during the disease course [43]. This switch in co-receptor usage is associated with increased viral loads, drops in CD4 counts and faster rates of progression to disease [44-46]. What drives this R5 to X4 coreceptor switch is relatively unknown and remains one of the major questions to be solved regarding understanding HIV-1 pathogenesis (as depicted Fig. 7). The molecular mechanisms promoting the switch in coreceptor usage are better understood with multiple changes in the V3 and V1V2 regions having been associated with coreceptor use [47-49]. Alterations in chemokine receptor expression patterns, CC- and CXC-

chemokine production and changes in immune responses have all been postulated to influence and ultimately drive the switch [50].

4. Sexual transmission of HIV-1

The vast majority of HIV-1 infections are initiated by a single or a limited number of viral variants (Figure 8), irrespective of transmission route or HIV-1 subtype indicating a severe bottleneck to transmission [51,52]. However, infection can also

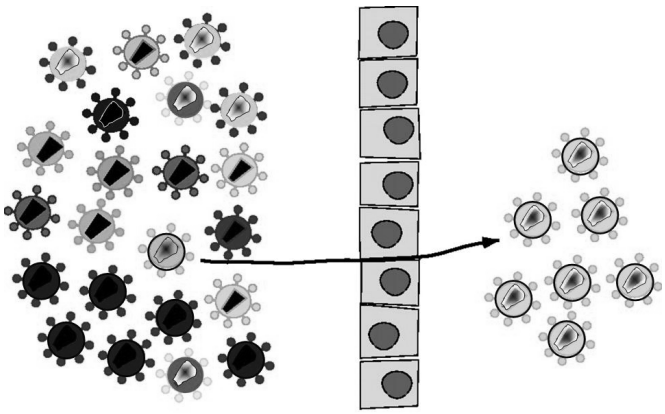

Figure 8. HIV-1 transmission bottleneck

be initiated by two to five or more distinct but highly related viral variants, associated with factors such as genital inflammation which has been postulated to transiently decrease the protective effect of the mucosal barrier of the recipient partner [53-57]. Whether multiple variants traverse the mucosal barrier, but only

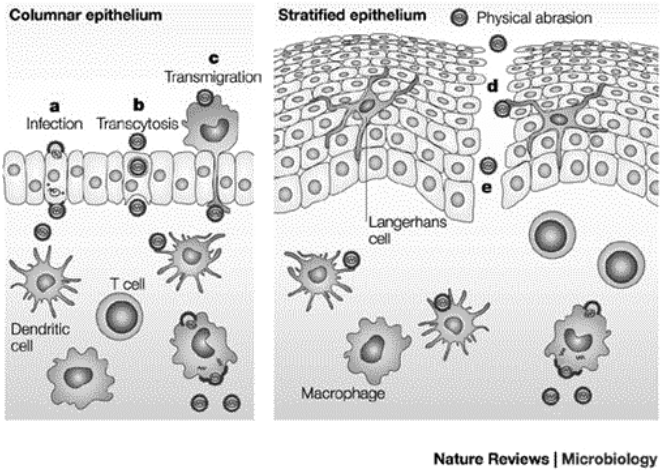

Figure 9. HIV-1 at the mucosa

one variant dominates during the ramp-up phase or alternatively a single variant is transmitted, is unknown and depicted in Figure 9. Finding common characteristics of newly transmitted variants able to establish a persistent infection would indicate which HIV-1 variants need to be targeted and blocked. Recently transmitted subtype A and C HIV-1 variants show shorter variable loops and less potential N-linked glycosylation sites (PNGS) in gp120 when compared with the donor quasi-species [58-61]. However, for subtype B viruses shorter, less glycosylated gp120 domains of transmitted viruses have not been observed. CXCR4 using viruses are rarely transmitted. The virus variants undergoing transmission predominantly utilize the CCR5 coreceptor [62]. A 32bp deletion in the CCR5 receptor, resulting in the reduced expression on the cell surface and a molecule non-functional for HIV-1 entry, provides considerable protection against HIV-1 transmission and has been associated with a delay in disease progression. Neither infection of macrophages nor alternate coreceptor usage seem to be advantageous for HIV-1 transmission [63]. Infectious molecular clones generated from subtype B- and C-infected individuals replicated to high titers in activated $CD4^+$ T-cells, whilst none of the transmitted viruses replicated in monocyte-derived macrophages [64].

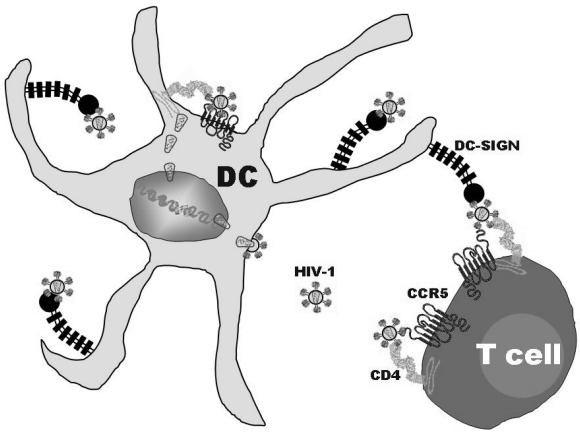

Figure 10. HIV-1 interaction with DCs

DCs play a significant role in HIV-1 transmission and are located within or under the mucosal surfaces and interact with many invading pathogens, which are internalized and degraded into peptides to be presented as antigens at the surface of the cell [65,66]. The DCs subsequently migrate to localized lymph nodes to present antigen to naïve T-helper cells and promote their differentiation into effector T-cells [65,67,68]. One of the receptors expressed on the surface of DCs is the dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin receptor (DC-SIGN or CD209) and recognizing a wide array of pathogens including HIV-1 [69-72]. HIV-1 can likely cross the epithelial border through ruptures or lesions in the epithelium or via a mechanism termed transcytosis [73]. After crossing the mucosal barrier the virus can interact with DC-SIGN expressed on DCs (depicted Figure 10). This interaction between HIV-1 and DC-SIGN is substantially influenced by the N-linked glycosylation profile of the gp120/gp41 Env protein [74]. A proportion of captured virus particles can be transported to a non-lysosomal compartment and thereby escape degradation. These viruses can subsequently infect $CD4^+$ T lymphocytes in a process known as *trans*-infection and which can greatly enhance HIV-1 infectivity [69]. It has also been postulated that the interaction of HIV-1 with DCs via binding of DC-SIGN can protect the virus against

the effects of antibodies (Abs) and antiretroviral drugs [75]. Interaction between DCs either infected with or having captured HIV-1 and $CD4^+$ lymphocytes is bridged by the infectious synapse, which supports cell-to-cell viral transmission [76-80]. This process has been shown to be very efficient and likely contributes to HIV-1 pathogenesis *in vivo*. It has also been demonstrated that HIV-1 in the presence of NAbs can be efficiently captured by DCs via DC-SIGN and after internalization be rendered infectious for $CD4^+$ T lymphocytes, providing a mechanism of immune evasion [81].

5. Antiretroviral therapy

In 1987 the U.S. Food and Drug Administration approved the antiretroviral drug Zidovudine (AZT), an RT inhibitor, as a treatment against HIV-1. Initially the AZT treatment looked promising but soon after drug resistant variants emerged and the use of the drug was halted [82]. Combination therapy with other newly introduced RT inhibitors and newly developed protease inhibitors changed the face of antiretroviral therapy with many fewer deaths occurring through infection with HIV-1 in the US from 1995 onwards (Figure 11) [83,84].

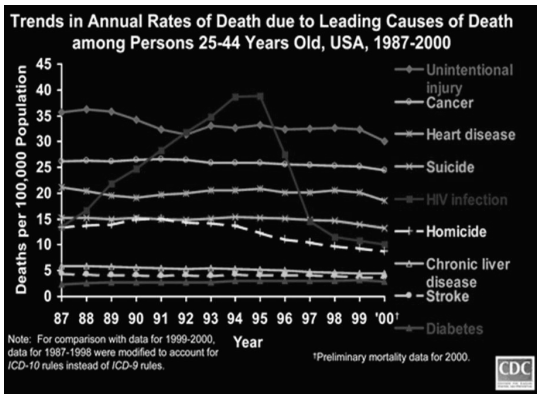

Figure 11. Effect of cART on reducing AIDS deaths

Combination antiretroviral therapy (cART) with two RT inhibitors and a protease inhibitor provided sufficient viral suppression and limited the emergence of resistant strains. Today, cART is potent, convenient and usually well tolerated and capable of reducing HIV blood concentrations to undetectable values within a few weeks of treatment initiation and resulting in robust and sustained CD4 T-cell gain as it is able to target many different stages of the HIV-1 replication cycle (Figure 12) [1, 2]. However, since current treatment strategies are unable to eradicate HIV-1 from infected individuals, therapy must be life-long, with the potential for short- and long-term side effects and with high costs for health care systems. HIV-1 suppression through cART prevents virus transmission from infected women to their newborn babies [85]. Similarly, sexual transmission in serodiscordant adult couples is less frequent in those with lower viral loads supporting the concept that treatment can prevent infection [86,87]. These findings suggest that initiating early HIV-1 treatment may prove to be an integral component of HIV-1 transmission prevention strategies. Treatment of HIV-1 uninfected but highly exposed individuals has been proposed as a means to prevent HIV-1 transmission [88]. The strategy, known as pre-exposure prophylaxis (PrEP), has been shown to be successful in a number of completed trials but with compliance being seen as a major drawback to such an

approach [88]. In addition, the costs and the implications of prescribing potentially harmful antiretroviral drugs to HIV-1 uninfected individuals' requires further ethical debate.

Several clinical trials have studied the effect of the anti-malarial drugs chloroquine (CQ) and hyroxychloroquine (HCQ) on modulating HIV-1 replication *in vivo* [89-91]. In two of these trials treatment with HCQ resulted in a decrease in VL with no measurable effect on the CD4 counts [90,91]. A decrease in plasma IL-6 expression levels was observed together with a decrease of total serum IgG [92]. These results make HCQ and CQ promising candidates in HIV-1 treatment strategies. Furthermore, CQ has been shown to accumulate in human milk and has been shown not to be toxic in breastfed infants [93]. These latter findings along with the low cost of the drug plus its wide availability have led to the speculation that the drug may be effective at reducing HIV-1 viral loads in human milk and prevent MTCT via the breastfeeding route.

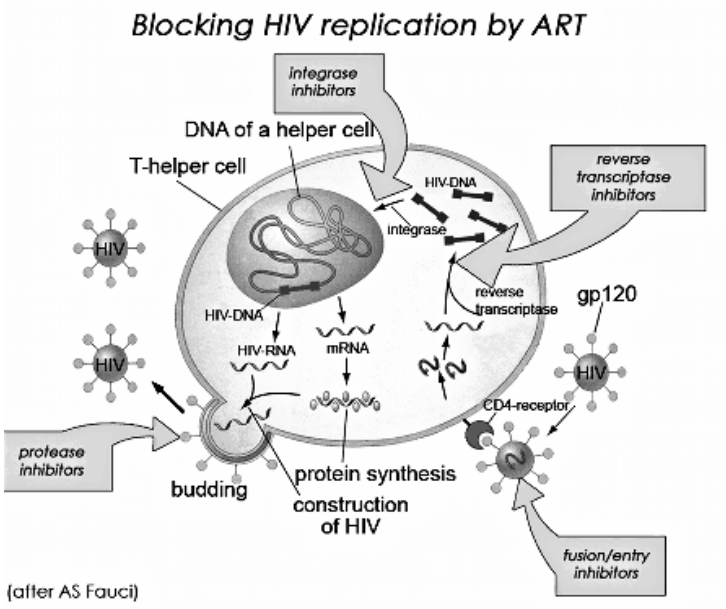

Figure 12. Effect of ARTs on HIV-1 Replication cycle

6. HIV-1 NAbs and vaccine design

HIV-1 eradication from an infected individual cannot be achieved by cART, which makes the search for a vaccine against the virus of high importance. To date, however, the most advanced HIV-1 vaccine trials have all proved to be ineffective and with two trials based on inducing potent cytotoxic T lymphocyte (CTL) responses (STEP and HVTN505) being stopped prematurely due to no protective effect being demonstrated [94]. The only vaccine trial deemed to have any beneficial effect is RV144, the Thai trial, where a modest reduction in HIV-1 transmission was claimed [95]. From the subsequent analysis of immune correlates it has been claimed that HIV-1 gp120/gp41 Env binding Abs (more so V1V2) are associated with providing protection against infection. This result indicates that HIV-1 binding Abs may be important in providing protection, however, no such effect was observed in the first large scale vaccine trial (VAXGEN) where such responses were induced. It still remains to be determined which types of Ab responses and their induced mechanisms of action will be required for an effective HIV-1 vaccine.

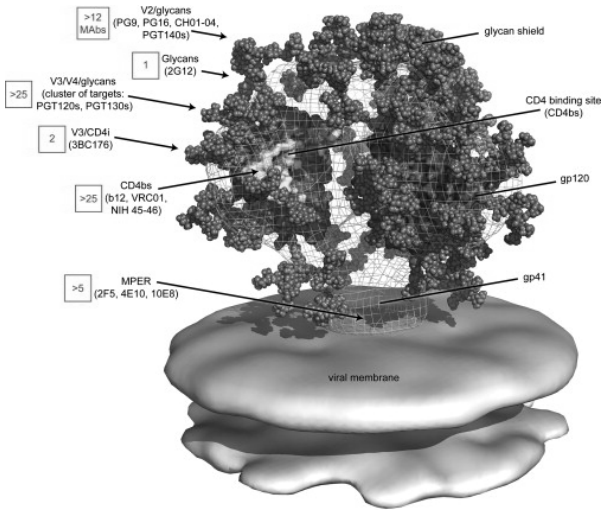

Figure 13. NAb recognition of HIV-1 Envelope

The gp120/gp41 Env molecule is the major target for NAbs and in naturally infected HIV-1 patients broadly NAbs (bNAbs) are not commonly induced. The Env regions against which the most studied bNAbs are directed are shown in Figure 13. Such NAbs are most often directed against strain-specific or non-neutralizing epitopes. Neutralizing activity of autologous serum against contemporary viruses is often low, and neutralizing activity is observed to be better against viruses from earlier time points suggesting viruses have been selected through the pressure of humoral immune pressure [34,60,96-98]. Immune escape results from alterations in the envelope gene, including point mutations, insertions, deletions and the masking of conserved epitopes through altered variable region structures as well as alterations in the composition of the protective glycan shield [99]. A vaccine should be able to neutralize HIV-1 variants that establish infections and the study of transmitted variants should therefore be able to identify their common Env characteristics. The development of an effective HIV-1 vaccine will likely depend on the successful design of an immunogen capable of eliciting bNAbs able to neutralize circulating strains of HIV-1 [100-103]. Recently, multiple studies have identified a number of monoclonal Abs with a broad neutralization potential, but how to induce such Abs still remains elusive.

7. Mother to child transmission of HIV-1

With no prevention strategies in place approximately 30% of HIV-1 infected pregnant woman will transmit virus to their infants either during gestation, during delivery or via breastfeeding and in approximately equal ratios (Figure 14). Maternal immunologic and clinical parameters modulate the risk of MTCT. High plasma viral load, $CD4^+$ T-cell counts less than 200 cells/mm^3 and clinical AIDS are clear risk factors associated with risk of transmission [104]. Maternal-infant HLA incompatibility has a protective effect against HIV-1 transmission [105]. Since the beginning of the epidemic much progress has been made in reducing HIV-1 MTCT. Transmission rates can be reduced to lower than 1.0 - 2.0% by use of cART during pregnancy as well as labor, by neonatal prophylaxis, elective caesarean delivery and avoiding breastfeeding. The exact timings and mechanisms associated with MTCT are largely unknown.

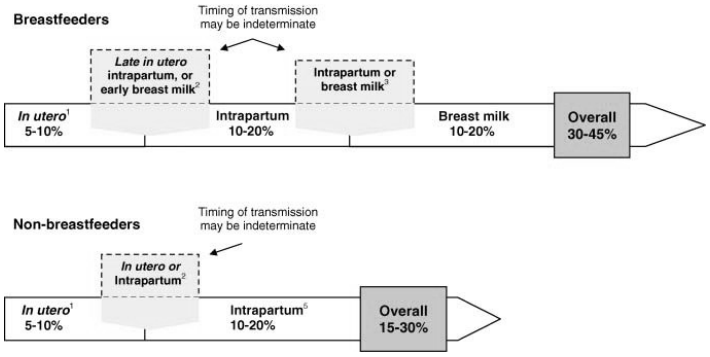

Figure 14. Rates for mother-to-child HIV-1 transmission among breastfeeders and non-breastfeeders

Studies using PCR have found very little or no HIV-1 positivity in lymphoid tissue of first and second trimester human fetuses [106-108]. Kourtis *et al* analyzed results from many prevention studies using cART at various stages during pregnancy and elective caesarean section performed before the onset of labor [109]. This study identified that approximately half of the *in utero* transmissions occur late in pregnancy and the authors claim this represents transmission during days prior to delivery and labor, when the placenta separates from the uterine wall. Placental insult, either through the physical stress of labor or via specific infections such as malaria or chorioamnionitis, may allow the passage of HIV-1 directly to the infants bloodstream leading to infection [110]. Infant exposure to maternal blood and other HIV-1-infected secretions may also support transmission whilst it passes through the birth canal. Cesarean section before labor onset provides an 80% reduction in risk of HIV-1 transmission [85,111], potentially due to preventing both micro-transfusions during active labor and avoidance of infant gut and conjunctival exposure to maternal blood and secretions, which can occur during vaginal delivery. However, a large study of twin births indicated that the order in birth did not influence the risk of infection, suggesting that birth channel exposure is not a major risk for the baby [112]. In approximately 18% of uninfected children born to HIV-1 infected mothers there was evidence of unintegrated virus present in their PBMCs suggesting that HIV-1 may enter the fetus but in the absence of activated lymphocytes may not integrate and establish infection until birth when lymphocyte activation is initiated. This window may provide an opportunity where ART intervention may be successfully used in order to prevent MTCT.

Breastfeeding provides another major risk for HIV-1 MTCT. It has been estimated that during a breastfeeding period of 2 years the HIV-1 transmission risk will reach 18% [109]. Breast abscesses as well as subclinical mastitis have also been associated with increased risk of transmission during lactation [113,114]. The biological mechanisms involved with HIV-1 transmission during breastfeeding have not yet been determined. What is known is that during the first days of life the infant may be at higher risk for HIV-1 transmission due to the lack of acidic gastric fluids, which can inactivate HIV-1, as well as ingestion by the infant of HIV-1-infected macrophages known to be present in colostrum. Early mixed feeding compared with exclusive breastfeeding during the first 3 months of life has been associated with an increased risk of HIV-1 infection among breastfed infants [115], whereas exclusive breastfeeding is associated with a lowered risk [116]. This increased transmission with mixed feeding may be caused by dilution of HIV-1 inhibitory components in milk from the mother, damage to the integrity of the infant intestinal mucosa or local inflammation, all of which may enhance the transfer of

HIV-1 across the lumen of the gut [117]. HIV-1 can likely interact with immature DCs (iDCs) at the gut mucosa through binding molecules such as DC-SIGN and where captured virus can then be transmitted to $CD4^+$ T-cells [118]. This would likely have to occur via breaches or tears in the gut mucosal lining and where such cells would be exposed. Several factors have been described in human breast milk which may provide some protection against transmission, including HIV-1-specific $CD8^+$ CTLs, HIV-1-specific IgG and IgA immunoglobulins as well as anti-DC-SIGN antibodies [119,120]. Two other compounds in human milk, MUC1 and bile-salt stimulated lipase (BSSL), have been shown to bind DC-SIGN and potently inhibit HIV-1 capture and *trans*-infection of $CD4^+$ T -cells *in vitro* [121]. The relative contribution of cell-free versus cell-associated virus to MTCT through breast milk ingestion is still not known but both will likely contribute to risk of infection via this route.

8. Role of NAbs in HIV-1 MTCT

A study utilizing rhesus macaques infected with SIV demonstrated that vaccination of pregnant animals resulted in decreased SIV infection during gestation or during birth, indicating that induced immune responses can provide protection against infection [122]. SIV hyper immune serum given subcutaneously prior to oral SIV inoculation also demonstrated decreased HIV-1 acquisition [123]. These results suggest that active immunization as well as anti-HIV immunoglobulin administration of human HIV-1 infected pregnant women may decrease the rate of perinatal HIV-1 infection. During the last month of gestation a considerable amount of maternal IgG Abs, including those directed against HIV-1, are transferred to the child. It could therefore be hypothesized that passively transmitted anti HIV-1 IgG Abs play a role in preventing infection, and that mothers who have better NAb responses will have a lower risk of passing virus to their infants. Indeed, there are reports on mother child pairs showing better neutralization by non-transmitting mothers (NTM) than by transmitting mothers (TM) [119,124]. Other reports demonstrate better neutralization by TM or find no differences between TM and NTM [125-130]. NAbs raised against HIV-1 may select variants undergoing transmission. Neutralization resistance of viruses generated from children against mother's plasma/serum has been reported suggesting transmission of neutralization escape mutants, but in contrast sensitivity to neutralization by plasma of the mother has also been found [119,127,128,131]. These discrepancies may depend on differences in viral subtype, mode of transmission, timing of transmission, timing of sampling or the selective study of autologous versus non-autologous viruses. Although the role of maternal NAbs in MTCT is controversial trials with HIV-Ig have been conducted. One such trial demonstrated protection against *in utero* transmission whilst the other revealed a significant increase in the number of infections at birth and 2 weeks after delivery in the treated versus untreated group [120,132].

9. Outline of the thesis

This thesis focuses on studying the role that the gp120/gp41 Env protein can play in relation to HIV-1 MTCT. The HIV-1 envelope protein provides the primary contact between the virus and host, and is the main target of the adaptive humoral immune response. The length of gp120 variable loops and the number of N-linked glycosylation events are key determinants for virus infectivity and NAb immune escape, whilst the V3 loop overall positive charge is known to affect co-receptor tropism. We also addressed whether the use of chloroquine (CQ), which has been shown previously to reduce HIV-1 viral loads *in vivo*, could be used to lower viral loads in breast milk and thereby limit HIV-1 transmission via breastfeeding. The relevant background to the studies is provided in **chapter 1**.

In **chapter 2** we describe two families in which both parents and two children had been infected with HIV-1 for nearly 10 years, but who demonstrated variable parameters of disease progression. We analyzed the gp120 envelope sequence and compared individuals that progressed to those that did not in order to decipher evolutionary alterations that are associated with disease progression when individuals are infected with genetically related virus strains. The analysis of the V3-positive charge demonstrated an association between higher charges with disease progression. The ratio between the number of amino acids and the number of potential N-linked glycosylation sites was also shown to be associated with disease progression with the healthier family members having a lower ratio. In conclusion, in individuals initially infected with genetically linked virus strains the V3-positive charges and N-linked glycosylation patterns are associated with HIV-1 disease progression and can follow varied evolutionary paths.

In **chapter 3** we describe the effects that CQ has on the *in vitro* infection and replication profile of HIV-1. It has been reported that CQ can decrease HIV-1 viral loads *in vivo* and can accumulate in breast milk of lactating women. We found that viruses generated in the presence of CQ are not hampered for infectivity and/or replication capacity. We did determine that viruses generated in the presence of CQ demonstrated a reduced capacity for DC-SIGN mediated *trans*-infection of $CD4^+$ lymphocytes. We analyzed the gp120 envelope protein sequences of viruses cultured in the presence or absence of CQ and observed a loss of two PNGS involved in binding of the monoclonal NAb 2G12 and in DC SIGN binding in viruses cultured with CQ. These results suggest that CQ may be a useful drug aimed at reducing HIV-1 MTCT via breastfeeding.

In **chapter 4** we describe the effect of CQ when given to breastfeeding mothers over a 16 week period from time of delivery. We observed a 2.5 fold higher concentration of CQ in breast milk compared with blood plasma but these higher concentrations of CQ did not result in a decline of viral load in the milk of the treated mothers. In contrast to previous reports, we found a significant increase in HIV-1 plasma viral loads in the CQ treated versus placebo mothers. When analyzing predicted gp120 Env protein sequences no variant characteristics were found that could explain the increased viral loads.

In **chapter 5** we studied the genotypic properties of the HIV-1 gp120 Env proteins from 30 mothers infected with HIV-1 subtype A or C viruses of whom 7 were known to have infected their children either during gestation or soon after birth. We analyzed and compared the sequences of the V1-V5 region of the gp120 envelopes from transmitting mothers (TM), non-transmitting mothers (NTM) and their infected children. No differences were found with respect to the lengths and number of PNGS in the V1-V5 region, but we identified that viruses with a PNGS at positions AA234 and AA339 were preferentially transmitted and that viruses with

PNGS-N295 showed a disadvantage for transmission. We also found that the frequency of PNGS-N339 in the viruses of TM and infected children from other cohorts was significantly higher than in viruses undergoing sexual transmission. These results provide evidence that the presence of the PNGS-N339 site in the gp120 envelope confers an advantage to HIV-1 when considering MTCT.

In **chapter 6** we describe the viral and immunologic correlates of the HIV-1 gp160/gp41 Env proteins of the 7 TM and child pairs and 4 NTM from the cohort studied in chapter 4. We found no differences in HIV-1 infectivity or interaction with DC-SIGN that could have facilitated HIV-1 MTCT but we did find that TM showed significantly higher plasma neutralization capacity than NTM. TM who infected *in utero* were found to neutralize their autologous Env's, and those of the corresponding children, to significantly higher levels than mothers that transmitted the virus *peri-partum* or through breastfeeding. Furthermore, plasma from children infected *in utero* was able to neutralize their autologous Env's as well as those from their mothers to higher levels in comparison to those infected *peri-partum* or by breastfeeding. These findings have consequences for the development of active or passive vaccination strategies aimed at preventing MTCT *in utero*.

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Chapter 2

HIV-1 gp120 envelope characteristics associated with disease progression differ in family members infected with genetically similar viruses

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ABSTRACT

The human immunodeficiency virus type 1 (HIV-1) envelope protein provides the primary contact between the virus and host and is the main target of the adaptive humoral immune response. The length of the gp120 variable loops and the number of N-linked glycosylation events are key determinants for virus infectivity and immune escape, while the V3 loop overall positive charge is known to affect co-receptor tropism. We selected two families in which both parents and two children had been infected with HIV-1 for nearly 10 years but who demonstrated variable parameters of disease progression. We analyzed the gp120 envelope sequence and compared individuals that progressed to those that did not in order to decipher evolutionary alterations that are associated with disease progression when individuals are infected with genetically related virus strains. The analysis of the V3 positive charge demonstrated an association between higher V3 positive charges with disease progression. The ratio between the amino acid length and the number of potential N-linked glycosylation sites was also shown to be associated with disease progression with the healthier family members having a lower ratio. In conclusion in individuals initially infected with genetically linked virus strains the V3 positive charges and N-linked glycosylation are associated with HIV-1 disease progression and follow varied evolutionary pathways for individuals with varied disease progression.

INTRODUCTION

Large differences have been described amongst humans in their susceptibility to infection with human immunodeficiency virus type 1 (HIV-1) as well as rates of disease progression. After infection some individuals progress slowly in their disease course (slow progressors/long-term non progressors) [1-4] some progress rapidly and develop disease in as little as 2-5 years (fast progressors) [5], whilst a small minority show no progression over 20 years of infection (Elite controllers) [6]. Many viral as well as host factors have been associated with this variation in viral transmission and disease progression [7]. One important host factor associated with both HIV-1 transmission and disease progression is the 32bp deletion in the CCR5 co-receptor (CCR5Δ32) for HIV-1 [8]. Individuals homozygous for the deletion are highly protected against infection, whilst individuals heterozygous for the deletion are not protected from infection but disease progress is attenuated once infected [9]. Other host factors involved with the chemokine/chemokine receptor (axis) have been associated with altered rates of HIV-1 disease progression [10,11]. Numerous allelic polymorphisms in genes involved in the adaptive immune recognition by T cells, mainly through human leukocyte antigen (HLA) restriction have shown to influence disease course, presumably through the elimination of infected cells and control of viral replication [12-14]. More recently, a number of genetic factors linked to the innate immune response were shown to be associated with both risk of transmission and disease progression [15].

The viral factor frequently associated with disease progression is the viral set-point (following the period of acute infection), with higher viral loads associated with faster progression to disease [16]. Additionally, HIV-1 lacking the Nef protein through a genetic deletion in the gene has been associated with decreased pathogenicity and longer survival [17]. However, the majority of research linking viral variation with HIV-1 transmission and disease progression has focused mainly on the gp120 envelope gene. One of the key characteristics to HIV-1 infection is the preferential transmission of viruses utilizing the CCR5

coreceptor (R5) [18] and the infrequent transmission of CXCR4 using (X4) or dual-tropic viruses (R5/X4) [19,20]. This restriction has been attributed to a number of factors including cell-type of infection and a better immune control of the X4 viruses. It has also been well documented that a switch from R5 to X4 viruses during disease is linked with increased HIV-1 RNA concentrations, reduced $CD4^+$ cell counts and faster progression [21]. What precise mechanisms control this switch in co-receptor activity is still poorly understood, but many studies have addressed this by comparing envelope sequences. The most obvious genetic alterations occur in the variable regions of the envelope. These alter both charge and potentially N-linked glycosylation patterns [22-25]. These alterations in N-linked glycosylation influence the extent to which effective neutralizing antibodies can bind to HIV-1 and thus prevent infection [22-28]. In all likelihood the diversity within the gp120 variable regions are contributing to both co-receptor activity and neutralizing antibody escape providing for the evolution observed during disease progression.

We have identified two families within which the siblings demonstrate major differences in HIV-1 RNA loads, $CD4^+$ cell counts and disease progression (as monitored through initiation of therapy) and who have in all likelihood been infected with viruses from a similar source. An increased insight in the genetic make-up of the transmitted HIV-1 strains can provide information on viral as well as host factors that can be associated with HIV-1 disease progression.

RESULTS

Subjects and their disease description

Both families were from central Africa but of different ethnicity. From family 1 the father's samples were not available for analysis. The mother (M1) when first seen at the clinic possessed a high HIV-1 RNA load ($\log_{10}$4.91 copies/ml) (Fig. 1a) and low $CD4^+$ cell count (20 cells/ml^3) (Fig. 1c). She was diagnosed with a WHO B3 classification and ART was initiated in March 2000 (IDV/3TC/d4T) and switched regime in August of the same year (NVP/3TC/AZT). This therapy subsequently failed due to non-compliance and a new regime was initiated resulting again in reduced viral loads after which viral blips were observed in 2003 and 2005 (Fig. 1). The mother experienced a slow but sustained increase in $CD4^+$ cell counts during her treatment period. Her first child (C1A), was considered to be a slow progressor ($\log_{10}$2.06 virus copies/ml and 780 $CD4^+$ cells/ml^3) at study entry. C1A was asymptomatic (WHO classification A) with no requirement for treatment. The subsequent viral load rose steadily over the following years while $CD4^+$ cell counts remained steady (Fig. 1a and b). The second child (C1B) was born five years after C1A and was diagnosed with a WHO classification B HIV-1 infection when first seen at the clinic. This child's baseline viral load was moderate ($\log_{10}$4.26 copies/ml) with high $CD4^+$ cell counts (680 cells/ml^3) and the patient was started on combination ART with nelfinavir/d4T/3TC, after which the viral load became undetectable with occasional blips (Fig. 1a). C1B demonstrated a sustained drop in $CD4^+$ cell counts over the years despite successful therapy (Fig. 1c) and demonstrated signs of progressing.

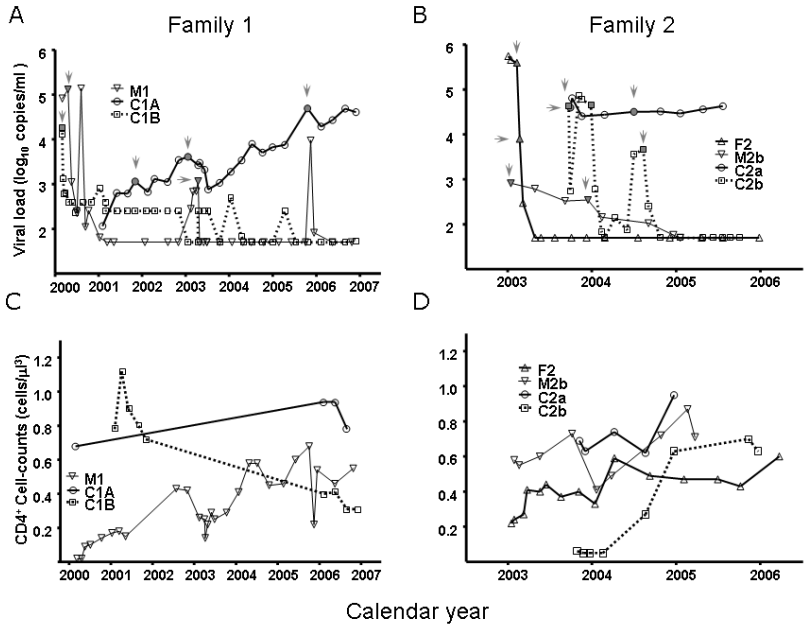

Figure 1.
Viral load (**A**, **B**) and $CD4^+$ cell counts (**C**, **D**) of family 1 (**A**, **C**) and family 2 (**B**, **D**). The calander year is shown in axis X. The full labels with the grey arrows in (**A**) and (**B**) indicate the time points of which the gp120 envelope virus RNA was isolated and the sequence was analyzed.

In family 2 the father 2 (F2) presented with a high viral load (log_{10}5.74 copies/ml) and low $CD4^+$ cell counts (220 cells/ml^3) (Fig.1b and d) and received successful ART. There were no records from his first wife (M2a) who presumably died of AIDS. The second wife (M2b) had low viral load (log_{10}2.91copies/ml) with high $CD4^+$ cell counts (580 cells/ml^3) and was considered a non-progressor. The first child (C2a) was born in 1991 to M2a (first wife) and was first seen in the clinic with a moderate viral load (log_{10}4.59copies/ml) and high $CD4^+$ cell counts (690 cells/ml^3) (WHO classification A2). No treatment was initiated and the viral load burden remained stable during the entire observation period (Fig. 1b). The second child (C2b) born to M2b two years after C2a had a similar viral load as C2a (log_{10}4.64 copies/ml) but with a much lower $CD4^+$ cell counts (60 cells/ml^3) when first seen at the clinic. C2b received ART (Kaletra/AZT/3TC) that successfully reduced viral loads throughout follow up (Fig. 1b).

All the children were HLA typed for their class A, B and C alleles but we did not observe any association with those alleles known to be involved with risk of transmission or alteration in disease progression [13,14] (data not shown). HLA data were not available for M2b who is clearly controlling viremia (Fig. 1b).

Phylogenetic analysis and genetic diversity

We chose to compare gp120 region sequences of viruses isolated from all study individuals. Phylogenetic inference utilizing the Kimura-2-parameter/neighbor joining model showed that the members of family 1 were infected with an HIV-1 subtype C virus equidistant within the topology of the overall subtype C HIV-1 phylogeny (Fig. 2). Family 2 harboured the CRF-01 recombinant form of HIV-1 that is more common in Southeast Asia and infrequent in central Africa. We therefore determined that all infected members within each family carried a virus of common origin. This analysis also confirmed that M2a and M2b had to have

been infected with a virus similar to F2, since C2a and C2b were also infected with closely related F2 strains, despite having different mothers.

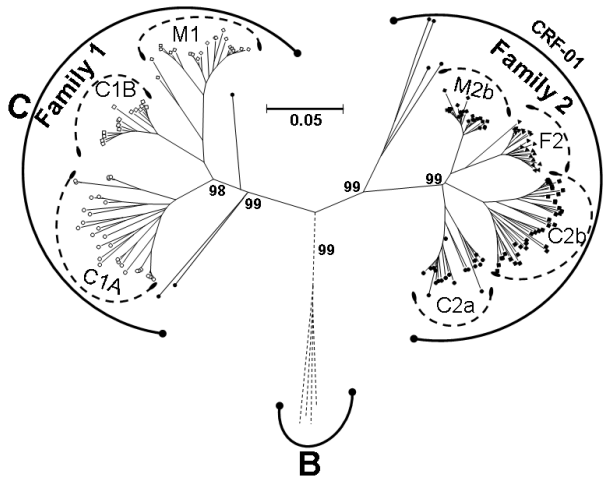

Figure 2.
Phylogenetic analysis of the envelope sequences isolated from the members of family 1 (M1, C1A and C1B) and family 2 (F2, M2b, C2a and C2b). The virus strains isolated from family 1 form a monophyletic group, infected by a subtype C virus strain. Family 2 harbors a CRF-01. Subtype reference sequences from the Los Alamos database [http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html] were also included and are indicated doted lines. The lines with the round black symbols are same subtype reference sequences as those circulating in each family taken from the Los Alamos HIV-1 database.

Greater diversity was observed amongst the viral envelope sequences in the children compared to the parents when the study began (ie arrival at the clinic) (Fig. 3a). However, no differences in sequence diversity were observed between the study subjects at this time based on requirement for ART initiation or not (Fig. 3b). The virus diversity did not correlate with viral load values (Fig. 3c), indicating that when the study began there was no association between intra-patient virus diversity and disease status.

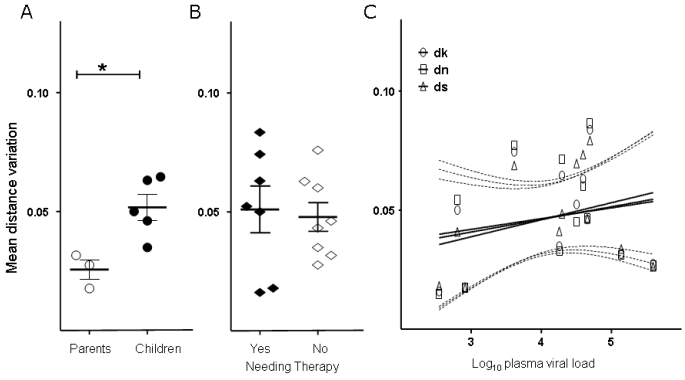

Figure 3.
Mean nucleotide variation of the gp120 envelope fraction of the virus strains: (**A**) comparison of the parents' strains, open circles, and their children, closed circles, (**B**) comparison of family members requiring therapy with those that did not. The analysis was performed using the using the neighbor-joining algorithm and the kimura-2-parameter method of the MEGA software package (*P<0.05). (**C**) Correlation of the plasma viral load to the mean nucleotide variation: dk is the overall diversity by the kimura-2-parameter method, dn is the non synonymous nucleotide variation and ds is the synonymous nucleotide variation.

V3 loop amino-acid analysis

We analyzed the gp120 sequences to identify associations with clinical outcome in those patients with varied disease status but who were infected with related virus strains. Initially we studied the overall predicted positive charges of the V3 region for each family member. In family 1, M1 harboured mainly +4 V3 charges at base-line and prior to receiving therapy. Child C1A had HIV viruses with lower V3 charges, mainly +2, which gradually increased over time, whilst C1B had viruses of a +3 charge at initial analysis. In family 2, the V3 charges of the father's HIV viruses ranged from +3 to +5 with the majority being +4. Mother M2b harboured viruses with lower V3 charges with the majority being +2 at the first time-point analyzed with attenuated charges of +1 a year later, coinciding with a lower viral load without therapy. When analyzing the children, C2a had mainly viruses with +2 charges with some being +3. The other child C2b possessed viruses with higher charges ranging from +3 - +6 and this was the child receiving ART. For this child we also observed that other V3 alterations were common, such as loss of a potential N-linked glycosylation site (PNGS) at the stem of the V3 loop (Fig. 4a), which had previously been associated with a switch in the R5 to X4 phenotype. Furthermore, virus strains from C2b showed a gain in positive amino acid charge at position 11 (E→S→K), and loss of negative charges in positions 25 and 29 that are also associated with a switch in coreceptor usage. The existing algorithms for predicting co-receptor usage have not been applied here since they are CRF-01 strains. When the WebPSSM bioinformatic tool was applied to viral sequences obtained from family I (infected with subtype C) all viruses were predicted to utilize the CCR5 co-receptor.

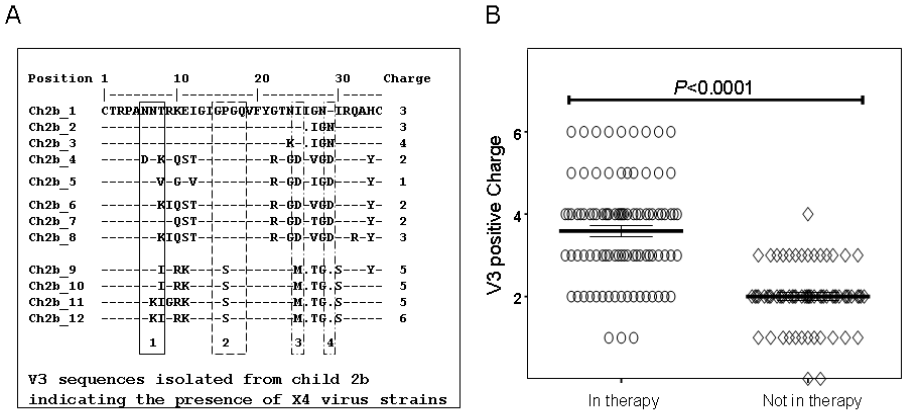

Figure 4.
V3 positive charge analysis: (**A**) Representative V3 loop virus sequences found in three time points analyzed of the Child C2a. The loss of the PNGS at position 9, changes at the GPGQ motif, the gain of a positive charge (K) and the loss of negative charges (D), shown in bold, are indicating the presence of X4 strains. The column at the right indicates the V3 positive charge. The numbers below are indicating, 1 the PNGS, 2 the GPGQ crown-motif, 3 and 4 the negative charge positions. (**B**) V3 positive charge comparison between family members requiring therapy with those that did not. Family members in both families requiring therapy had significantly lower V3 positive charges as demonstrated by the Mann-Whitney test.

In general, those individuals who did not receive ART tended to possess virus variants with the lowest positive V3 charges. With high V3 charge, the individual tended to possess higher HIV-1 viral loads or lower $CD4^+$ cell counts. Furthermore higher V3 positive charges were observed in family members that were in need of ART in comparison to those that did not (Fig. 4b, $P<0.0001$). The

difference remained significant when C2b sequences, suspected of CXC4 coreceptor usage, were excluded from the analysis (P<0.0001). Interestingly C1A, who initially harboured viruses with low viral loads but which were shown to steadily increase over time, demonstrated a corresponding increase to the V3 charge. M2b who presented with very low viral loads and which dropped over time in the absence of therapy showed a decrease in the V3 loop positive charge.

Amino-acid length and N-linked glycosylation analysis

We wished to identify whether alterations in the amino acid lengths of the various g120 envelope regions or alterations to the number of PNGS would be associated with the disease status of the two family members. We analyzed the V1V4 sequences isolated and observed a marked difference between the two families when the amino acid lengths were plotted against the number of PNGS. Overall there was a strong positive correlation (P<000.1) between the amino acid length and number of PNGS (Fig. 5a). However, when the sequences were reanalyzed, taking into account who received therapy and who did not, we found differences between family members requiring ART. A strong positive correlation between amino acid length and number of PNGS was observed in those not requiring therapy (P<000.1/r^2=0.7681) while the correlation was poor for those who were in need of ART (P=0.0044/r^2=0.0952) (Fig. 5a). We calculated and compared the ratio of amino acid length to number of PNGS (aa/PNGS) between

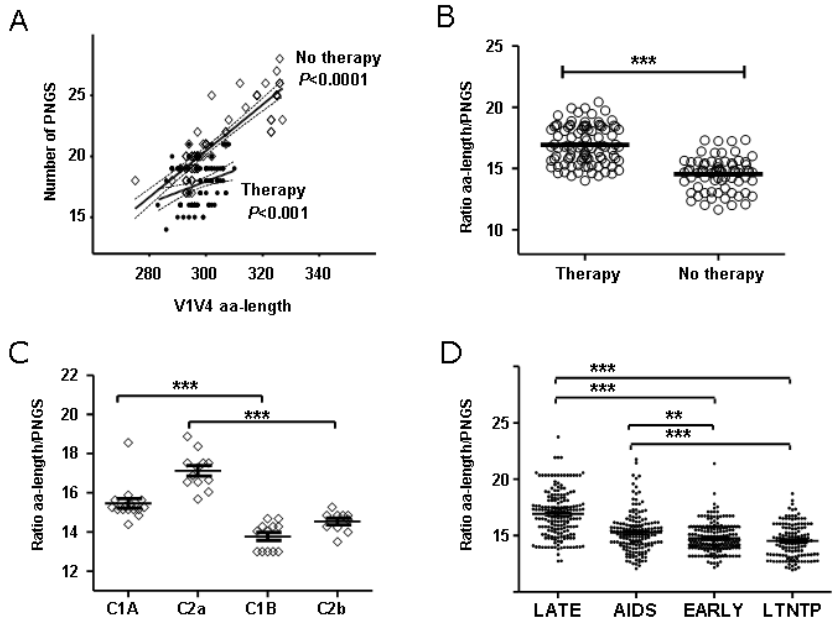

Figure 5.
Ratio of amino-acid length of the V1V4 fraction of the envelope gene to the number of PNGS. (**A**) Correlation between the V1V4 amino-acid length and the number of PNGS of family members receiving therapy (closed symbols) and those not (open symbols). (**B**) Comparison of the ratio of V1V4 amino acid length to number PNGS between family members requiring therapy with those that did not (Mann-Whitney applied). (**C**) Base line comparison of siblings that progressed and those that did not (Mann-Whitney applied). (**D**) Comparison of the ratio of V1V4 amino-acid length to number PNGS of sequences taken from the Los Alamos data-base collected either 'early' or 'late' in infection, from individuals that have progressed in disease (AIDS) and those that were infected for a long period without progressing (LTNP) (**P<0.001 and ***P<0.0001).

family members requiring ART and those not and found those requiring ART possessed a significantly higher ratio (Fig 5b). When the analysis was performed including only the HIV-1 virus sequences obtained from children at base-line equally significant differences were found between C1B and C2b (fast disease progressors) and C1A and C2a (slow progressors) (P<000.1) (Fig. 5c). We conclude from the above that differences in the ratio between the length and the number of PNGS associate with disease progression.

In addition to the HIV-1 gp120 sequence analysis of the virus strains isolated from the study participants we extracted and analyzed gp120 sequences from the Los Alamos HIV-1 sequence database. We compared the sequences obtained during early infection to those obtained late. Our assumption being that early sequences were collected from individuals that had not yet progressed in their disease whilst late sequences were from individuals who had. We calculated the V1V4 amino acid length to number of PNGS and compared the two groups (Fig. 5d). Early sequences showed a lower ratio when compared to sequences collected later in disease from individuals expected to have progressed in their disease course (P<0.0001). Furthermore, comparison of early sequences with those obtained from individuals whom had AIDS also revealed a difference, although not as pronounced (P=0.001) as seen with late patients, likely due to the loss of immune control hence selection pressure occurring in AIDS. We also studied long-term non-progressors (LTNPs) and found a difference in comparison to sequences from late patients (P=0.001), but no difference with viruses found early in disease. This analysis supports the findings obtained from the sequence comparisons from the two families studied here indicating that the aa/PNGS ratio is associated with disease progression.

DISCUSSION

Here we describe individuals infected with genetically related HIV-1 strains and who have progressed differently in their disease course. We study members of two families with variant ethnicities, although both from sub-Saharan Africa, one which are infected with different HIV-1 subtypes which are genetically distinct (C and CRF01-AE). Through comparing viral gp120 envelope sequences from progressors and non-progressors in both families we find similar findings for both subtypes.

Numerous reasons have been proposed to explain why disease progression within HIV-1 infected individuals is so varied. Host genetic factors have been associated with variant disease course, such as co-receptor availability [10] or HLA allelic variation [29]. A few studies have associated virus characteristics with slower progression such as a defective *nef* gene [17] or decreased HIV-1 replication capacity [30]. Nevertheless, few studies have investigated genotypic differences of viruses circulating in individuals with varied progression. The vast sequence diversity of the HIV-1 renders such studies difficult and often there is no apparent consistency in the trends observed, with a lack of clearly identifiable patterns of amino acid sequence changes among patients [29]. Here the individuals studied were infected with genetically related virus strains rendering such comparisons possible despite the small number of study subjects included. The fact that the study participants were originally infected with genetically related viruses means that relationships can be drawn between subsequent viral evolution and markers of infection and course of disease progression in sibling pairs.

The overall V3 loop positive charge has been linked with co-receptor switch and this has been associated with faster disease progression. Our study is small in size but we have identified increased V3 positive charges in the viruses circulating in the members that have progressed in their disease and for both families. We could not determine whether viruses with higher V3 positive charge have switched co-receptor usage phenotypes from R5 to X4 but with the exception of child C2b this is unlikely. In family 1 the members are infected with HIV-1 subtype C which is characterized by a very low frequency of R5 to X4 phenotype switching [31,32] and from the study of large numbers of isolates such charge increases have not been related with a switch in coreceptor usage [24]. In family 2 the members harbour a CRF01-AE virus which has been more associated with corecepor switching potential, but with the exception of child C2b all members had V3 positive charges of +4 or less without loss of the V3 stem glycosylation, again highly indicative of CCR5 utilization [24]. In conclusion our data suggest that V3 charge increase is associated with faster disease progression and is observed in both families. It should be noted that it is not possible to state whether the increase in overall V3 positive charge leads to disease progression or whether disease progression attenuates the immune defences allowing the virus to evolve towards higher charges.

It has previously been described that the length of the V1V2 region can vary during the course of HIV-1 infection influencing the ability of the virus to infect CCR5 expressing cells or alter the potential for antibody directed neutralization [26]. In this study we have demonstrated an association between disease progression and the length of the envelope protein together with the number of PNGS. The aa length or the number of PNGS varied between the study subjects and showing no association with disease progression. It could be that these parameters are strain dependent or that they evolve differently in separate hosts, depending on the potency of the immune responses mounted. When we calculated the aa length/PNGS ratio we could identify differences for subjects progressing versus not progressing in their disease course. This indicates that V1V2 length and glycosylation are interdependent parameters each contributing to the plasticity of the virus, potentially providing advantages for evading host restrictions. Again, the fact that individuals from one family were all infected with virus strains of common origin indicates that the host's environment can drive viral evolution which results in variant disease outcomes. In a previous study identical twin brothers infected with HIV-1 at the same time showed parallel progression [33]. Other studies, investigating fraternal and identical twins, have shown that HIV-1 infected individuals can be born with very similar viral loads with subsequent envelope sequence divergence being dependent on quasi-species stability [26,34]. In our study the two sets of family members have variant compositions with mother M1 progressing whilst M2b controlled disease. Among the two sets of siblings, C1A and C1B demonstrated discordant viral loads at baseline while C2a and C2b showed comparable plasma loads. In both families the second child progressed which has been reported in other studies [33,35]. Most viral genes have been associated with varied disease progression and in a previous study of slow progressing sibling's modifications in viral *rev* has been postulated to associate with disease progression [34,36].

Here we have identified that HIV-1 originating from the same gene pool can evolve in very different ways within individuals that are close relatives. These individuals have varied disease course, however, it is unclear whether subtle differences within the incoming virus strain, the host genetic background or the time of infection determine the final clinical outcome. What is unclear from this

study, but would be pertinent to answer, is which variant immune responses or host actors can drive the virus to evolve in the specific directions described. Both the variable loop aa length and glycan numbers have been shown to play a role in HIV-1 immunity [8,26,37], a role that may be lost in late stages of the disease when immune responses are impaired [26,38]

The comparison of gp120 sequences from progressing versus non-progressing individuals demonstrated that the ratio between the amino-acid length and the number of PNGS differs in such individuals. The analysis of gp120 sequences from the Los Alamos database supported this finding although in the latter analysis we made the assumption that sequences collected late or early corresponded to individuals that had subsequently progressed or not. The gp120 variable domains can tolerate up to 35% divergence [35,36] especially within the variable domains with dramatic insertions, deletions and varied glycosylation patterns identified [27] and these changes have also been associated with neutralization resistance against autologous plasma [39-41]. Here we show that in terms of disease progression there is a strong interplay between the envelope characteristics described (amino-acid length and number of PNGS) and in all likelihood represent variations in host induced immunity. The better understanding of these differences, even within closely related individuals (such as siblings), needs to be deciphered to aide in the development of future HIV-1 vaccines that need to induce broad protective immunity covering many viral variants in unrelated hosts.

MATERIALS AND METHODS

Study subjects

We studied HIV-1 infection in two families where the members (parents and children) have been infected for ten years or more and some members had progressed while other had not. Both families are of African origin and in each family two infants were HIV-1 infected, presumably with related virus strains, but with variant viral loads and $CD4^+$ cell counts (Fig. 1). In both families, the youngest child had progressed and required antiretroviral treatment upon their visit to the clinic. Time of seroconversion is not known for the parents and it is assumed that all children were vertically infected by the mother either *in-utero* or *intra-partum*. Blood samples were collected between 2001 and 2007 with consent obtained from participating parents and on behalf of their infants with all experiments conducted under medical ethical review relevant to the Amsterdam Cohorts Studies.

Viral RNA amplification and sequence analysis

For all individuals, longitudinal plasma samples were available with time points as indicated (Fig. 1). The viral genomic RNA was isolated as described (Boom *et al.*, 1990). A region of the gp120 *env* was reverse-transcribed utilizing SuperScript® III Reverse Transcriptase (Invitrogen) and amplified under standard PCR conditions with AmpliTaq® DNA Polymerase (Appliedbiosystems). The outer primers corresponding to the HXB2-positions 6203-6228 and 7834-7863 and the inner primers corresponding to the HXB2-positions 6540-6562 and 7789-7810 were used for the amplification. The PCR-amplicon was cloned into the TOPO-TA vector (Invitrogen) and bacterial colonies carrying the HIV-1 *env* region were screened and sequenced (between 10 and 16 per time point). Nucleotide sequences were aligned in respect to the predicted amino-acid

sequence of the corresponding reference-alignment extracted form the Los Alamos HIV database (http://www.hiv.lanl.gov/content/hiv-db/ALIGN_CURRENT/ALIGN-INDEX.html).

Phylogenetic analysis was performed using MEGA version 3.0 (Kumar *et al.*, 2004) and confirmed by the DNADIST, NEIGHBOR, and DRAWTREE options of the PHYLIP software package (http://evolution.Genetics. Washington.edu /phylip.html). The distance matrix was generated by Kimura's two-parameter estimation (Kumar *et al.*, 2004). The number of potential N-linked glycosylation NX[S/T] positions was determined using the GLYCOSITE link (http://www.hiv.lanl.gov/content/hiv-db/GLYCOSITE/glycosite.html) provided through the Los Alamos site (Zhang *et al.*, 2004). The WebPSSM bioinformatic tool (http://indra.mullins.microbiol.washington.edu/pssm/) was applied to predict HIV-1 co-receptor usage patterns (Jensen *et al.*, 2003).

Sequences of the V1-V4 region of the gp120 envelope protein were extracted from the Los Alamos HIV-1 sequence database (http://www.hiv. lanl.gov/components/sequence/HIV/-search/search.html) for comparative analysis. We defined 4 groups according to timing of infection, early (*N*=227) or late (*N*=191) and progression, namely AIDS (*N*=176) or LTNPs (*N*=143) as defined in the patient information section of the website.

$CD4^+$ T cell-count and viral load analyses

All subjects were tested for HIV-1 antibodies using the HIVSPOT assay (Genelabs Diagnostics, Singapore) and Vironostika ELISA (Organon, The Netherlands). Results were confirmed by Western Blot (HIV Blot 2.2, Genelabs Diagnostics, Singapore). Blood was drawn at six month-intervals. $CD4^+$ T-lymphocyte count was carried with standard fluorescent activated cell sorting (FACS), using commercially available fluorescent-labelled antibodies (Becton-Dickinson Immunocytometry, CA, USA). HIV-RNA was measured with the bDNA 3.0 assay (Bayer Diagnostics, California, USA).

Statistical analyses

Descriptive statistics were performed utilizing the Prism package software (GraphPad software Inc., San Diego, Calif. version 4.0) (http://www.graphpad.com/prism/Prism.htm). The tests performed were two tailed and chosen upon the outcome of the normality test and are indicated in Figure legends.

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Chapter 3

Effect of chloroquine on reducing HIV-1 replication *in vitro* and the DC-SIGN mediated transfer of virus to $CD4^+$ T-lymphocytes

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Retrovirology (2007) 30;4:6.

ABSTRACT

Background: Chloroquine (CQ) has been shown to inhibit HIV-1 replication *in vitro* as well as *in vivo* and has been proposed to alter the glycosylation pattern of the gp120 envelope. These activities indicate that the compound can be used not only as an effective HIV-1 therapeutic agent but also as a modulator of the gp120 envelope protein structure enabling for the production of broader neutralizing Ab responses. Results: We confirm here that HIV-1 replication in $CD4^+$ T-lymphocytes can be reduced in the presence of CQ and show that the reduced replication is producer cell mediated, with viruses generated in the presence of CQ not being inhibited for subsequent infectivity and replication. By analysing the gp120 envelope protein sequences from viruses cultured long-term in the absence or presence of CQ we demonstrate variant evolution patterns. One noticeable change is the reduction in the number of potential N-linked glycosylation sites in the V3 region as well as within the 2G12 Ab binding and neutralization epitope. We also demonstrate that HIV-1 produced in the presence of CQ has a reduced capacity for transfer by Raji-DC-SIGN cells to $CD4^+$ T-lymphocytes, indicating another means whereby virus transmission or replication may be reduced *in vivo*. Conclusions: These results indicate that CQ should be considered as an HIV-1 therapeutic agent with its influence exerted through a number of mechanisms *in vivo*, including modulation of the gp120 structure.

INTRODUCTION

The anti-malarial drug chloroquine (CQ) and its hydroxyl analogue hydroxychloroquine (HCQ) have both been shown to inhibit the *in vitro* replication of HIV-1 and HIV-2 [1]. The low cost and wide-availability in resource restricted settings make them prime candidates as antiretroviral agents, most likely to be used in conjunction with other anti-HIV-1 medications. A previous report has indicated that CQ may mediate its effect through modulating glycosylation patterns of the HIV-1 gp120 envelope protein [2]. Since HIV-1 neutralizing Ab responses can be modulated by alterations in the potential N-linked glycosylation (PNG) sites of gp120 [3-5], CQ and HCQ may therefore have the beneficial effect of changing the immunogenicity of the molecule and induce a broader Ab response.

The HIV-1 inhibitory effect of CQ and HCQ is likely mediated by variant properties of the drugs. As a weak base CQ is known to increase pH in lysosomal and trans-Golgi network vesicles [6], thereby disrupting the cellular acid hydrolase enzymes and altering the level of post-translational modification of newly synthesized proteins and reducing the level of gp120 glycosylation. The cellular endosomal pH has also been shown to be increased through CQ treatment which can lower IL-6 synthesis [7]. Down-modulation of IL-6 has been shown to diminish HIV-1 production from chronically infected T-cells and monocyte cell-lines [8], providing an additional HIV-1 suppressing effect. CQ has also been shown to decrease Tat-mediated transactivation of the HIV-1 LTR *in vitro*, thereby decreasing HIV-1 production [9].

Dendritic cells (DCs) have been implicated to play an important role in the transmission of HIV-1 and the establishment of infection through capturing virus and enhancing infection of $CD4^+$ T-lymphocytes [10-12]. DC-SIGN has been shown to specifically interact with HIV-1 and allow for the enhancement to infection [13-15], although an array of C-type lectins have been postulated to perform the same function [16,17]. The interaction of HIV-1 with DC-SIGN can

lead to either infection of DCs or internalization of the virus and subsequent transfer [18;19]. The interaction of HIV-1 and DC-SIGN is mainly dependent on the glycosylation of gp120 and in particular the V3 region of the protein [20].

Several clinical trials have been performed where CQ or HCQ was given to HIV-1 infected individuals. In one study a decrease in HIV-1 viral load measurements was observed [21] whilst in another a decrease in plasma CA-p24 levels was noted in comparison to the control group [22]. No alterations to $CD4^+$ T-lymphocyte counts were identified in either study. In one trial a decrease in IL-6 and immunoglobulin G levels were found, suggesting a further means whereby HIV-1 viral loads can be modulated [22].

RESULTS

Inhibition of HIV-1 replication by CQ

To confirm that CQ has an inhibitory effect on the *in vitro* replication of HIV-1 we separately cultured an R5 (JR-CSF) and X4 (LAI) virus on $CD4^+$ T-lymphocytes and monitored replication in the presence of variant concentrations of CQ (200, 100 and 50 μM). We observe that CQ inhibits the replication profile of both viruses in comparison to the control cells (Fig. 1). When comparing the dose dependent inhibitory effect of CQ on viral replication the R5 virus (Fig. 1A) appears more sensitive than the X4 virus (Fig. 1B), suggesting a co-receptor phenotype restriction to inhibition by CQ. The observed inhibition by CQ was not due to enhanced cell death since cell counts and viabilities were identical in the 100 and 200 μM CQ cultures to the non-CQ treated control cells during one week of culture (data not shown).

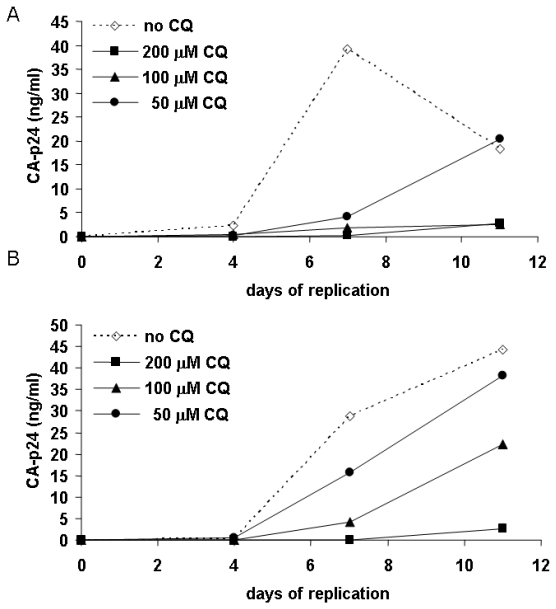

Figure 1. Viral replication in the presence of CQ
(**A**) JR-CSF (R5) virus (**B**) LAI (X4) virus replication was monitored in the presence of 200 μM, 100 μM, 50 μM of CQ or in the absence of CQ. Viral input for the replication assay was 100 $TCID_{50}$/ml with the CA-p24 concentration determined during the course of the infection.

In order to determine whether the inhibitory effect of CQ was mediated through altered infectivity of generated virus particles we analyzed replication on $CD4^+$ T-lymphocytes of HIV-1 produced in C33A cells pre-treated with 100 μM CQ. The viruses JR-CSF (R5), 299.10 (R5/X4) and LAI (X4) produced from cells not treated with CQ showed higher CA-p24 levels than viruses produced from cells treated with CQ (data not shown). When we studied the replication kinetics of the viruses away from CQ with a set CA-p24 viral input there was no difference in replication of the viruses generated in the presence or absence of CQ (Fig. 2). $TCID_{50}$/ml values were identical for all three viruses generated in the presence or absence of CQ (data not shown). These results indicate that viruses produced in the presence of CQ are as equally infectious as those produced in its absence and that the effect of the drug on lowering CA-p24 production is mediated at the cellular level.

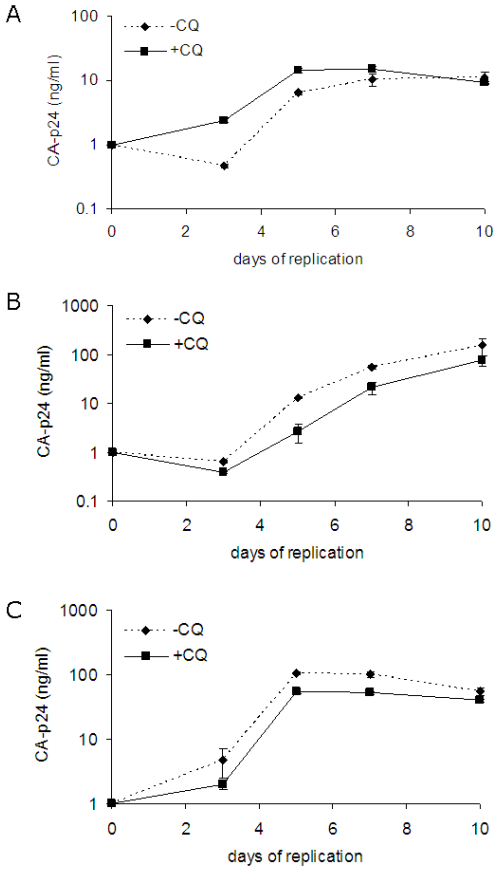

Figure 2. Viral replication of C33A produced viruses in the presence of CQ
(**A**) JR-CSF (R5) replication, (**B**) 299.10 (R5X4) replication and (**C**) LAI (X4) replication. All three viruses were produced by transfection of C33A cells pre-treated with 100 μM CQ or in its absence as a control. The replication capacity of the produced viruses were determined on $CD4^+$ T-lymphocytes in the absence of CQ. CA-p24 at 1 ng/ml was used as viral input with the CA-p24 concentration determined during the course of the infection. Standard deviations are depicted in all panels. All replications were performed in triplicate.

Prolonged culture in the presence of CQ does not alter replication of HIV-1

To monitor the effect of long-term culturing of HIV-1 in the presence of CQ we passaged virus 293.10 (R5X4) for 30 weeks either in the absence or presence of CQ (100 μM). Each week, or when CA-p24 levels were sufficiently high, a set viral load (15 ng/ml) was transferred to fresh $CD4^+$ T-lymphocytes and cultured. CA-p24 production was consistently lower for the virus passaged in the presence of CQ compared to the control passaged virus (Fig. 3), even after 206 days of culture (30 passages). These results demonstrate that HIV-1 does not evolve to escape the inhibitory effects of CQ.

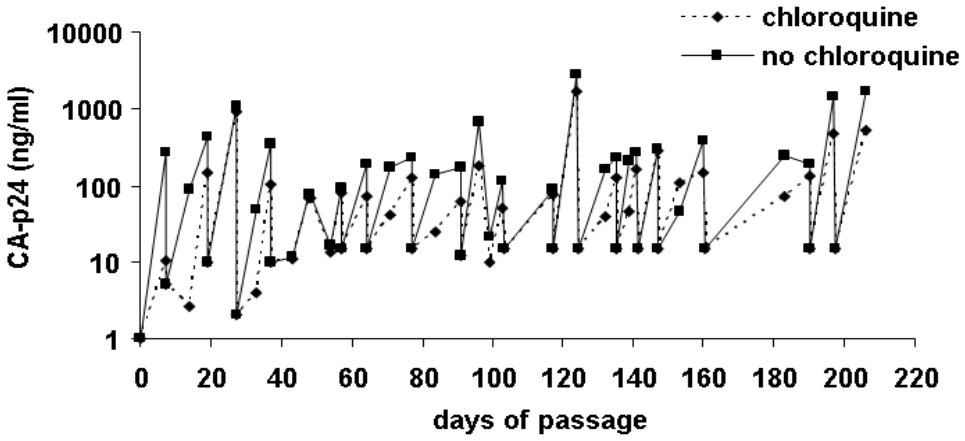

Figure 3. Prolonged passage of HIV-1 in the presence of CQ
An R5X4 virus (293.10) was cultured for 206 days in the presence or absence of CQ (100 μM). The concentration of CA-p24 was determined in culture supernatants on either day 7 or 10 of culture and 15 ng/ml CA-p24 was added to fresh $CD4^+$ T-lymphocytes. The culture was monitored for CA-p24 and the culture in the absence of CQ is depicted with a solid line and the culture in the presence of CQ is depicted as a broken line.

Since we have shown previously that viral replication was not altered after HIV-1 production on C33A cells in the presence of CQ we wished to identify whether this was the same for the long-term cultured virus stocks. The replication profile of harvested viruses from various time-points during the passage in the presence or absence of CQ was determined on $CD4^+$ T-lymphocytes (Fig. 4). The replication at day 37 showed an increase in replication for the CQ passaged virus population versus the non-CQ treated culture (Fig. 4A). On the contrary for day 77 (Fig. 4B), day 103 (Fig. 4C), day 147 (Fig. 4D), day 183 (Fig. 4E) and day 206 (Fig. 4F) no differences in replication between the CQ passaged viruses and the non-CQ passaged viruses were observed. These results indicate that there is no difference in replication of HIV-1 after long-term culture in the presence or absence of CQ, although the virus from the CQ treated day 37 culture showed an enhanced replication over the non-CQ treated stock. The fact that the later viruses did not show such an increase indicates that the result observed for the day 37 CQ passaged virus was most likely due to experimental variation and reflects the poor infectivity of the viruses from that time-point. However, the main finding is that CQ did not diminish the replication

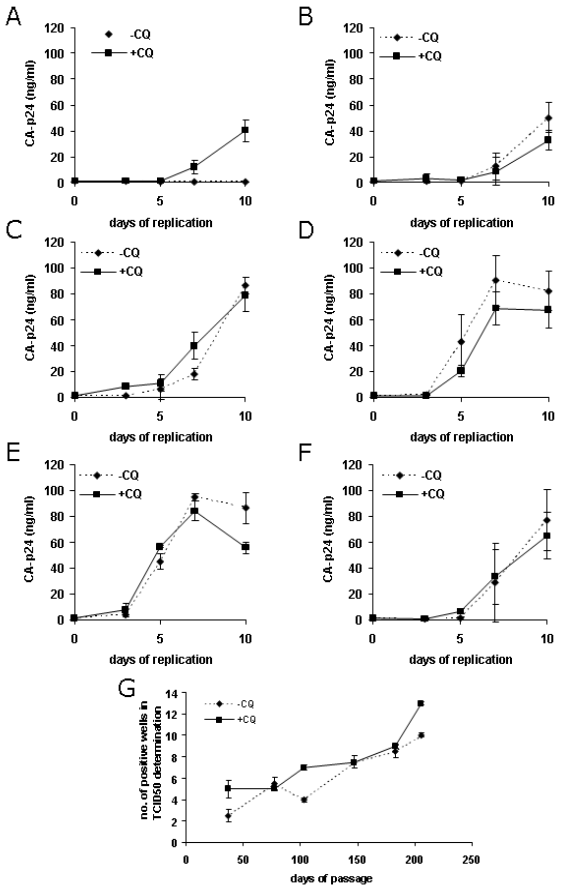

Figure 4. Replication of CQ passaged virus
The replication of CQ passaged or the control passaged 293.10 viruses were tested for their replication in the absence of CQ. CA-p24 or 1 ng/ml was used as input for monitoring replication (**A**) day 37 of passage, (**B**) day 77 of passage, (**C**) day 103 of passage, (**D**) day 147 of passage, (**E**) day 183 of passage and (**F**) day 206 of passage. Standard deviations are depicted in all panels. All virus replications were performed in triplicate. (**G**) Determination of $TCID_{50}$/ml values of passaged viruses in the absence or presence of CQ. Viral infectivity of the viruses passaged in the absence or presence of CQ (days 37, 77, 103, 147, 183 and 206) was measured on $CD4^+$ lymphocytes. Standard deviations are depicted.

capacity of HIV-1. $TCID_{50/ml}$ values were determined for stocks generated on days 37, 77, 103, 147, 183 and 206 during the prolonged passage in the absence or presence of CQ. Both culture conditions demonstrated an increased infectivity of virus over time (Fig. 4G), indicating that viruses in the presence of CQ adapt as efficiently as non-CQ treated cultures. This again reiterates that CQ exerts a cellular restriction to viral production and not a direct effect on viral infectivity.

Sequence analysis of the viruses passaged in the presence of CQ

A previous study has suggested that CQ can modify the PNG patterns of the gp120 envelope [2]. We therefore wished to determine whether HIV-1 passaged in the presence of CQ had a similar gp120 envelope sequence to virus passaged in the absence of CQ. DNA sequence analysis of a number of cloned PCR

products of gp120 identified that the overall amino acid charge of the V1V2 region (Fig. 5A) is significantly higher for the CQ passaged virus compared to the

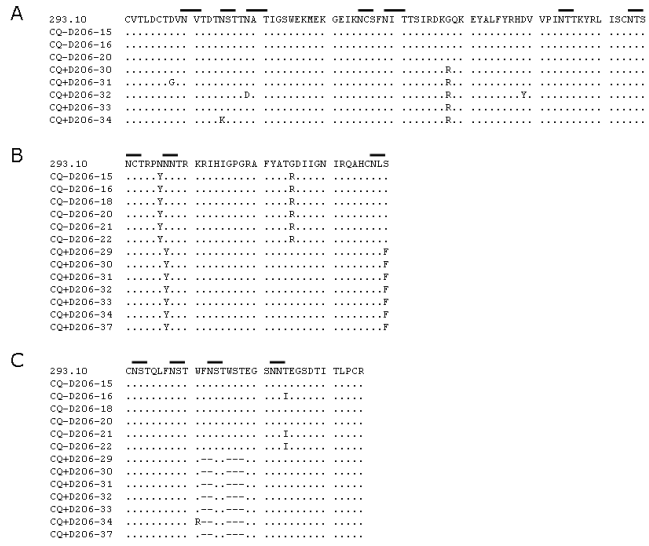

Figure 5. Sequence analysis of passages viruses in the presence or absence of CQ
HIV-1 RNA was isolated from culture supernatant and viral RNA was converted to cDNA and then subjected to a nested PCR in order to amplify a fragment covering the V1V2 - C4 region of the gp120 gene. Sequence analysis was performed on several clones of the CQ and control passages. The sequence of the original virus 293.10 is shown. (**A**) V1V2 region. (**B**) V3 region including the PNGS at the base of the loop. (**C**) V4 region. The black lines above the original sequence represent PNG sites.

control passage (P = 0.001), or the original virus (P = 0.001) (Table 1). On the contrary, the overall positive charge of the gp120 V3 region (Fig. 5B) is significantly lower (P < 0.0001) in the CQ passaged virus but equal to the original virus (Table 1). A significant decrease in V4 region length (Fig. 5C) is also identified in the CQ passaged virus in comparison to the control (P < 0.0001), or the original 293.10 virus (Table 1). Of particular interest is the observation that the PNG profile of the V3 region (Fig. 5B) was significantly reduced after passage of the 293.10 virus in the presence of CQ with the virus reducing the number of PNG sites in V3 region from 2 to 0 (P < 0.0001), whilst in the non-CQ treated culture it is reduced from 2 to 1.7 (Table 1). Overall the sequence analysis reveals that there are differences in the envelope sequences of viruses cultured in the presence of CQ that may have an influence on the virus phenotype or the immunogenic properties of gp120.

Prolonged passage of HIV-1 in the presence of CQ results in a loss of PNG sites important for 2G12 binding

We compared the gp120 sequences of the passaged viruses with what is known for the 2G12 binding site, a monoclonal Ab with broad neutralizing activity against HIV-1 subtype B isolates. This antibody has a known PNG component to its recognition epitope [23]. For the virus passaged in the presence of CQ we

Table 1 Sequence comparison between the passage of 293.10 for 206 days with or without CQ

			day 206 of passage		
	gp120 region	**293.10**	**CQ (#)**	**Control (#)**	**P value**
Charge	V1V2	0	1.33 (±0.52)	0 (±0)	0.001 *
	V3	4	4 (±0)	4.85 (± 0.36)	< 0.0001 *
	V4	-2	-2 (± 0)	-2 (± 0)	equal
Length	V1V2	76	76 (± 0)	76 (± 0)	equal
	V3	36	36 (± 0)	36 (± 0)	equal
	V4	28	23(± 0)	28 (± 0)	< 0.0001 *
no.of N-glycosylation sites	V1V2	7	6.67 (± 0.52)	7 (± 0)	0.24
	V3	2	0 (± 0)	1.69 (± 0.75)	< 0.0001 *
	V4	3	2 (± 0)	2.5 (± 0.58)	0.09

charge, length and potential N-glycosylation sites were determined from the amino acid sequences
Standard deviations
* P values are considered significant

observed a loss of two PNG sites (332 and 397) that have been shown to express carbohydrates important for 2G12 binding [23], as well as an additional site in the V3 region of gp120 (data not shown). The PNG site expressing carbohydrates involved in 2G12 binding (397) is lost in the V4 region due to a deletion of 5 amino acids. Loss of a PNG site in the V4 region is also observed in the control passage (Table 1) but does not involve this specific site under question since the deletion is 7 amino acids upstream from position 397.

DC-SIGN mediated transfer of HIV-1 is decreased for both C33A generated viruses and after prolonged culture in the presence of CQ

Since PNG sites were altered in the CQ passaged viruses and these events are known to be involved with HIV-1 binding to DC-SIGN [20,23] we tested the efficiency by which the viruses were transferred by Raji-DC-SIGN cells to $CD4^+$ T-lymphocytes. When comparing the viruses produced on days 14, 77, 135 and 197 we observe that for viruses produced in the absence of CQ there is a significantly higher level of DC-SIGN mediated transfer than viruses produced in the presence of CQ (day 14, P = 0.001; day 77, P = 0.004; day 197, P = 0.005) (Fig. 6A). These results indicate that the alteration of the PNG sequence of gp120 may alter its binding to DC-SIGN, or alternatively the glycosylation machinery of the cell can influence the interaction of the virus with DC-SIGN. To test the latter we monitored the Raji-DC-SIGN mediated transfer to $CD4^+$ T-lymphocytes of viruses produced on C33A cells (CQ or non-CQ treated). All three viruses were shown to have a reduced capacity for DC-SIGN mediated transfer when produced in cells treated with CQ over viruses generated in non-CQ treated cells (JR-CSF, P = 0.0009; 299.10, P = 0.002; LAI, P = 0.003) (Fig. 6B). This result indicates that the same virus produced in the presence of CQ has a reduced capacity for transfer by DC-SIGN expressing cells to $CD4^+$ T-lymphocytes.

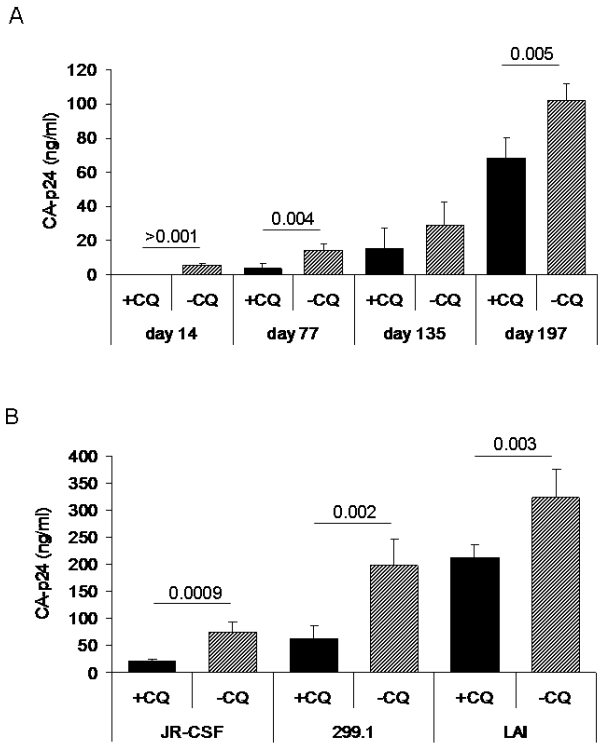

Figure 6. DC-SIGN mediated transfer of CQ passaged viruses and C33A derived viruses in the presence of CQ
Raji and Raji-DC-SIGN cells were incubated with viruses before washing with PBS and addition of $CD4^+$ T-lymphocytes. CA-p24 levels were determined at day 7 by standard ELISA. The CA-p24 levels of transfer by Raji cells alone were subtracted from the CA-p24 values of transfer observed with Raji-DC-SIGN cells. (**A**) DC-SIGN dependent transfer of viruses cultured long-term in the presence or absence of CQ (days 14, 77 and 197). (**B**) DC-SIGN dependent transfer of JR-CSF, 299.10 and LAI virus produced in C33A cells either in the presence or absence of CQ. Standard deviations are depicted in both panels and P-values given.

DISCUSSION

We demonstrate, in support of previous *in vitro* and *in vivo* studies [1,21,22,24-27], that CQ has an inhibitory effect on HIV-1 production. We further demonstrate that viruses produced in C33A cells or which have been extensively passaged through $CD4^+$ T-lymphocytes in the presence or absence of CQ show no difference in their infectivity profile and $TCID_{50}$/ml values when cultured away from CQ, indicating that the inhibitory effect on viral replication is provided at the level of the producer cell. Sequence analysis of the viruses after prolonged passage in the presence or absence of CQ demonstrates a loss of PNG sites in the gp120 region. Previous results have shown that N-glycosylation is of importance for the pathogenesis of HIV-1 but does not alter replication or infection of target cells [28], which is in correspondence to our results. CQ has been shown previously to reduce viral yield *in vitro* [1,25,27], but also viral infectivity [1,27]. However, in our study we do not observe inhibition of infection of $CD4^+$ T-lymphocytes. This may be explained by the fact that in our experiments we compensate for the presence of CQ in the produced virus stock, thereby eliminating the possibility of CQ transfer inhibiting viral replication in the stocks produced in the presence of the drug. It also should be noted that the

inhibitions observed in the two previous reports are low, varying between 5 - 50% of viral inhibition.

Sequence analysis of HIV-1 extensively passaged through $CD4^+$ T-lymphocytes revealed a number of genotypic differences between the CQ and the control passaged virus, including an increased V1V2 charge, a lack of increase in the V3 overall charge, a shortened V4 region and modulation of the PNG patterns in the variable loops, suggesting some pressure on the envelope structure exerted through culturing in the presence of CQ. Interestingly, it has been reported that CQ may modulate the PNG sites of the gp120 envelope [1,2,27], which is supported by our results. When we specifically analyze the epitope of the 2G12 neutralizing Ab, which is known to be expressed by PNG sites [23], we observe a high degree of variation with a number of PNG sites lost. Whether this modulation at the genetic level increases or decreases the capacity of the virus to be neutralized by 2G12 remains to be elucidated. This would support our hypothesis that CQ could be considered as a therapeutic agent that does not only reduce viral load but which can also modify the gp120 envelope to induce a broader array of neutralizing Abs. Previous reports have indicated that alterations to PNG sites of the gp120 structure can provide for altered immune escape [3-5]. The PNG events on the gp120 molecule have been referred to as providing a "glycan shield", whereby the epitopes responsible for neutralization are protected. Modulating the gp120 envelope glycosylation patterns through treatment with CQ may have the benefit of broadening the Ab repertoire in treated individuals and hence providing better control of *in vivo* viral replication.

CQ has been shown to impair the formation of glycosylated epitopes on gp120 which are known to be involved with the binding of 2G12 [1,2]. The epitopes on the gp120 envelope that are involved with the 2G12 interaction are at amino acid positions 295, 332, 386, 392, 397 and 448 [23]. It is known that the binding sites on gp120 that interact with 2G12 and DC-SIGN are overlapping and encompass PNG events. Binding of cellular DC-SIGN can be reduced by the 2G12 Ab [29], although there have been reports demonstrating that 2G12 does not block the DC-SIGN interaction [30]. Our results with the CQ passaged virus show a loss of PNG sites at positions 332 and 397 of gp120, which have been shown to be an integral part of the 2G12 binding epitope. The loss of these amino acids may also explain the reduction in the DC-SIGN mediated transfer of the CQ passaged virus. Variation in the V1V2 and V3 regions have also been shown to be involved with altered DC-SIGN interactions [20], hence the genotypic alterations observed in the long-term culture may well be expected to alter the ability of the virus to be transferred to $CD4^+$ T-lymphocytes by cells expressing DC-SIGN. Our results with the C33A produced viruses indicate, however, that the decrease in DC-SIGN mediated viral transfer can also be exerted through single-cycle production of virus suggesting that CQ can affect the post-translational modification of the gp120 molecule. The similar infectivity phenotype of these viruses on $CD4^+$ T-lymphocytes alone suggests that the reduction in infectivity in the presence of Raji-DC-SIGN cells is mediated via the interaction with the DC-SIGN molecule.

The observed reduction of DC-SIGN mediated transfer could have implication for HIV-1 transmission. DC-SIGN has been implicated to play a role in the sexual transmission of HIV-1 and presumably other mucosal transmission routes, such as via breast-feeding [10,14,31-34]. The virus can interact with DC-SIGN and other C-type lectins expressed by DCs, which results in internalization of the virus. Maturation of the DCs results in migration to the lymph nodes where HIV-1 can be presented to a pool of $CD4^+$ T-lymphocytes and establish infection.

Transmission of viruses from a CQ treated patient may therefore be more difficult to transmit via this route due to weaker DC-SIGN interactions. Although the reduction we observe in DC-SIGN mediated transfer of HIV-1 to $CD4^+$ lymphocytes is low any reduction in DC-SIGN mediated capture of virus at sites of exposure may have a significant repercussion on lowering rates of transmission, given the relative inefficiency of infection [35,36]. The effect of CQ on DC-SIGN binding *in vivo* remains to be determined, but if the DC-SIGN binding is indeed reduced than CQ treatment could be considered as a strategy to reduce transmission of HIV-1, again advocating for the use of the drug in specific cases where infection is more likely to occur.

CONCLUSIONS

We have shown in this study that the effects exerted by CQ on reducing HIV-1 replication *in vitro* of both R5 and X4 viruses is exerted at the cellular level and that viruses produced via single round replications or via multiple passage are as infectious and replicate as efficiently as those produced in the absence of CQ. We have shown that HIV-1 passaged with CQ or produced in a single cycle production assay are less efficiently transferred to $CD4^+$ T-lymphocytes via DC-SIGN expressing cells than viruses produced in the absence of the drug. These results indicate that the effectiveness of CQ in reducing viral loads may have its effects exerted through multiple mechanisms. Additionally, we have identified that PNG patterns of the virus can alter when passaged in CQ indicating that *in vivo* the drug could be utilized as an agent to alter the immunogenic properties of gp120 in order to induce a broader range of neutralizing antibody responses and hence aide in the lowering of viral loads. The significance of these findings to the *in vivo* setting will be identified through the study of HIV-1 infected individuals treated with CQ.

MATERIALS AND METHODS

Cells

Raji and Raji-DC-SIGN cells were cultured and utilized as previously described [10,37]. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from three buffy coats from different HIV-1 uninfected donors by standard Ficol-Hypaque density centrifugation, pooled and frozen in multiple vials. After thawing, PBMCs were activated with phytohemagglutinin (2 μg/ml) and cultured in RPMI medium containing 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) with recombinant interleukin-2 (100 units/ml). On day 3 the cells underwent $CD8^+$ cell depletion using CD8 immunomagnetic beads according to the manufacturers instructions and $CD4^+$ T-lymphocytes were cultured with IL-2 (100 units/ml). The human carcinoma cell line C33A was cultured in DMEM, with 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Generation of HIV-1 stocks

Replication competent HIV-1 stocks of JR-CSF (R5), LAI (X4) and 293.10 (R5X4) molecular cloned viruses (previously described in ref [38] were generated by passaging virus through isolated $CD4^+$ T-lymphocytes. Virus stocks were also produced by transfection of C33A cells with plasmid expressing the specific virus strain to be analyzed and using the assay described below. The CA-p24 levels in

the culture supernatants were determined using a standard ELISA protocol. Virus stocks were generated either in the presence (100 μM) or absence of CQ.

Long-term passage of HIV-1 in the presence and absence of CQ

The R5X4 dual-tropic molecular cloned virus (293.10) was the starting virus for the passage and has been previously described [38]. Fifteen ng/ml of CA-p24 was added to $10x10^6$ $CD4^+$ T-lymphocytes in a volume of 5.0 ml RPMI medium with 100 μM CQ being added to the CQ passage culture flask. HIV-1 CA-p24 concentration was determined once a week with new virus being added to fresh $CD4^+$ T-lymphocytes. If the CA-p24 levels were too low then the CA-p24 was re-determined 3 days later with 15 ng/ml subsequently added to fresh cells. Remaining culture supernatants and cell pellets after each passage were stored at -80°C until required for analysis.

$TCID_{50}$/ml determination of generated viral stocks

$TCID_{50}$/ml values were determined by limiting dilution of the viral stock on $CD4^+$ T-lymphocytes, as previously described [38]. In short the $CD4^+$ T-lymphocytes were plated at $2x10^5$ cells/well in 96 well plates with 5 fold serial dilution of the virus. On day 7 the wells were scored for CA-p24 levels and the number of positive wells determined. These values were used to determine the $TCID_{50}$/ml values for each virus. For the determination of the $TCID_{50}$/ml for the C33A generated viruses and the viruses after prolonged culture, the input was standardized at 105 ng/ml CA-p24 and 10.5 ng/ml CA-p24, respectively.

Replication curves of HIV-1 stocks

$CD4^+$ T-lymphocytes were plated at $1x10^5$ cells/well in 96 well plates. One hundred $TCID_{50}$ of virus stock was utilized with CQ being added either at 200 μM, 100 μM or 50 μm/well. For replication analysis of the C33A generated viruses and the viruses obtained from the prolonged CQ passage 1 ng/ml of CA-p24 was utilized as virus input. When analyzing viruses obtained from CQ cultures the level of CQ in the culture supernatant was compensated for in the control culture or $TCID_{50}$/ml determination assay. CA-p24 values were determined using a standard ELISA assay for the culture supernatants obtained from the infection assay collected over time. All experiments were performed in triplicate with the standard means depicted.

Transfection of C33A cells with virus expressing plasmids

Transfection of C33A cells was performed with 10.0 μg of plasmid DNA expressing HIV-1 using the $CaCl_2$ precipitation method. All plasmid DNA used was prepared using Qiagen kits. The DNA precipitate was split between two wells of C33A cells plated 24 hours earlier at $1.5x10^6$ cells/well in a 6 well tissue culture plate in DMEM medium either in the absence or presence of CQ (100 μM). The transfections were performed in a final concentration of 6 ml of DMEM, with penicillin (100 units/ml), streptomycin (100μg/ml) and 10% FCS. The following day the cells were washed with PBS and fresh media was added, the viral stock was harvested on day 3 of culture with the viral CA-p24 levels determined by standard ELISA.

DC-SIGN mediated HIV-1 transfer assay

The assay was performed as previously described [37]. The Raji and Raji-DC-SIGN cells were plated at a concentration of $2x10^4$ cells/well in a 96 well format. Four hundred ng/ml of the appropriate virus was added to the Raji-DC-SIGN or Raji cells when studying the C33A produced virus stocks. For the CQ passaged viruses a set CA-p24 input of virus was utilized for each virus set (range 100 – 400 ng/ml). For the CQ passaged viruses the presence of CQ in the supernatant was compensated for in the control virus stock with an equal concentration of CQ added. After 2 hr incubation the culture was washed with PBS before addition of $CD4^+$ T-lymphocytes at a concentration of $1x10^5$ cells/well. CA-p24 values were determined on day 7 using a standard ELISA protocol and all experiments were performed in triplicate.

Sequencing and sequence analysis

HIV-1 RNA was isolated from culture supernatant according to the method of Boom [39]. Viral RNA was converted to cDNA and then subjected to a nested PCR to amplify a fragment covering the V1V2 to C4 region of the gp120 gene. DNA sequence alignments were performed manually. Positions containing an alignment gap were included for the pair-wise sequence analysis. Phylogenetic analysis of the amplified region was performed with the neighborhood-joining (N-J) analysis of MEGA [40]. The PNGS were analyzed using the program available at the HIV-1 sequence database [41].

Statistics

All statistical comparisons were performed using ANOVA with $P < 0.01$ being considered as statistically significant.

LIST OF ABBREVIATIONS

Ab, antibody; CQ, chloroquine; DC, dendritic cell; DC-SIGN, DC-pecific ICAM3 grabbing non-intergrin; ECD, extra-cellular domain; HCQ, hydroxychloroquine; R5, CCR5 coreceptor using HIV-1 isolate; R5X4, CCR5 and CXCR4 coreceptor using HIV-1 isolate; X4, CXCR4 using HIV-1 isolate; PNG; potential N-linked glycosylation.

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Chapter 4

Chloroquine treatment increases HIV-1 plasma viral loads in breastfeeding mothers without influencing the gp120 envelope genotype

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ABSTRACT

Chloroquine (CQ) treatment has previously been shown to decrease human immunodeficiency virus type 1 (HIV-1) viral load in infected individuals. It is also shown that CQ accumulates in breast milk cells with the predicted potential to reduce viral load in breast milk (BM) and thereby restrict HIV-1 transmission via breastfeeding. Here a Phase I/II, randomized controlled study was conducted to evaluate the effect of CQ on modulating HIV-1 activity in breastfeeding mothers: the CHARGE study. Thirty mothers from Rwanda (CQ treated n=20; placebo n=10) were enrolled in a 16 week study. CQ levels in plasma correlated to those in BM, and CQ levels were 2.5 fold higher in BM. We found a positive correlation between CQ concentrations and $CD4^+$ T-cell counts in plasma of the mothers. We found HIV-1 plasma loads increased significantly in mothers treated with CQ ($p=0.01$) and with no change observed in the placebo group. Three children were infected during birth or the breast feeding period; all of them in the group of CQ treated mothers. No major differences in amino acid sequences relating to charge, length or number of potential N-linked glycosylation sites were observed in the variable regions of the HIV-1 gp120 envelope gene. Additionally, no differences in neutralization potential of gp120 Env viruses were found following CQ treatment. These data caution against the use of CQ in reducing HIV-1 MTCT via breastfeeding.

INTRODUCTION

The anti-malarial drugs Chloroquine (CQ) and Hydroxychloroquine (HCQ) are cheap and widely available. These are both weak bases that affect acid vesicles leading to dysfunction of several enzymes such as those involved in protein post translational modification. In HIV-1 infected individuals CQ treatment may therefore lead to impaired glycosylation of the HIV-1 envelope protein [1-5]. This could decrease the capacity by which HIV-1 can undergo viral capture and transfer to $CD4^+$ T lymphocytes via cells expressing DC-SIGN [6], modulate neutralizing antibody (NAb) epitopes (as seen for 2G12 [1] as well as reduce the glycan shield protecting against Abs binding gp120. We amongst others have shown that in cell culture CQ can interfere with virus replication in $CD4^+$ T lymphocytes and that this inhibition is likely conferred at the cellular level [6]. The altered endosomal pH also reduces IL-6 production [7], followed by down-regulation of HIV-1 production in chronically infected T-cells and monocyte cell lines [2,8]. CQ also inhibits Tat mediated transactivation of HIV-1 transcription [9]. For these reasons it has been postulated that the treatment of HIV-1 infected individuals with CQ or HCQ may result in lower viral loads as well as a reduction in their capacity to transmit HIV-1.

Several clinical trials have studied the effect of CQ and HCQ on HIV-1 replication *in vivo* [3,4,10]. In two of these trials treatment with HCQ resulted in a decrease in viral loads with no measurable effect on the CD4 counts [3,4]. A decrease in plasma IL-6 expression levels was observed together with a decrease of total serum IgG [4]. These results make HCQ and CQ promising candidates in HIV-1 treatment strategies. CQ has been shown to accumulate in human milk [11-13] and has been shown not to be toxic in breastfed infants [14]. According to the 2011 UNAIDS Progress report 390,000 children were newly infected with HIV-1 in 2010 with the majority resulting from mother-to-child transmission (MTCT). Known maternal risk factors associated with MTCT are high plasma viral loads, low CD4 T-cell numbers coinciding with advanced maternal immune deficiency and prolonged labor [15]. In populations where replacement feeding is

not feasible it has been estimated that 41% of MTCT occur *in utero* (IU), 20% *peri-partum* (PP) and the remaining 39% during prolonged breastfeeding (BF) [16]. We performed a study "CHARGE" to assess the potential role of CQ in modulating HIV-1 activity in breastfeeding mothers.

MATERIALS AND METHODS

Study participants

The study was approved by an Independent Ethics Committees (IEC) in the Netherlands, the STEG-METC (ref no R01-089). In the absence of an operational IEC in Rwanda at the time, the Ministry of Health's Treatment and Research AIDS Center (TRAC), the Rwandan National Malaria program (PNLP) and the "Cellule de recherché" at the CHK acknowledged the approval of the Dutch IEC. All women provided written informed consent for both themselves and their children. Thirty Rwandese HIV-1 infected pregnant women were randomized (2:1) to receive either placebo (n=10) or CQ 200 mg once daily from the day of birth for a duration of 16 weeks (n=20) (Table 1). All women received a single-dose of 200 mg Nevirapine at the start of labor to reduce *intra-partum* transmission of HIV-1. Regular counseling on exclusive breastfeeding was provided. At inclusion the patients had to complete the following conditions: at least 18 years of age, between 32 and 35 weeks of gestation, antiretroviral therapy-naive, intending to breastfeed their child(ren), able to provide written informed consent for both themselves and their expected infant(s) and likely to complete the 18 weeks. Women were excluded if they had received any CQ treatment within 6 weeks prior to study drug administration, if there was evidence of severe fetal anomalies, serious disease or laboratory abnormalities incompatible with study participation or any CQ related ocular toxicity or other adverse effects that occurred due to previous CQ administration. The study was approved by an Independent Ethics Committee (IEC) in the Netherlands. In the absence of an operational IEC in Rwanda at that time, the Ministry of Health's Treatment and Research AIDS Center (TRAC) the Rwandan National Malaria program (PNLP) and the "Cellule de recherché" in CHK acknowledged the approval of the Dutch IEC.

Viral load and $CD4^+$ T-cell count

At intake blood from pregnant women was screened for HIV-1 infection using a rapid assay. At the day of delivery and 8 and 16 weeks blood, plasma and breast milk samples were taken and stored. HIV-1 viral loads were measured using the RNA PCR assay from Roche Diagnostics (Mannheim, Germany) with a lower limit of detection of 25 copies/ml (Table 2). Immunologic and virologic testing of mothers was performed in two laboratories (National HIV/AIDS Reference Laboratory, Kigali, Rwanda and the Joint Clinical Research Centre, JCRC, Kampala, Uganda).

Measuring CQ concentration

To a 1.0 ml plasma sample (patient samples were supplemented with blank plasma to this volume if required) in a glass conical tube, 25 µl of internal standard solution (50 µg/ml diaminonaphthalene in methanol), 1 ml of 1M sodium hydroxide and 6 ml diethylether were added. After shaking for 10 min, the mixture was centrifuged for 5 min at ca. $2x10^3$ *g*. After storage of the tube

for 1 h at -30°C, the organic layer was poured into a second tube and evaporated at 40°C under a gentle stream of nitrogen. Finally, the residue was reconstituted in 200 µl eluent using vortex-mixing and 50 µl was injected onto the HPLC column using partial loop filling.

To a 1 ml milk sample (patient samples were supplemented with drug-free cow milk to this volume if required) in a glass conical tube, 6 ml *n*-hexane was added. The tube was shaken for 10 min and centrifuged for 5 min at ca. $2x10^3$ *g*. After storage of the tube for 1 h at -30°C, the organic layer was discarded. After thawing the aqueous layer, 1 ml of 1M sodium hydroxide and 6 ml diethylether were added and the procedure was continued according to the pre-treatment of plasma samples. Chromatographic conditions: Fifty µl injections were made on a Symmetry C_{18} column (100×4.6 mm, d_p = 3.5 µm, average pore diameter = 10 nm, Waters), protected by a Symmetry C_{18} pre-column (20×3.8 mm, d_p = 5 µm, Waters). The column temperature was maintained at ambient temperature (23-29°C). The eluent comprised 9% (v/v) acetonitrile and 91 % (v/v) phosphate buffer (45mM, pH 3.0) and the eluent flow rate was 1.0 mL/min. The UV detection wavelength was 340 nm.

HIV-1 infection status of the children

Presence of HIV-1 viral RNA in the plasma at the day of birth was tested by RT-PCR for the HIV-1 V3 region using primer 3'V3Not (GCG CGG CCG CCC CCT CTA CAA TTA AAA CTG TG) in the RT reaction, followed by the first PCR using 3'V3Not and 5'V3Not (GCG CGG CCG CAC AGT ACA ATG TAC ACA TGG). In the second PCR primers 5'Ksi (ATA AGC TTG CAG TCT AGC AGA AGA AGA) and 3'Ksi (ATG AAT TCT GGG TCC CCT CCT GAG GA) were used. At week 16 the infection of the infants was established by viral load measurements as described above.

Sequencing of the HIV-1 gp120 envelope region and analysis

Viral RNA was isolated from plasma using a silica-based method [17]. The C1-C4 envelope region was amplified by RT-PCR as previously described [18]. Viral RNA was converted to cDNA and then subjected to a nested PCR amplifying a fragment covering the V1 to C4 region of the gp120 gene. The primers that were used for the first PCR were A1053 (5'-GAAAGAGCAGAAGACAGTGGCAATGA-3') and A1262 (5'-CTGACGGTACAGGCCAGACAATTATTGTC-3'). For the Nested PCR A1322 (5'-TCTTGGGAGCAGCAGGAAGCAC-3') and A0385 (5'-GAGGATATAATCAGTTTATGGGA were used as primers. PCR products were cloned into the TOPO II vector (Invitrogen, Carlsbad, CA, USA) and sequenced bi-directionally using the BigDye Terminator Cycle Sequencing kit (ABI, Foster City CA, USA) and analyzed using an ABI 377 automated sequencer (ABI) or the Thermo Sequenase fluorescence-labeled primer cycle-sequencing kit (Amersham International, Little Chalfont, UK) according to the manufacturer's instructions. The primers used for the sequencing were A1360 (5'-GAGCCAATTCCYATACAT TATTG-3'), SP6 (5'-ATTTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCACTATAGGG-3'). Sequences were assembled using CodonCode Aligner (CodonCode Corporation, Dedham, MA) and aligned with ClustalW and manually edited using Textpad (Helios Software Solutions). Phylogenetic analysis of the aligned sequences was performed using the neighbor-joining method of MEGA36 (Tamura, Dudley, Nei, and Kumar 2007). The distance matrix was generated by Kimura's two-parameter estimation and the tree topology was

confirmed by the maximum-likelihood option. Bootstrap values greater than 70% were considered significant based on 100 replications. Sequences obtained from the Los Alamos database were included as references. The presence of potential N-linked glycosylation sites (PNGS) was determined using the program available from the HIV sequence database (http://hiv-web.lanl.gov/content/hiv-db/GLYCOSITE/glycosite.html).

Subtype analyses

HIV-1 subtype was determined through analyzing the gp120 V3 region sequences. The Subtype Reference Alignments of the Los Alamos Data Base (www.hiv.lanl.gov/content/sequence/NEWALIGN/align.htm) year 2005 were used as reference sequences.

Determination of IgG concentrations

IgG concentrations in plasma were measured using the Cobas C502 (Roche, Roche Diagnostics, Darmstadt, Germany) according to the instructions of the manufacturer**.**

Statistics

All statistical comparisons were performed with the Mann-Whitney test or the paired t-test using GraphPad Prism version 5.00. P-values < 0.05 were considered statistically significant.

RESULTS

Clinical parameters of study individuals

Thirty HIV-1 positive mothers were included in this study (Table 1). Each mother received a single dose of Nevirapine at time of delivery with the 20 CQ treated mothers receiving daily doses of 200 mg CQ for a period of 16 weeks. Base-line, week 0, week 8 and week 16 plasma and BM samples were taken from each mother, with a week 0 and week 16 plasma sample taken from each infant. CD4 counts of the mothers were measured at baseline, week 0, week 8 and week 16. Patients 100, 150, 160, 400, 220, 260 and 280 stopped treatment between week 8 and 16. Three children died of unknown causes within the 16 week period (101, 161 and 401), 2 children born from mothers in the control group and 1 from a CQ treated mother. Seven mothers were lost for follow up during the 16 week period, 2 CQ treated and 5 placebos, indicating that dropout is not CQ treatment related. The HIV-1 subtype was determined by analyzing the V3 region of gp120. One CQ treated mother (250) carried an A/C recombinant, (Table 1) and the subtype of 2 mothers (020 and 240) could not be determined. The remaining 27 mothers were infected with either subtype A (n=20, 12 CQ treated and 8 placebo) or subtype C (n=7, 6 CQ treated and 1 placebo) reflecting the viral subtypes circulating within the geographical region. Four children were found to be positive at the day of birth indicating HIV-1 transmission *in utero*. Three children were infected either during delivery or during the breastfeeding period, notably all from CQ treated mothers.

Table 1. Characterization of study participants

Mothers							Children			
			Plasma IgG g/L		Plasma	Sequenc	RNA		Viral Load	Sequence
Mother	CQ	Subtype	Week 0	Week 16	neutralization	analysis	Week 0	Week 16	Week 16	analysis
20	+	ND	ND	ND	-	-	-	-	-	-
30	+	A	ND	ND	-	-	-	-	-	-
40	+	A	ND	ND	-	-	-	-	-	-
50	+	A	ND	ND	-	+	-	-	-	+
60	-	A	ND	ND	-	+	-	-	-	+
90	-	A	17.400	26.500	-	+	+	+	750000	+
100	-	A	29.400	38.200	+	-	+	+	ND	-
110	-	A	ND	ND	-	+	-	-	-	+
130	+	C	19.500	25.900	+	+	-	+	7590	+
150	-	A	ND	ND	-	-	-	-	-	-
160	+	A	23.000	37.000	+	+	-	-	-	+
180	+	C	ND	ND	-	-	-	-	-	-
190	+	C	ND	ND	-	+	-	-	-	+
200	-	A	20.400	31.100	+	+	-	-	-	+
210	+	C	ND	ND	-	-	-	-	-	-
220	+	A	ND	ND	-	-	-	-	-	-
240	-	nd	ND	ND	-	-	-	-	-	-
250	+	A/C	36.100	79.300	+	+	+	+	269000	+
260	-	A	ND	ND	-	-	-	-	-	-
270	+	C	16.500	31.300	+	-	-	-	-	-
280	-	C	ND	ND	-	-	-	-	-	-
290	+	A	20.600	29.500	+	+	+	+	531000	+
300	+	A	27.900	40.100	+	+	-	+	747000	+
340	+	A	ND	ND	-	+	-	-	-	+
350	+	A	ND	ND	-	-	-	-	-	-
360	+	A	ND	ND	-	-	-	-	-	-
370	+	A	20.200	40.300	+	+	-	+	144000	+
380	+	A	16.600	28.500	+	+	-	-	-	+
390	+	C	ND	ND	-	-	-	-	-	-
400	-	A	ND	ND	-	-	-	-	-	-

CQ concentrations higher in BM than in plasma

In previous studies patients received a daily dose of 800 mg HCQ, which corresponds to 500 mg CQ [3,4]. The mean plasma HCQ concentrations found in these trials were 316.3 and 347.1 ng HCQ /ml respectively at week 8, corresponding with 197.7 CQ and 216.9 ng CQ /ml and 435 ng HCQ /ml at week 16 corresponding with 271.9 ng CQ /ml. In our trial the mothers received a daily dose of 200 mg CQ, with mean plasma CQ concentrations of 759.7 ng CQ /ml (95%CI 467.8-1052) at week 8 and 478.2 ng CQ /ml (95%CI 351.7-604.6) at week 16 (Fig 1A and 1B). The CQ levels were comparable with those in the previous trials at week 16 and higher at week 8, thereby indicating differences in efficiency of uptake between HCQ and CQ.

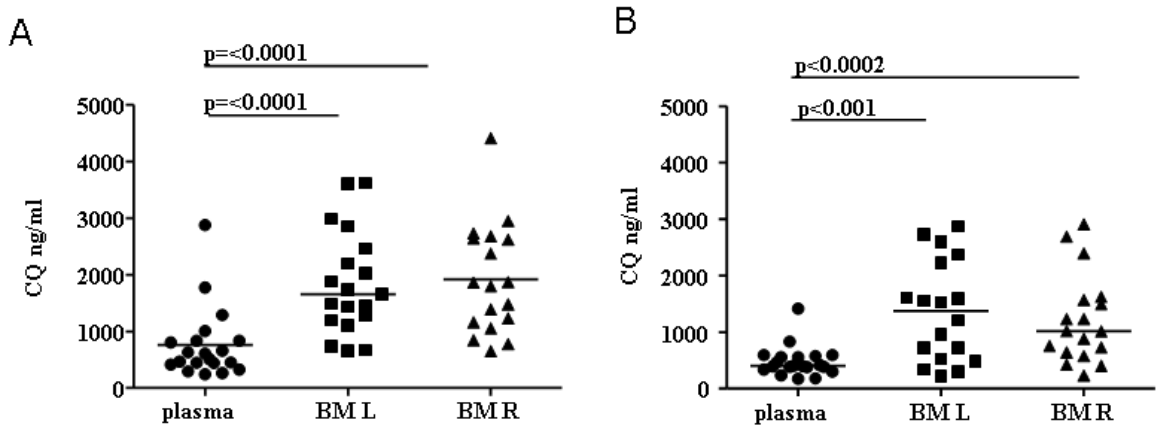

Figure 1. CQ concentration in ng/ml in plasma and breast milk after 8 weeks (**A**) and 16 weeks (**B**) of treatment.

Earlier reports demonstrated accumulation of CQ in BM cells [19]. We measured the CQ levels of milk of both right and left breast at 8 and 16 weeks after initiation of treatment and compared these with the CQ levels in blood plasma at the same time point. The CQ concentrations in plasma correlated with those in BM at week 8 (r^2 0.50, $p<0.0001$) and at week 16 (r^2 0.49, $p<0.0001$) (data not shown). The CQ levels in the right and left breast were found to be similar at both time points ($p=0.94$ and 0.79 respectively). The mean CQ concentrations were 1831 ng/ml (95%CI 1134-2648) and 1658 ng/ml (95%CI 1198-2457), respectively at week 8 and 1017 ng/ml (95%CI 232.0-1581) and 1369 ng/ml (95%CI 512.3-2265) at week 16 (Fig. 1A and Fig. 1B), making the CQ level in BM higher than observed in plasma ($p<0.0001$ at week 8 and $p<0.001$ at week 16). These results indicate that the CQ concentration in BM was approximately 2.5 fold higher in BM than in blood plasma supporting earlier findings that CQ accumulates in BM.

CQ treatment did not decrease BM HIV-1 load

It has been hypothesized that the accumulation of CQ in BM would lower VL and therefore lower the risk of HIV-1 transmission through breast feeding [19]. We measured HIV-1 VL in BM from both the right and left breast at baseline and after 8 and 16 weeks of CQ treatment. The VL load of the left and right breast correlated at all time points (r^2 0.80 $p<0.0001$, r^2 0.81 $p<0.0001$ and r^2 0.56 $p<0.0005$ respectively). We found no significant difference in BM VL between baseline and week 8 and 16 samples in the CQ treated mothers (Fig. 2A and 2B). No correlation was found between the CQ concentration and the VL in BM (data not shown). The three mothers who transmitted HIV-1 to their child either during delivery or via breast feeding (130, 300 and 370) were all from the CQ treated group. They had a mean BM-VL of 214 RNA copies/ml (95%CI 25-367) and a mean BM-CQ concentration of 1542 ng/ml (95%CI 845-2620) which was not different from the remainder within group.

CQ treatment increased plasma HIV-1 load

We determined the effect CQ treatment had on influencing plasma HIV-1 VL (Table 2). We performed paired t-tests of baseline versus week 8 and week 16 VL from treated and non-treated mothers. We found no significant increase or decrease in both the groups. There was no correlation between CQ levels and VL (data not shown), Three mothers (210, 290, 360) with high CD4 T-cell counts at

Table 2. Synonymous and nonsynonymous analysis of gp120 sequence

	ΔS*			ΔN*			ΔS/ΔN		
	Placebo (#)	CQ (#)	p value	Placebo (#)	CQ (#)	p value	Placebo (#)	CQ (#)	p value
V1V2	0.940 (± 0.72)	0.936 (± 0.83)	0.99	0.862 (± 0.61)	0.919 (± 0.95)	0.92	0.857 (± 0.68)	3.032 (± 4.21)	0.34
V3	0.406 (± 0.48)	2.023 (± 3.99)	0.44	1.602 (± 2.13)	1.526 (± 2.77)	0.96	0.603 (± 0.86)	1.960 (± 3.91)	0.51
V4	1.176 (± 1.21)	0.336 (± 0.59)	0.11	0.617 (± 0.55)	1.713 (± 2.00)	0.38	1.355 (± 1.53)	0.453 (± 0.73)	0.17

* ΔS and ΔN were determined by dividing the average distance of week 0 by week 16
\# standard deviations

baseline (1355, 1168 and 846 cells/µl respectively) showed CQ levels >1200ng/ml in their plasma at week 8. Two of these mothers showed a more than tenfold increase in plasma VL between baseline and week 8. We found no significant difference in VL between baseline and the week 16 samples in the placebo group but we did find a significant increase (P=0.010 and 0.012, respectively) of viral load within the CQ treated group at 8 and 16 weeks after treatment (Fig. 3). CQ treatment in our cohort associated with increased HIV-1 VL in plasma. To investigate if CQ treatment has a different effect on the two viral subtypes in our group, we compared the VL of subtype A and C infected, treated mothers and found no difference (data not shown) indicating that the effect of CQ is not subtype specific.

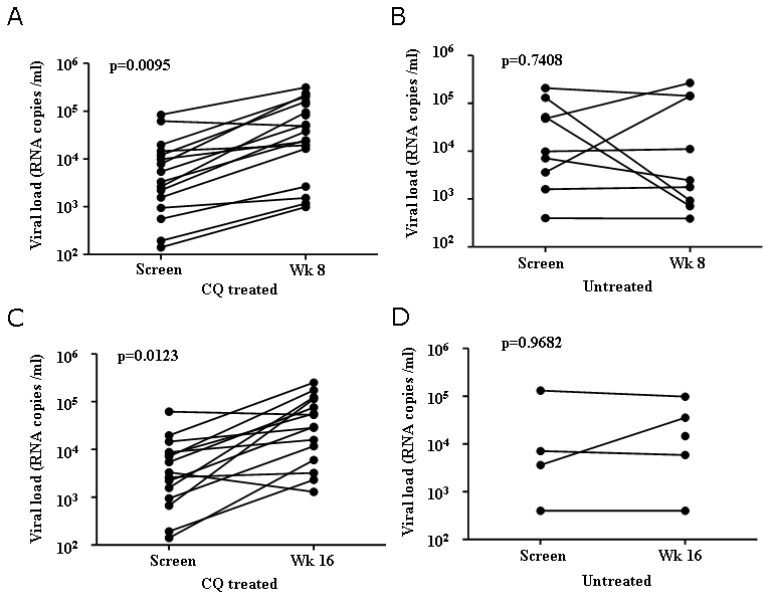

Figure 3. Viral loads in plasma after 8 weeks CQ (**A**) or placebo treatment (B) or 16 weeks CQ (**C**) or placebo treatment (**D**).

No effect of CQ treatment on CD4 T-cell counts

The increase in VL in our cohort could be the result of an increase in number of target cells for HIV-1 replication. In two earlier studies with HCQ treatment no measurable effects were seen regarding changes to CD4 counts [3,4]. The mean $CD4^+$ T-cell count at week 8 of both the treated and the placebo mothers were shown to increase, but not significantly (p= 0.145 and p= 0.076 respectively), confirming the earlier data (Fig. 4).

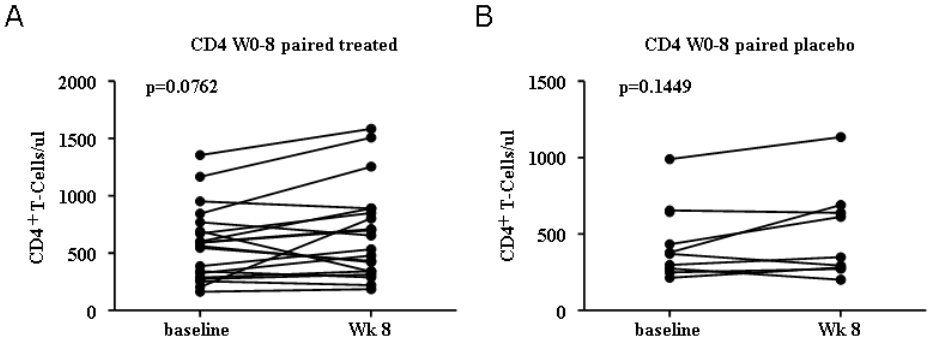

Figure 4. CD4+ T-cell counts in the CQ (**A**) and the placebo group (**B**) after 8 weeks of treatment.

The effect of CQ treatment on the gp120 envelope gene

The observed increase in HIV-1 VL could be the result of genotypic, hence phenotypic, changes in the viral population within the CQ treated group. Changes have been previously reported for HIV-1 cultured in the presence of CQ [1,6]. We have shown that HIV-1 cultured in the presence of CQ can increase the V1V2 charge, decrease the overall length of the V4 region, and induce loss of a PNGS in the V3 region [6]. To determine the effect of CQ treatment *in vivo*, we amplified gp120 envelope protein genes from plasma from mothers receiving CQ (n=10) and from placebo mothers (n=4) at baseline and 16 weeks after therapy initiation. In addition, we amplified the gp120 *env* genes from BM sample of 2 CQ treated mothers (130 and 160,) and 1 placebo mother (090) for both time-points. Ten clones from each sample were sequenced. We analyzed the V1V2, V3 and V4 regions for alterations in charge, length and PNGS patterns. The data acquired from the BM samples were similar to those from the corresponding plasma samples in all analyses.

i) Charge of variable regions

In the V1V2 region, the V3 region as well as in the V4 region there was variation in charge with decreases as well as increases in both the CQ treated and placebo mothers. No statistical differences were found between the baseline and the week 16 sample in overall charge in either of the variable regions analyzed.

ii) Length of variable regions

We found no statistical differences in the length of the V1V2 regions between baseline and week 16 samples in the CQ treated as well as placebo mothers The V3 length did not alter in any of the mothers in either the treated or the placebo group. Three of the CQ treated mothers acquired an insertion within the V4 region between baseline and week 16. Mother 190 had an insertion of 7 Amino Acids (aa), mother 300 of 8aa and mother 380 of 5aa. No insertions were found in the plasma of the mothers and in BM. The VL in the 3 mothers showing an insert in the V4 region increased with 1.4 log (14.3 (95% CI 11.0 – 17.7) fold) at week 8 and 1.15 log at week 18. The VL in the CQ treated mothers with no insert in V4 increased 0.27 log (12.6 (95% CI -0.06 – 25.3) fold) (p=0.05) at week 8 and 0.28 at week 16. When tested we found no overall correlation between the length of V4 and viral load in all mothers (data not shown).

iii) Alterations in variable region PNGS patterns

We determined loss or gain of PNGS in all three variable regions studied. We found no major changes in number of PNGS between baseline and week 16 in the V1V2 region in either the CQ treated or the placebo mothers. All baseline clones of mother 160 had a V3 PNGS at position 301 (HXB2 numbering) and which was absent at week 16. All clones from mother 200 also demonstrated the absence of PNGS on position 301 indicating that the loss of this PNGS is not associated with CQ treatment. No difference was observed in the number of PNGS in the V4 region, but in the CQ treated mothers the mean number of PNGS increased significantly between baseline and week 16 from 4.2 (95%CI 3.6 – 4.9) to 4.9 (90%CI 4.1 – 5.6) (p=0.014). No correlation was found between the number of PNGS and VL.

iv) No difference in the synonymous versus non-synonymous mutation rates of gp120 after CQ treatment

Overall our results indicate that CQ does not alter charge, length or PNGS patterns of the gp120 envelope protein *in vivo*. The ratio between non-

synonymous and synonymous mutations in a gene over time is a measure for positive or negative pressure on these mutations. We therefore calculated the synonymous (ΔS) as well as the non-synonymous (ΔN) mutation rates and determined the ΔS/ΔN between baseline and week 16 samples for both the CQ and the placebo group. We found no statistical differences between the two groups in ΔS, ΔN or the ΔS/ΔN ratio, indicating that there was no evolutionary pressure on the gp120 protein by CQ (Table 2).

Plasma IgG levels are not different between CQ and placebo treated mothers

The lower endosomal pH caused by CQ treatment is postulated to reduce IL-6 production [7] along with a decrease of total serum IgG [4]. We measured the plasma levels of total IgG in a random selection of mothers in our cohort. In both the CQ mothers and the placebo treated mothers the IgG levels were enhanced at 16 weeks after delivery compared with the baseline levels ($p < 0.005$ and $p < 0.004$ respectively). This increase of IgG levels was not different between the CQ treated and placebo mothers ($p = 0.19$), indicating CQ does not modulate IgG production (Fig. 5).

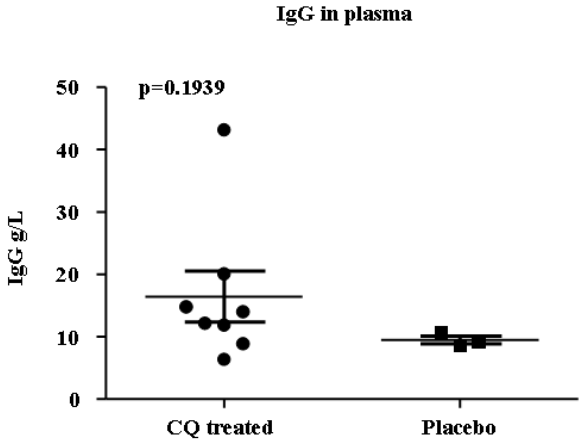

Figure 5. Increase of plasma IgG concentrations from the day of delivery to 16 weeks later in the CQ and the placebo treated group

CQ did not alter the neutralizing antibody responses

To identify whether the NAb responses were altered by CQ treatment we analyzed data from chapter 5. When comparing neutralization of the JRFL gp120 Env pseudo-typed virus with mother plasma (baseline and week 16 - 18 weeks after delivery) no differences were found between the early and late samples (data not shown). We also analyzed the autologous virus neutralization capacity and found no differences between early and late plasma samples for their ability to neutralize virus, again indicating that CQ treatment did not modify HIV-1 neutralization potential.

DISCUSSION

Here we present the results of the CHARGE study which was performed to study the influence of CQ treatment on modulating HIV-1 activity during the breast feeding period. Earlier reports had demonstrated the accumulation of CQ in cells present in BM [19]. Our findings show that the CQ concentration is approximately 2.5 fold enhanced in BM compared to blood plasma and confirm that CQ accumulates to higher levels in BM. In spite of higher CQ levels, no change was observed in BM viral loads between baseline and either week 8 or week 16 after treatment initiation. In contrast to previous reports describing a decrease in HIV-1 viral loads after HCQ treatment [3,4] our study demonstrated a significant increase in VL in the CQ treated mothers whilst no change was observed in the placebo group. The discrepancy in VL changes between our study and the earlier reports cannot be readily explained by the fact that our mothers received a lower daily dose of CQ. On the contrary, the concentration of CQ in the plasma of the mothers after 8 weeks of treatment was higher than in the earlier studies, possibly relating to differences in efficiency of uptake between HCQ and CQ. In concordance with earlier reports we fount no effect of CQ on $CD4^+$ T-cell counts.

On comparing the gp120 envelope sequences of CQ treated and placebo mothers we observed no overall differences in the amino acid composition of the variable regions. However, we did find numerous insertions within the V4 region in 3 of our CQ treated mothers after 16 weeks of treatment. It is not clear if these insertions are induced by CQ, but these inserts in V4 were not observed in the non-treated mothers nor were they observed in BM clones. Interestingly, the mothers demonstrating insertions within V4 showed an increase in VL of 1.15 logs after 16 weeks of treatment, higher than the increase of 0.28 log seen for the remaining CQ treated mothers. Inserts in the V4 region have previously been associated with immune evasion [20] and we suggest that the higher increase in VL in the mothers with these inserts may be caused by immune evasion and partly explain the results. There was also no significant change in the ΔS/ΔN ratio between the different groups of individuals, indicating that the increase in viral load does not stem from CQ exerting pressure on the gp120 envelope resulting in altered genotypes, hence phenotypes. We identified that the alterations observed *in vitro* culture under CQ pressure did not translate to differences *in vivo.*

It has previously been reported that CQ treatment can decrease the glycosylation pattern of the HIV-1 envelope [1]. We have also shown that the *in vitro* passaging of HIV-1 in the presence of CQ can provide alterations to HIV-1 that can be linked to alteration within the glycosylation patterning of the gp120 envelope [6]. We did not find such alterations *in vivo*, however, it should be noted that we only looked at alterations to envelope amino acid sequences and may have missed post-translational modification differences. The lack of observed genotypic differences could be due to the short course of CQ treatment (16 weeks in comparison to the *in vitro* culturing of 30 weeks). Longer treatment may see the emergence of viruses with selected mutations that are preferentially fixed. Alternatively, the alterations *in vivo* could be directed by host genetic or immune factors and therefore the alterations will be specific to the host environment in which they are selected. Since we have shown that the modifications observed with *in vitro* culturing can modify regions associated with escape from neutralizing antibody responses, namely alterations in the length of variable regions and PNGS patterns, each individual may select different viruses based on pre-existing immunity etc. Decrease of the number of PNGS for

example could lead to better Ab recognition of the virus resulting in enhanced neutralization of HIV-1, possible explaining why these variants are not found in patient plasma. Apart from an insertion in the V4 region we did not find differences in the viral population between the CQ treated and placebo groups that could explain for the increase in viral load. Another possibility is that some of the patients developed resistance to CQ, which results from mutations outside the gp120 region, however, this is highly unlikely given timings and the number of mothers demonstrating an increased in viral loads. We also show that NAb responses of the plasma's have not changed and it will be interesting to further analyze the viruses from the patients in this study to identify whether the viruses isolated have altered capacities to be neutralized with either monoclonal Abs or the longitudinal sera from their respective host.

It has been shown for $CD8^+$ T-lymphocytes that CQ can induce their activation state through the increased presentation of antigen [21]. It could be argued that if the same scenario prevails for $CD4^+$ T-lymphocytes then the increase in viral load observed could reflect an enhanced presentation of antigen via the MHC-class II molecule by APC, resulting in heightened cellular activation and increased viral replication. However, this would not explain the lack of increase of VL in earlier CQ or HCQ studies [3,4]. Our results support a study where mothers receiving CQ did not show a decrease in plasma VL or BM VL [10]. Since the previous studies, where reduction in plasma HIV-1 viral loads were shown, were composed predominantly of male participants we may be looking at a male versus female phenomenon and more so one which is determined by hormonal differences brought about by pregnancy or child delivery and lactation. Our results would suggest for the analysis of larger cohorts, however, ethical issues naturally will prevent such studies from being performed and we will have to rely on the information available from the limited individuals studied to-date.

The adverse effect of CQ on HIV-1 viral load observed in plasma could have implications for the potential use of CQ as a prophylaxis for HIV-1, especially in breastfeeding women. It will be interesting to decipher the specific cause and/or mechanism leading to the increased viral loads observed. CQ is now considered to be a safe and cheap alternative to conventional HIV-1 treatments, and is being considered for large-scale use in resource-limited settings. Our results indicate caution when treating HIV-1 patients with CQ and especially pregnant women and those breastfeeding where the likelihood of transmitting HIV-1 to their infants may be increased. Although all mothers received Nevirapine, which reduces transmission during delivery by approximately 50% [22], we can not identify which transmissions took place via breast feeding. Remarkably all three *post partum* transmissions were in the group of CQ treated mothers.

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Chapter 5

HIV-1 mother to child transmission facilitated by distinctive glycosylation sites in the gp120 envelope glycoprotein

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ABSTRACT

The human immunodeficiency virus type 1 (HIV-1) characteristics associated with mother to child transmission (MTCT) are still poorly understood. We studied a cohort of 30 mothers from Rwanda infected with HIV-1 subtype A or C viruses of whom seven infected their children either during gestation or soon after birth. CD4 counts and viral load did not significantly differ between non-transmitting mother (NTM) versus transmitting mother (TM) groups. In contrast to earlier studies we not only analyzed and compared the genotypic characteristics of the V1-V5 region of the gp120 envelope of viruses found in TM and their infected children, but also included data from the NTM. No differences were found with respect to length and number of potential N-glycosylation sites (PNGS) in the V1-V2 and the V1-V5 region. We identified that viruses with a PNGS on positions AA234 and AA339 were preferably transmitted and that viruses with PNGS-N295 showing a disadvantage in transmission. We also showed that the frequency of PNGS-N339 in the viruses of TM and infected children was significantly higher than the frequency in NTM in our cohort and in viruses undergoing sexual transmission whilst the frequency of PNGS-N295 in children was significantly lower than the frequency in TM and acute horizontal infections. Collectively, our results provide evidence that the presence of the PNGS-N339 site and absence of the PNGS-N295 site in the gp120 envelope confers an advantage to HIV-1 when considering MTCT.

INTRODUCTION

Transmission of HIV-1 from mother to child accounts for a significant proportion of transmissions in those countries where antiviral therapy is still limited. For example, in 2009 over 370,000 children less than 15 years of age were infected with human immunodeficiency virus, with the vast majority via mother to child transmission (MTCT) (www.unaidstoday.org). Known maternal risk factors associated with MTCT are high plasma viral loads, low CD4 T-cell numbers coinciding with advanced maternal immune deficiency and prolonged labor. In the absence of antiretroviral therapy approximately a third of mothers will transmit HIV-1 to their children during pregnancy, labor or through breastfeeding, and at equal ratios [1].

A number of studies focused on analyzing the molecular characteristics of viruses that get transmitted, either through MTCT or sexual intercourse, show that the majority of infections are characterized by the presence of a highly homogeneous viral population [2]. These viruses are predicted to exclusively utilize the CCR5 coreceptor (termed R5 viruses) for infection even when the mothers are shown to carry CXCR4 using variants (termed R5/X4 or X4 viruses) [3], indicative of a strong R5 selection pressure. This is somewhat surprising in children given the predominance of CXCR4 expressing naïve $CD4^+$ lymphocytes in the circulation, a consequence of limited antigen exposure [4,5].

Virus selection during MTCT may be influenced by the binding affinity of the gp120 envelope glycoprotein to a number of cell receptors. A higher affinity for the CD4 receptor or the CCR5 co-receptor may be of advantage for the infecting virus particle. A low net charge within the V3 variable loop of gp120 and the presence of a potential N linked glycosylation site (PNGS) on position 301 of the V3 loop [6-8] are variables that have been associated with higher CCR5 affinity and being the variants preferentially transmitted [9,10].

In MTCT the selection of HIV-1 variants transmitted to children tend to be more resistant to maternal plasma at time of delivery in comparison to viruses from the mother [11,12] Some studies suggest that the properties and function of maternal antibodies (Abs) in MTCT can prevent perinatal transmission or mediate viral selection [13] A number of gp120 envelope characteristics have been associated with resistance against neutralizing Abs, such as the V1-V2-region of the envelope which has been postulated to regulate neutralization sensitivity by occluding conserved epitopes such as the CD4 and/or co-receptor binding sites [14,15]. The V1-V2 length of subtype C viruses has been shown to positively correlate with resistance to broadly cross-neutralizing sera [16-21].

It has been postulated that HIV-1 transmission can be enhanced by binding of the gp120 glycan moiety to the C-type lectin DC-SIGN which is expressed to high levels on immature dendritic cells (DCs) and facilitate infection of $CD4^+$ T lymphocytes either locally or in distal lymph nodes [22-24]. DC-SIGN expressed by a subset of B-cells in the tonsils and blood may also contribute to the infection of $CD4^+$ lymphocytes.[25] If this mode of transmission plays a role in MTCT, selection of the transmitting virus may be driven by more efficient binding of the virus to DC-SIGN.

Here we study a group of 30 pregnant mothers from Rwanda infected with either HIV-1 subtype A or C viruses of whom seven transmitted virus to their children. No correlation was identified between viral loads or $CD4^+$ lymphocyte counts for TM or NTM. We analyzed the gp120 envelope sequences for TM, NTM and infected children. From this analysis we identified two potential glycosylation sites, PNGS-N234 and PNGS-N339, which were associated with viruses that are preferentially transmitted from mother to child and a disadvantage in transmission for viruses with PNGS-N295.

MATERIALS AND METHODS

Study population

This is a sub-analysis as part of a clinical study performed in Kigali, Rwanda as previously described [26]. In brief, the study is composed of a group of 30 HIV-1 infected women from Rwanda and their children. Plasma samples were obtained from both mothers and children at time of delivery and at week 16. Seven children tested HIV-1 positive at 16 weeks after delivery. All the women were given a single dose of nevirapine at the start of labor to reduce *peri-partum* transmission of HIV-1. All children were breastfed during the 16 week period.

Viral load and $CD4^+$ cell count assessment

Blood plasma samples were analyzed for HIV-1 viral loads using the Roche diagnostics RNA PCR quantification assay version 1.5, with a lower limit of detection of 400 copies / ml. Immunologic testing of mothers was performed in two laboratories (National HIV/AIDS Reference Laboratory, Kigali, Rwanda and the Joint Clinical Research Center JCRC, Kampala, Uganda).

HIV-1 RNA extraction

HIV-1 RNA was isolated from blood plasma samples according to the method of Boom *et al* [27].

Children's infection status at birth and week 16

Plasma samples taken at birth were tested in a RT PCR amplification assay covering the HIV-1 gp120 V3 region. Initially, viral RNA was converted to cDNA using Superscript III Reverse Transcriptase (Invitrogen) and the 3' primer V3Not (GCG CGG CCG CCC CCT CTA CAA TTA AAA CTG TG), followed by PCR using Taq DNA Polymerase (Roche), 3'primer 3'V3Not and 5'primer 5'V3Not (GCG CGG CCG CAC AGT ACA ATG TAC ACA TGG). A nested PCR was subsequently performed using 5'primer 5'KSI (ATA AGC TTG CAG TCT AGC AGA AGA AGA) and 3'primer 3'KSI (ATG AAT TCT GGG TCC CCT CCT GAG GA) and the PCR product was analyzed by agarose / ethidium bromide electrophoreses. At week 16 the children's HIV-1 infection status was determined using standard viral load assessment as described above.

Amplification of the V1-V5 envelope region

To amplify the V1-V5 *envelope* region viral RNA was converted into cDNA using the Superscript III Reverse Transcriptase Kit (Invitrogen) and 3'primer A1262 (CTG ACG GTA CAG GCC AGA CAA TTA TTG TC) followed by a PCR using the Expand Long Template PCR system (Roche), according to the manufacturers instructions. For the first PCR 5' primer A1053 (GAA AGA GCA GAA GAC AGT GGC AAT GA) and 3' primer A1262 were used, and for the second PCR the 3'primer was A1322 (TCT TGG GAG CAG CAG GAA GCA C) and the 5'primer A0358 (GAG GAT ATA ATC AGT TTA TGG GA).

Sequencing and analysis

PCR products were sequenced using the ABI PRISM Big Dye Terminator 1.1 Cycle Sequencing Kit and the ABI 3730 XL DNA Analyzer (Applied Biosystems). Sequences were assembled using the CodonCode Aligner program (CodonCode Corporation). Alignments were performed taking the translation codons into account and the sequences were translated into amino acids using the BioEdit program (Ibis Therapeutics). Analysis of the amplified region was performed utilizing the Neighbor-Joining (N-J) method of MEGA version 4. Positions containing an alignment gap were included for pair-wise sequence analysis**.**

Subtype determination

To determine HIV-1 subtype the V1-V5 *envelope* PCR products were sequenced using primers 5' A0385 (GAG GAT ATA ATC AGT TTA TGG GA), 3'A1322 (TCT TGG GAG CAG CAG GAA GCA C), 5'A1360 (GAG CCA ATT CCY ATA CAT TAT TG) and 3'A1191 (ATG GGA GGG GCA TAC ATT GC). The Subtype Reference Alignments of the Los Alamos Data Base (www.hiv.lanl.gov/content/sequence/NEWALIGN/align.htm) year 2005 were used as reference sequences.

Cloning and sequence analysis

V1-V5 envelope PCR products were cloned into the pCRII-TOPO vector (Invitrogen). For each subject and time point between 5 and 15 clones were sequenced using the primers 5'SP6 (ATT TAG GTG ACA CTA TAG), 3'T7 (TAA TAC GAC TCA CTA TAG G), 5'A1360 and 3'A1191. The number of PNGS was

determined using the tool N-GlycoSite of the HIV sequence Database (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). The mean intra-patient diversity was calculated with the use of MEGA version 4. The amino acid length and number of PNGS of the viral envelopes were compared using GraphPad Prism version 5.00 using the Mann-Whitney test, with P-values < 0.05 being considered statistically significant. The overall V3 net positive charge was calculated by counting the number of positive charged amino acid residues R and K and the number of negative charged amino acid residues D and E being subtracted [28].

Direct sequencing of the C2-C3 region

To expand the group of NTM for comparison of the frequency of PNGS-N295 and PNGS-N339 we performed direct sequencing on the V1-V5 *envelope* PCR products of 10 additional mothers encompassing amino acids 245 till 370 (HXB2 numbering) using 5'primer (GCG CGG CCG CAC AGT ACA ATG TAC ACA TGG) and 3'primer (AT TTC TAA GTC CCC TCC TGA).

Comparison of the frequency of PNGS-N295 and PNGS-N339

The percentages of glycosylation on pos. AA295 and AA339 (HXB2 numbering) for subtype A and C were calculated separately for the envelope sequences from the group of NTM, children infected in uterine (C2)[29], children infected by breastfeeding (C3)[30], breastfeeding TM (TM2)[31], acute horizontal infections (contr.1)[32-34] and a random selection of chronic infections acquired by horizontal transmission from the Search Interface of the HIV Sequence Database (contr.2). (http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html). In accord with the proportions of subtype A (5 out of 7) and C (2 out of 7) in our group of TM and children, percentages of individuals positive for glycosylation site N295 or N339 in the groups C2, C3, TM2, NTM, contr.1 and contr.2 were adjusted and the results analysed using The Supervised Comparison of Subsets.[35] P-values < 0.05 were considered statistically significant.

RESULTS

High viral loads and low $CD4^+$ T-cell counts were not the only determinants for HIV-1 transmission

Viral loads and $CD4^+$ T-cell counts were measured and compared for the 23 NTM versus seven TM (Table 1) (Fig. 1A and 1B). Four mothers gave birth to HIV-1 positive children as determined by positive HIV-1 RT-PCR reactions. This indicates three of 26 mothers (11.5%) were infected either *intra-partum* or during early breastfeeding, fitting with known HIV-1 transmission rates when mothers given nevirapine at time of delivery [36]. TM were not found exclusively in the group with high viral loads and low $CD4^+$ T-cell counts. Three out of the seven TM had $CD4^+$ cell counts >500 cells/µl, and three of seven had viral loads lower than 10,000 copies/ml, with one mother in both categories. These data suggest that factors other than viral load and $CD4^+$ cell counts can influence HIV-1 transmission.

Table 1. Characteristics of the not transmitting mothers

pat.nr	Subtype	Viral load W00 cies/ml	CD4 count W00 cells/ul
20	?	561	562
30	A	3780	628
40	A	3377	672
50	A	12703	258
60	A	11463	434
110	A	8329	990
150	A	2594	299
160	A	396915	164
180	C	4325	952
190	C	1900	342
200	A	17213	370
210	C	2194	1355
220	A	2092	275
240	?	252	644
260	A	19376	655
270	C	111701	387
280	C	654	214
340	A	6827	546
350	A	320	692
360	A	1197	846
380	A	4339	602
390	C	1459	602
400	A	62453	381

Subjects indicated by gray shadows are used in the analysis

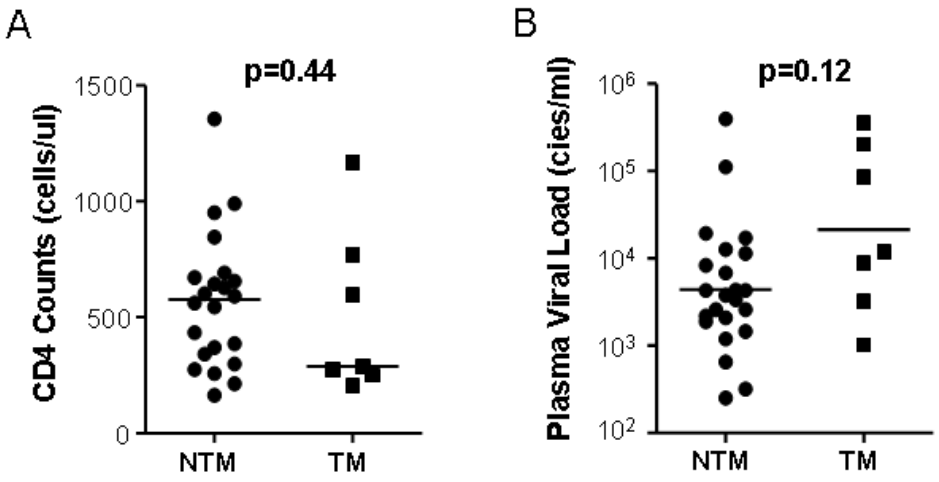

Figure 1. Comparison of CD4 T-cell counts and viral loads in TM and NTM.
The CD4 T-cell counts in cells/ul (**A**) and the viral loads in number of copies/ml (**B**) from NTM and TM are not significantly different. P-values were calculated using the Mann-Whitney test. The horizontal lines indicate the median for each set.

Phylogenetic analyses of sequences of mothers and children

The V1-V5 region of the gp120 envelopes of NTM and TM was sequenced. Out of the seven TM two (29%) were infected with subtype C viruses while five (71%) were infected with subtype A viruses. Of the group of 23 NTM, six (26%) were infected with subtype C and 15 (65%) with subtype A viruses and for two mothers the virus subtype was undetermined.

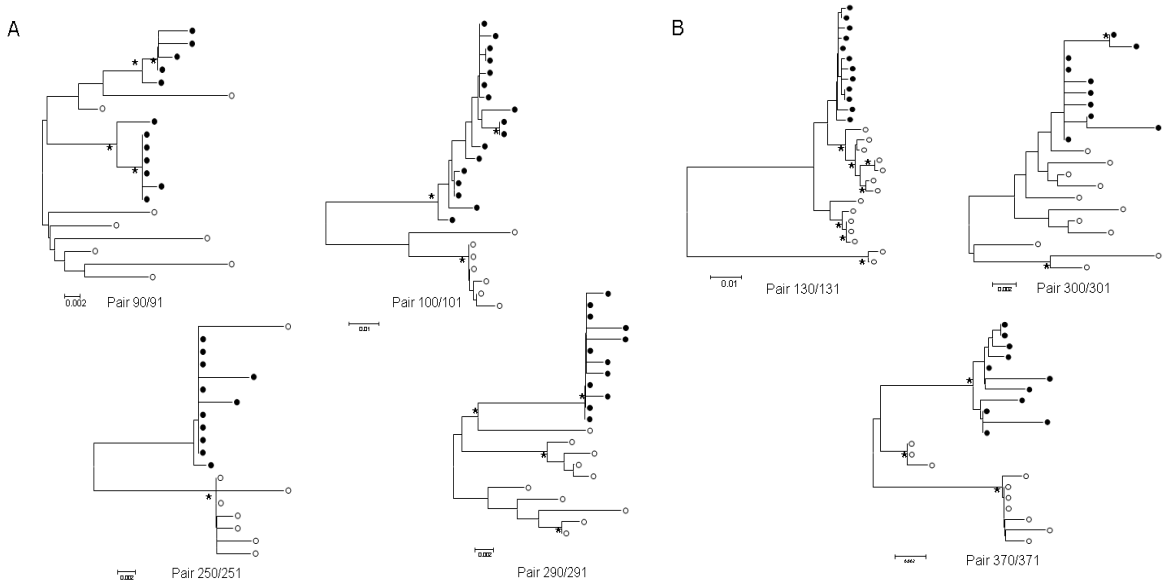

Figure 2. Phylogenetic analysis V1-V5 *envelope* sequences
The unrooted neighbor-joining trees show sequences of 7 mother-child pairs. (**A**): Sequences of day of birth of four mother-child pairs of which the children tested positive at day of birth. (**B**): Sequences of 16 weeks after birth of three mother-child pairs of which the children tested negative at the day of birth, but positive at week 16. In one case transmission of two variants occurred (pair 090/091) and in the six other cases a single variant was transmitted. Mother's sequences are depicted with open circles and children's sequences with closed circles. Bootstrap values higher than 85 are indicated with an asterix**.**

We cloned and sequenced the V1-V5 regions of eight NTM and seven TM and their infected children. Phylogenetic analysis of the sequences of the V1-V5 regions of mothers and children confirmed the relatedness of the virus circulating in the respective mother child pairs (data not shown). Four of the seven children were infected *in utero* of whom child 091 demonstrated two separate variants (Fig. 2A). All other infected children had only one HIV-1 variant while the viral quasi-species from the TM contained multiple variants (Fig. 2B). The intra patient mean genetic distances of the V1-V5 region were analyzed (Fig. 3A). The mean genetic distance in NTM was 0.022 (95% CI 0.007-0.037; n=8) and was similar to that of TM [0.025 (95% CI 0.004-0.046; n=6)] (p=0.82) or from the children [0.007 (95 CI 0.003-0.012; n=6)] (p=0.12), however, the mean intra-patient diversity in the children was significantly lower than in the TM (p=0.007), which was in agreement with the transmission of one virus variant in six out of seven children and two variants from one child.

V3 regions with a PNGS on position AA301 and a charge of +3 or +4 are transmitted

We next analyzed the number of PNGS and the overall V3 charge in the three groups. PNGS-N301 (HXB2 numbering) which is associated with CCR5 usage was found in the majority of clones from TM (100% in 6/7 and 90% in 1/7), the NTM (100% in 6/8, 25% and 0% in 1/8) and was present in 100% of the clones of all children. The mean V3 charges of viruses from NTM varied between 1.89 - 4.03 (average 3.17) and for TM between 2.58 - 5.00 (average 3.69) and was found to be narrower at between 3.00 - 3.91 (average 3.40) in the children (Fig. 3B).

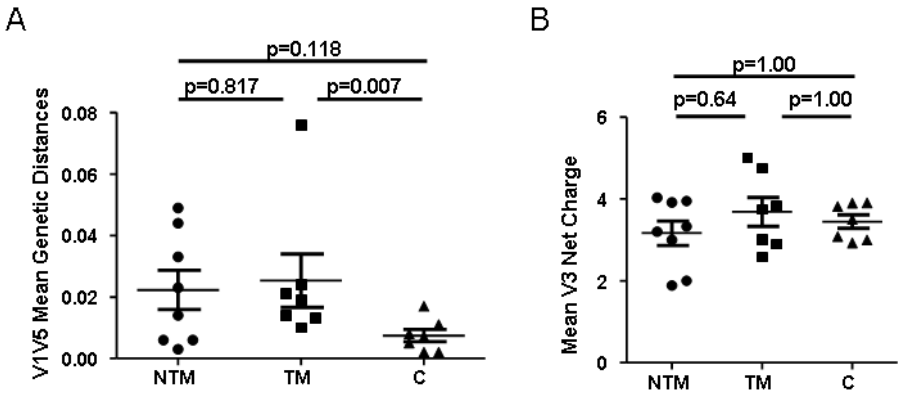

Figure 3. Viral characteristics between NTM, TM and children
(**A**) The Mean Intra Patient Diversity of the V1-V5 envelope region in the Children (C) was significantly lower than in TM, but not significantly different from the NTM. P-values were calculated using the Mann-Whitney test. The horizontal lines represent the mean and the Standard Error of the Mean. **(B)** The mean net charges of the V3 loop were not significantly different between NTM, TM and Children (C), but the range of the mean V3 charges of viruses from children was narrower than in the mothers. The horizontal lines represent the mean and the Standard Error of the Mean. P-values were calculated using the Mann-Whitney test.

No differences in V1-V2 and V1-V5 length and number of PNGS

To identify differences between the virus variants in the NTM and TM or children we analyzed the envelope sequences. We compared the size and the number of PNGS of the V1-V5 region and the V1-V2 region. The mean length of the V1-V5 region of amplified products from the NTM was 342.1 (95% CI 336.6-347.5) amino acids, in the TM 342.3 (95% CI 337.0-347.5) amino acids (p=0.95) and in the children 340.0 (95% CI 334.8-345.1) (p=0.70) amino acids (Fig. 4A). The mean number of PNGS in the V1V5 region of the viruses in the NTM was 22.0 (95% CI 20.5-23.6), in the TM 22.2 (95% CI 20.6-23.7) (p=1.00), and in the children 23.0 (95% CI 21.2-24.8) (p=0.39) (Fig. 4B). The mean size of the V1-V2 of NTM was 73.0 (95% CI 66.4-79.7) amino acids, in the TM 68.8 (95% CI 64.9-72.8) (p=0.24) and in the children 68.6 (95% CI 64.5-72.7) (p=0.22). Overall, we did not find significant differences between the NTM, the TM or the children in length and number of PNGS in either the V1-V2 or V1-V5 envelope regions.

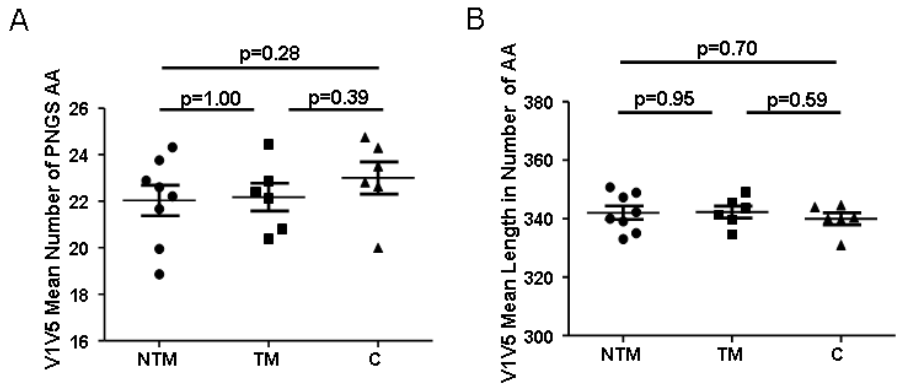

Figure 4. V1-V5 envelope length and number of PNGS in NTM, TM and children
No significant differences in V1-V5 length (**A**) and number of PNGS (**B**) between NTM, TM and children (**C**) are observed. P-values were calculated using the Mann-Whitney test. The horizontal lines represent the mean and the standard error of the mean.

Selective transmission of viruses with a PNGS on N234 and N339

In order to further identify envelope PNGS differences we mapped the sites in NTM, TM and children. Six PNGS were found conserved in all variants for all groups (HXB2 numbering N136, N156, N198, N262, N332 and N447), with three sites showing a difference between the groups (Table 2). PNGS-N234 (in C2) was

Table 2. Characteristics of the mother-child pairs

Transmitting Mothers				Children		
pat.nr	clade	Viral load W00 cies/ml	CD4 count W00 cells/ul	pat.nr	PCR W00	Viral load W16 cies/ul
90	A	203333	250	91	pos.	750000
100	A	355714	275	101	pos.	sna
130	C	85173	595	131	neg.	7590
250	C	3214	206	251	pos.	269000
290	A	1018	1168	291	pos.	531000
300	A	12104	770	301	neg.	747000
370	A	8803	289	371	neg.	144000

pos., positive.
neg., negative.
sna, sample not available

present in all of the children's clones, in the majority of the clones of TM (86%) and less abundant in the NTM (62%). Only 60% of the clones of TM 290 had a PNGS at position N234, whereas 100% of her child's (291) clones possessed this PNGS, suggesting an advantage in transmission. PNGS-N295 (between C2 and V3) was present in all NTM clones, the majority of clones in four/seven TM and in four/seven children, which would suggest a disadvantage for transmission, however, when the PNGS is present transmission can occur. Six of seven children demonstrated PNGS-N339 in all virus variants which was present in the majority of clones from TM (five/seven) and was found in only two/eight NTM. PNGS-N339 was absent in all the clones of mother 370 and the corresponding child 371. Only 13% of the clones of mother 250 held PNGS-N339 whereas her child (251) had

100% of viruses carrying this PNGS. The above findings suggest that variants with PNGS-N234 and/or PNGS-N339 were preferentially transmitted and that variants with PNGS-N295 had a disadvantage.

To verify our above findings we compared our results with data from different Source's (Search Interface of the HIV Sequence Database) where we could compare sequences of subtype A or C viruses from children infected *in utero* (C2/ N=40), from children infected through breastfeeding (C3/ N=40), as well as from mothers who transmitted the virus by breastfeeding (TM2/ N=83). Data from the virus populations of NTM were not available in the database. We performed a direct sequence analysis of 10 additional NTM from our cohort to increase the overall study group size. Viral sequences from acute infection (AI) patients of subtype A and C were included to verify if this PNGS is associated with recently sexual intercourse transmitted viruses (control 1/ N=90). We also included a random selection of sequences from drug naïve African individuals of subtype A and C (control 2/ N=318) to verify the prevalence of the PNGS at position 295 and 339 in individuals other than mothers or children (Fig. 5A and 5B, respectively). If more than one sequence of an individual was available we calculated the consensus of these sequences. We matched the percentages of individuals positive for the PNGS to equivalent proportions of subtype A and C in the groups of TM and children that we investigated (C1 and TM1). In order to determine significance we performed a Supervised Comparison of the Subjects with the children groups C1, C2 and C3 as the reference group for the G-standard (Fig. 5).

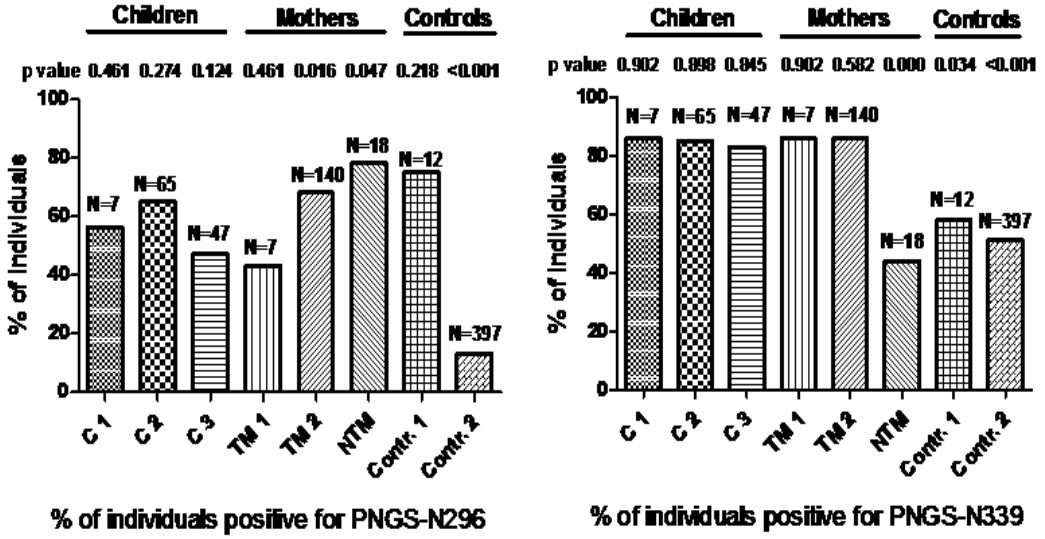

Figure 5. PNGS percentages at positions AA295 and AA339 (HXB2 numbering)
The subtype A and subtype C sequences from our TM and children were compared to an equivalent proportion of subtype A and subtype C viruses from the literature. C1 - Children infected in uterine, peri-natal or by breastfeeding. C2 - Children infected in uterine. C3 - Children infected by breastfeeding. TM1 - in uterine, peri-natal or by breastfeeding TM. TM2 - by breastfeeding TM. NTM - Non-transmitting mothers. Contr.1 - acute horizontal infections. Contr.2 - random selection of chronic infections acquired by horizontal transmission. The data for C1, TM1 and NTM are from our cohort; the data C2, C3, TM2, Contr.1 and Contr.2 are from the Search Interface of the HIV Sequence Database. P-values were calculated using The Supervised Comparison of Subsets [35] with the children groups C1, C2 and C3 used as the reference group for the G-standard.

Due to the fact that sequences of groups C2, C3 and TM2 did not encompass PNGS-N234 we could not analyze this PNGS. When analyzing further PNGS-N295 we found it to be present in 50-70% children (C1, C2, C3), which was significantly reduced when compared with TM2 (70%) and the acute horizontal transmissions (control 1) (69%) (both p=0.001) which is in concordance with a disadvantage in MTCT for envelopes carrying PNGS-N295 (Fig. 5A). For PNGS-N339 we found this site to be present in over 79-86% of the consensus sequences for both TM and children, whereas for NTM and control 1 and control 2 the frequency was significantly reduced (43%) (p=<0.001), 44% (p=<0.001) and 51% (p=<0.001) respectively, indicating a strong positive selection in MTCT for virus variants with a PNGS on position AA339 in the C3 region of the viral envelope (Fig. 5B).

DISCUSSION

From our V1-V5 gp120 sequence analysis of viruses preferentially transmitted between mothers and their children and in comparison to non-transmitting viruses we identified a higher frequency of PNGS at positions AA234 and AA339 and a lower frequency of PNGS at position AA295 in transmitted viruses. We confirmed the results of preferential transmission of viruses with PNGS at position N339 and the disadvantage in transmission for viruses with PNGS-N295 in MTCT through analyzing additional cohorts of MTCT as well as in individuals in their acute infection period and infected through sexual intercourse. We should highlight that for PNGS-295 the observation is more group based than at the individual transmission pair level.

In sexual transmission HIV-1 subtype A and C variants with shorter envelope and fewer PNGS have been shown to be more efficiently transmitted and more susceptible to neutralizing antibodies present within the donor [37-39]. One report concludes that variants transmitted from mother to child did not have shorter V1-V5 regions but did have fewer PNGS [11], whilst another study showed no association between transmitted variants and the length of the variable loops or the number of PNGS [40]. We did not find differences in the number and the length of the V1-V2 and the V1-V5 regions between NTM, TM and children in our cohort. Probably resistant viruses are preferentially transmitted from mother to child but it is not clear how changes in length of the envelope and in the number of PNGS correlate with MTCT [11,12,37]

A low overall net charge and a PNGS at N301 within the gp120 V3 variable loop have been heavily associated with CCR5 usage [7]. In agreement with other reports that show selection of CCR5 using variants in transmission we found that all transmitted viruses contained PNGS-N301 and that the V3 charge of these viruses was restricted to +3 and +4, although the viruses of both mothers' groups had V3 charges ranging from +2 to +5. The majority of studies to date have reported on viruses with low V3 charges being preferentially transmitted and here we describe a narrow range in V3 charges within children even when mothers contain quasi-species of viruses with lower charges. This could result from such viruses being the major variants in the TM, but could also reflect a less stable electrostatic interaction between gp120 and the array of receptors to which it binds and which may influence HIV-1 transmission, including CD4, CCR5 or DC-SIGN [39,43]. PNGS N197, N230, N241, N301, N332, N386, N392, N397 (HXB2 numbering) have been identified as candidates to play a role in DC-SIGN binding and may therefore exert such an influence through modulating this specific interaction [44,45].

The selection for viruses with PNGS-N339 in viruses undergoing MTCT and not in acute horizontal infections could be explained by neutralizing antibody selection. Children will possess antibodies from the mothers whereas individuals sexually exposed to HIV-1 will not have such antibodies and can therefore be infected with viruses lacking PNGS-N339. The function of PNGS-N339 in the envelope of the children remains to be determined, however, PNGS-N339 is situated in the α2-helix region of C3 and several reports have demonstrated that residues within the α2-helix of subtype C viruses are under strong positive selection and that unique mutational patterns in the α2-helix and acquisition of length in gp120 hyper variable domains are associated with resistance to autologous neutralization of HIV-1 subtype C [46]. The disadvantage in MTCT for PNGS-N295 can be explained by the generation of resistance against neutralizing antibodies such as 2G12 [47].

The further clarification of mechanisms of HIV-1 MTCT and the viruses involved will provide insights into which gp120 envelope regions may need to be targeted in a vaccine aimed at restricting transmission. Therapeutic vaccination of HIV-1 positive mothers during their pregnancy against such variants may have the benefit of restricting MTCT during all possible transmission periods and allow for lower rates of infection. We demonstrate that PNGS-N234 and PNGS-N339 within the gp120 envelope associate with risk of viral transmission from mother to child. Such knowledge on which viruses are preferentially transmitted may aide in the future design of HIV-1 vaccine immunogens aimed at restricting MTCT.

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Chapter 6

HIV-1 autologous antibody neutralization associates with mother to child transmission

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ABSTRACT

The HIV-1 characteristics associated with mother to child transmission (MTCT) are still poorly understood and if known would indicate where intervention strategies should be targeted. In contrast to horizontally infected individuals, exposed infants possess inherited antibodies (Abs) from their mother with the potential to protect against infection. We investigated the HIV-1 gp160 envelope proteins from seven transmitting mothers (TM) whose children were infected either during gestation or soon after delivery and from four non-transmitting mothers (NTM) with similar viral loads and CD4 counts. Using pseudo-typed viruses we tested gp160 envelope glycoproteins for TZM-bl infectivity, CD4 and CCR5 interactions, DC-SIGN capture and transfer and neutralization with an array of common neutralizing Abs (NAbs) (2F5, 2G12, 4E10 and b12) as well as mother and infant plasma. We found no viral correlates associated with HIV-1 MTCT nor did we find differences in neutralization with the panel of NAbs. We did, however, find that TM possessed significantly higher plasma neutralization capacities than NTM (P=0.002). Furthermore, we found that *in utero* (IU) TM had a higher neutralization capacity than mothers transmitting either *peri-partum* (PP) or via breastfeeding (BF) (P=0.002). Plasma from children infected IU neutralized viruses carrying autologous gp160 viral envelopes as well as those from their corresponding mothers whilst plasma from children infected PP and/or BF demonstrated poor neutralizing capacity. Our results demonstrate heightened autologous NAb responses against gp120/gp41 can associate with a greater risk of HIV-1 MTCT and more specifically in those infants infected IU. Although the number of HIV-1 transmitting pairs is low our results indicate that autologous NAb responses in mothers and infants do not protect against MTCT and may in fact be detrimental when considering IU HIV-1 transmissions.

INTRODUCTION

According to the 2011 UNAIDS Progress report an estimated 2.7 million people worldwide were newly infected with HIV-1 in 2010. Of this 390,000 were infants with the majority resulting from mother-to-child transmission (MTCT). Known maternal risk factors associated with MTCT are high plasma viral loads, low CD4 T-cell numbers coinciding with advanced maternal immune deficiency and prolonged labor [1]. In populations where replacement feeding is not feasible it has been estimated that 41% of MTCT occur *in utero* (IU), 20% *peri-partum* (PP) and the remaining 39% during prolonged breastfeeding (BF) [2]. The majority of transmissions are found in regions where antiretroviral therapy availability is limited, such as sub-Saharan Africa (UNAIDS Progress report 2011) and specifically regions where HIV-1 subtype A and C predominate, including the growing number of infections in Russia [3]. Little is known regarding mechanisms determining risk of MTCT but better understanding of such events will be critical in designing effective means to limit transmissions.

As seen with HIV-1 sexual transmission the established viruses in MTCT predominantly utilize the CCR5 coreceptor (R5) for cell entry and rarely CXCR4 (X4) [4,5]. Earlier studies have indicated that HIV-1 transmissions are initiated by a single or limited number of donor viruses, often a minor variant, indicating a bottleneck in transmission or selective outgrowth of transmitted variants [6,7]. Much attention has focused on defining the genetic and phenotypic properties of the HIV-1 gp120/gp41 envelope glycoprotein (Env) of HIV-1 since this directs the receptor and coreceptor interactions that determine infection. The Env is also the major target of the host immune response and induces binding antibodies

(Abs), some of which are neutralizing (NAbs) that can control or prevent infection [8-10]. There has been much speculation that viral fitness may determine MTCT with some studies showing that viruses from transmitting mothers (TM) possess higher replication capacities than viruses generated from non-transmitting mothers (NTM) [11,12]. Two studies found no difference in infectivity between mothers and children's *env* clones tested in a single-cycle assay [13,14]. A study comparing Env pseudo-typed viruses generated from subtype C infected MTCT pairs demonstrated that Env from children have a higher replication capacity than Env from the mothers which is V1V5 restricted [15]. Additionally, no differences were found between transmitted and non-transmitted viruses for their capacity to utilize CD4 or the CCR5 coreceptor [13,14,16].

Studies of adult HIV-1 transmission pairs in Africa have shown that viruses undergoing horizontal transmission possess Env genotypes with shorter variable loops and fewer numbers of potential N-linked glycosylation sites (PNGS) which can associate with the development of anti-HIV-1 Ab responses [17]. Correlations between variable loop length and number of putative PNGS have been reported for MTCT. In some studies fewer Env PNGS are found in the transmitted viral variants whilst other studies do not find differences in total number but have found the position of the PNGS to associate with risk of transmission [14,18-20].

In MTCT Abs are present in the exposed child having been passed from the mother. The common perception is that these Abs protect against HIV-1 infection or select variants undergoing transmission. In agreement with this notion, animal models indicate that Abs can reduce or prevent MTCT [21-23]. Reports on human mother child pairs have shown better neutralization by NTM than by TM suggesting a protective role by Abs [24,25]. Others report better neutralization by TM or find no differences between TM and NTM [12,14,18,24,26-29]. Neutralization resistance in children against mother's plasma or serum has been reported suggesting transmission of neutralization escape mutants, but, in contrast, sensitivity for neutralization by plasma of the mother has also been found [18,24,26,27,30,31]. These discrepancies may depend on differences in viral subtype, mode of transmission, timing of transmission, timing of sampling or the selective study of autologous versus non-autologous viruses. Although the role of maternal NAbs in MTCT is controversial trials with HIV-Ig have been conducted. One demonstrated protection against IU transmission whilst the other revealed a significant increase in the number of infections at birth and 2 weeks after delivery in the treated versus untreated group [32,33].

IgG transport from the placenta to the fetus during gestation is mediated by the FcRn receptors expressed on the syncytiotrophoblast followed by transcytosis [34]. The FcRn receptor is also expressed on intestinal mucosa and functions in the uptake of IgG from breast milk. Although the FcRn receptor binds monomeric IgG more avidly than aggregated or immunocomplexed IgG the mechanism may provide a route whereby HIV-1/anti-HIV-1 IgG complexes can be transferred to the fetus [35]. Total IgG binding to gp160, gp120 glycoproteins and/or peptides derived from V3 or gp41 was measured in TM and NTM individuals with better binding found in the TM, which may be in line with heightened enhancement to transcytosis of HIV-1 via HIV/IgG complexes [12,36-38]. In addition, it has been demonstrated in studies utilizing a trophoblast monolayer model mimicking the barrier between the placenta and fetal blood that the fusion of HIV-1 infected PBMCs, monocytes and macrophages at the apical side of the monolayer can be followed by HIV-1 transcytosis to the lateral side [35,39]

HIV-1 MTCT requires passage of virus across a mucosal barrier, be it via the amniotic fluid during IU, from blood and vaginal secretions during IP and/or virus present in breast milk during feeding. Experiments using *ex vivo* fetal oral and intestinal tissue model systems have indicated that infectious cell free HIV-1 can traverse the oral as well as intestinal mucosa and that HIV-1 infected macrophages can also transmigrate across these surfaces [40]. To a lesser extent HIV-1 infected lymphocytes were able to cross the intestinal epithelium and establish infection. It has been widely postulated that HIV-1 mucosal infection can be heightened through the interaction of virus with an array of C-type lectins, including DC-SIGN, expressed on dendritic cells (DCs) lying below the mucosal epithelium [41-43]. One study associating a specific DC-SIGN genetic polymorphism with risk of HIV-1 infection indeed suggests that this molecule has a role to play in viral transmission [44]. DC-SIGN allows for the capture of virus and its heightened transfer to $CD4^+$ lymphocytes, termed *trans*-infection. We have shown that this mechanism can be enhanced when virus is coated with Abs and taken up via the Fc receptor on DCs and that the neutralization effects of numerous Abs can be negated through such an interaction [45]. A number of glycoproteins present in human bodily secretions, including milk, have been reported to bind DC-SIGN and block HIV-1 capture [46-49]. One such molecule, bile-salt stimulated lipase (BSSL) from milk, is highly polymorphic with the variant forms differentially inhibiting viral capture and infection of $CD4^+$ lymphocytes [49]. How well virus interacts with DC-SIGN or escapes natural inhibitors may provide the virus with a phenotypic advantage in MTCT.

Here we study Env pseudo-typed viruses generated from TM, NTM and their children to identify phenotypes that associate with the risk of MTCT. From this analysis we conclude that strong autologous neutralization activity can associate with risk of transmission indicating that high gp120/gp41 NAb responses may in fact be detrimental.

RESULTS

Selection of *env* clones for study

From a cohort of 30 pregnant HIV-1 positive women from Rwanda we generated Env pseudo-typed viruses from seven MTCT pairs (090/091, 100/101, 130/131, 250/251, 290/291, 300/301 and 370/371), four infected IU and three PP or BF (Table 1). We included a group of four NTM (160, 200, 270 and 380) infected with similar viral loads and CD4 counts. We generated pseudo-typed viruses expressing whole gp160 Env derived from plasma of mothers and children and screened for infectivity on TZM-bl cells. The *env* clones that gave rise to infectious pseudo-typed viruses were sequenced and subjected to phylogenetic analysis (Fig. 1). This analysis confirmed the relatedness of the virus circulating in the respective mother child pairs. One transmission pair (130/131) was infected with subtype C virus, five pairs were infected with subtype A (090/091, 100/101, 290/291, 300/301 and 370/371) and one pair (250/251) carried an AC recombinant. Three NTM (160, 200, and 380) harbored a subtype A virus and one (270) subtype C. Earlier analysis on this cohort showed that six out of the seven pairs demonstrated transmission of a single virus variant, whilst pair 90/91 showed transmission of two variants [20] (Fig. 1). Since the viral population in each child was homogenous we selected one clone from each for analysis, with exception of child 091 where both variants were analyzed. For each TM and NTM we randomly selected two variants. From three TM and two

Table 1. Characteristics Transmitting Mothers, Non-Transmitting Mothers and Children

Mothers							Children				
Subject	NTM/TM	Subtype	Viral load screen plasma RNA cies/ml	Viral load week 0 plasma RNA cies/ml	CD4 count week 0 cells/ul	Time point clones (weeks after birth)	Time point late plasma	Subject	PCR week 0	Viral load week 16 RNA cies/ml	Time point clones (weeks after birth)	Time point plasma (weeks after birth)
90	TM	A	131,000	203,333	250	4	week 17	91	Pos	750,000	0	16
100	TM	A	208,000	355,714	275	0	week 16	101	Pos	sna	0	sna
130	TM	C	61,900	851,73	595	0	week 18	131	Neg	7,590	16	16
250	TM	A/C	8,820	3,214	206	4	week 18	251	Pos	269,000	0	16
290	TM	A	1,570	1,018	1168	16	week 18	291	Pos	531,000	0	16
300	TM	A	7,130	12,104	770	8	week 18	301	Neg	747,000	16	16
370	TM	A	5,430	8,803	289	4	week 16	371	Neg	144,000	16	16
160	NTM	A	84,400	396,915	164	4	week 16	161	-	-	-	sna
200	NTM	A	7,110	17,213	370	4	week 18	201	-	-	-	16
270	NTM	C	19,700	111,701	387	4	week 16	271	-	-	-	16
380	NTM	A	7,840	4,339	602	8	week 18	381	-	-	-	16

TM: transmitting mother, NTM: non-transmitting mother, Pos: positive, Neg: negative, sna: sample not available.

NTM the clones were shown to closely cluster and from mothers 300, 290, 370 and 090 only two variants were available and both were utilized. From mother 100 we selected a third variant (100P37) based on sequence prediction indicating it to be a R5/X4 dual-tropic virus. We investigated the constructed Env pseudo-typed viruses from NTM, TM and IC for properties such as entry, receptor and coreceptor usage, interaction with DC-SIGN as well as neutralization sensitivity.

Env pseudo-typed viruses from TM and NTM more infectious than viruses from children

We tested the infectivity of all pseudo-typed viruses from NTM, TM and children in the TZM-bl single-cycle infection assay. Limiting dilutions of the viral stock was performed to determine that the readings were within the linear range when testing up to 5ng CA-p24 input. The infectivity median was determined by measuring the Relative Luciferase Units (RLU) normalized to the amount of capsid p24 (CA-p24) (Fig. 2). We found no difference between viruses of NTM and TM but the children's virus clones showed significantly lower entry efficiency than the TM (P = 0.04) indicating that high infectivity is not associated with virus selection during transmission.

No differences in virus affinity for CD4 between TM, NTM or children

We examined the affinity for the CD4 receptor by incubating the selected pseudo-typed viruses with increasing concentrations of sCD4 on TZM-bl cells (Fig. 3A). The median 50% inhibitory concentration (IC_{50}) of sCD4 in the pseudo-typed viruses from TM tended to be lower than the median in the NTM (P = 0.06) suggesting higher affinity for the CD4 receptor in the TM group. The median IC_{50} of sCD4 in TM and children was not significantly different. We also measured the effect of CD4 directed monoclonal Ab OKT4 by incubating TZM-bl cells with increasing concentrations of OKT4 before infection with pseudo-typed viruses (Fig. 3B). No difference was found in the median

IC_{50} of OKT4 in the NTM compared with the IC_{50} in TM, but the median IC_{50} in the children tended to be lower than in TM (P = 0.06), suggesting lower affinity for the CD4 receptor of viruses from children compared to mothers. In conclusion, no evidence was found for transmission selection of viruses with higher affinity for the CD4 receptor in our cohort.

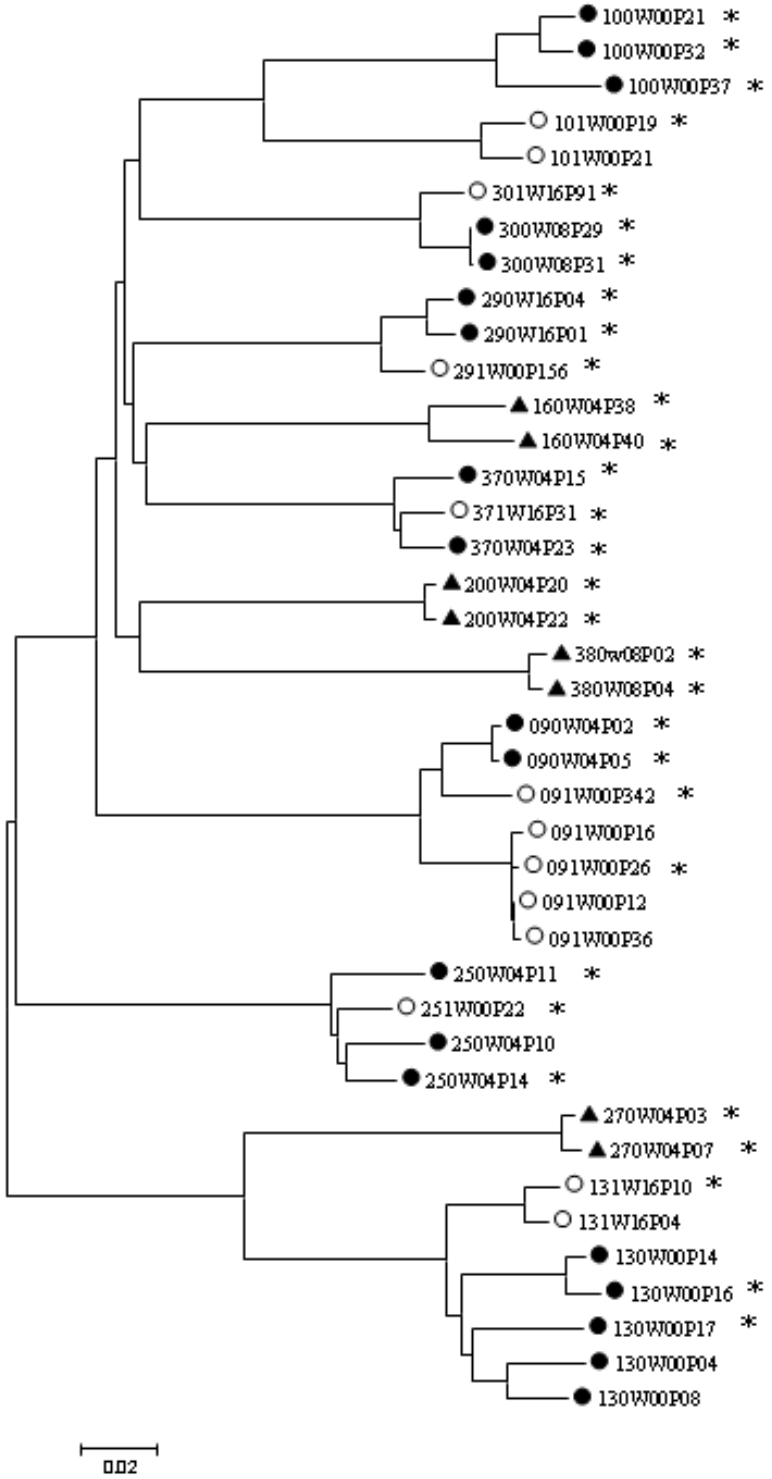

Figure 1. Phylogenetic analysis of the gp160 envelope region
The analysis was performed using the Neighbor-Joining (N-J) method of MEGA version 4. Positions containing an alignment gap were included for pair-wise sequence analysis. TM depicted by closed circles, IC by open circles and NTM by triangles. The clones selected for further analyses are indicated with an asterix.

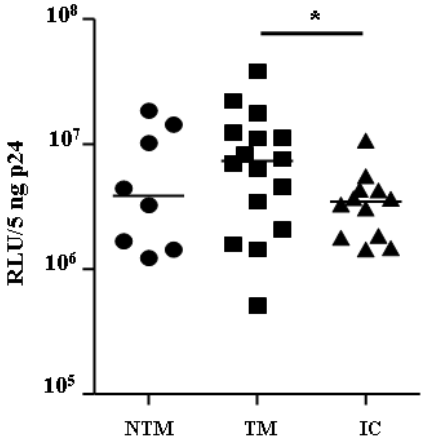

Figure 2. Infectivity of TZM-bl cells with Env pseudo-typed viruses
Viruses were tested from NTM, TM and infected children (IC) with results expressed in relative luciferase units (RLU). *Probability that the groups are similar is <0.05.

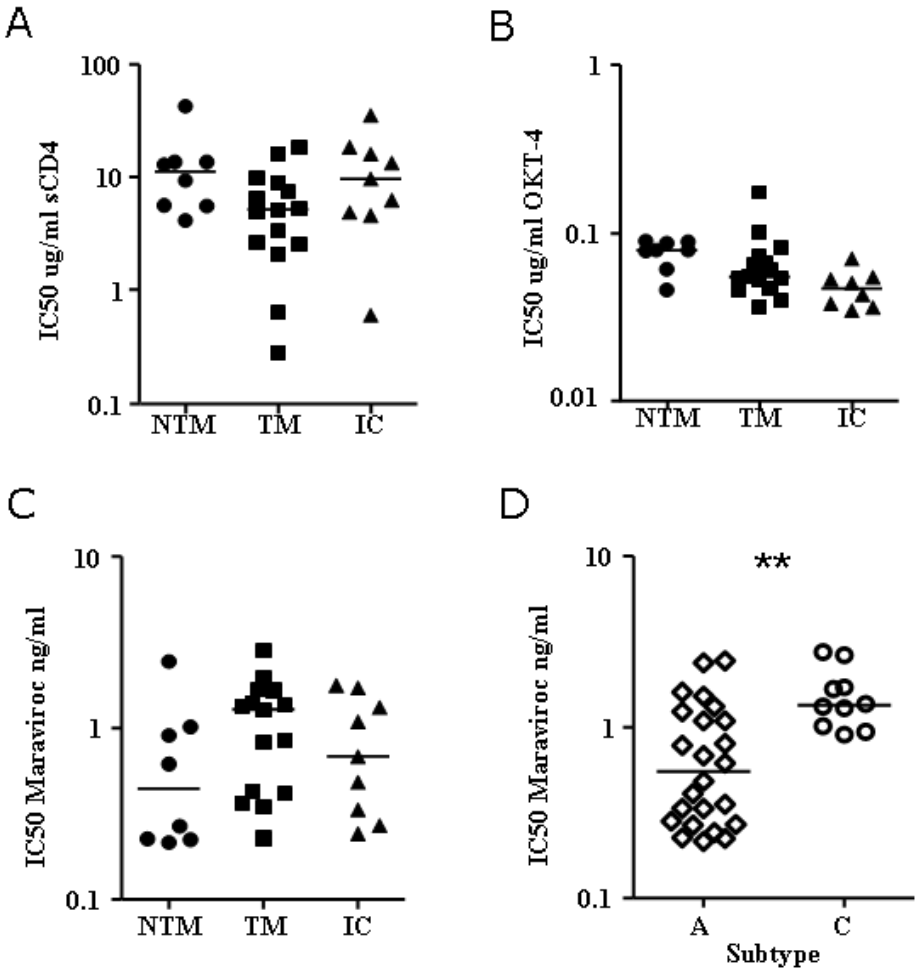

Figure 3. Inhibition of Env pseudo-typed viruses from NTM, TM and infected children (IC) expressed as IC_{50}'s
(**A**) sCD4, (**B**) αCD4 Ab OKT4 and (**C**) CCR5 antagonist maraviroc. NTM are represented by dots, TM by squares, and IC by triangles. Competition with maraviroc of subtype A (open diamonds) versus subtype C (open dots) viruses is shown in (**D**). **Probability that the groups are similar is < 0.01.

No differences in NTM, TM or children's virus interacting with the CCR5 coreceptor

We next determined whether there were differences in virus coreceptor utilization between the different groups. Initially we determined the coreceptor usage of the Env pseudo-typed viruses by incubating TZM-bl cells with the CXCR4 inhibitor ADM3100, with the CCR5 inhibitor maraviroc and with both before infection with the selected pseudo-typed viruses. One variant from TM 100 was found to be dual-tropic for CCR5 and CXCR4 whilst all other Env's from NTM, TM and children were exclusively CCR5 using. We then examined the affinity of the Env pseudo-typed viruses for the CCR5 receptor by incubating TZM-bl cells with increasing concentrations of maraviroc and determining IC_{50} values (Fig. 3C). The dual-tropic clone of mother 100 was excluded from this analysis. We found no significant differences between the three groups, but the median IC_{50} of subtype A gp160s were significantly lower than for subtype C (P = 0.006) (Fig 3D). When we analyzed the gp160s of the two subtypes separately no significant differences in IC_{50} values were found between NTM, TM and children. We can conclude that enhanced affinity for the CCR5 coreceptor was not providing selection in MTCT.

No differences in Env interaction with DC-SIGN or efficiency of virus transfer from DC-SIGN bound viruses to TZM-bl cells from TM, NTM or children

Since it has previously been shown that DC-SIGN polymorphisms can associate with risk of transmission [44] and that factors found in bodily secretions, such as breast milk, can bind this molecule [49] we choose to study how our HIV-1 pseudo-typed viruses interacted with this specific C-type lectin. To determine whether there is a role of DC-SIGN selection in MTCT in our cohort we examined the interaction of the selected pseudo-typed viruses in a DC-SIGN capture assay (Fig. 4A). We found no significant differences between Env pseudo-typed viruses between NTM, TM and children. We subsequently tested the transfer of DC-SIGN captured pseudo-typed viruses to TZM-bl cells to mimic *in vivo* transfer to $CD4^+$ lymphocytes (Fig. 4B). We found no differences in infection of transferred pseudo-typed viruses between NTM and TM, but the transfer by children viruses tended to be lower (P = 0.07) reflecting the lower direct infectivity previously observed with the same viruses (Fig. 2). Since DC-SIGN binding is influenced by the gp120 glycan composition we investigated the correlation between the number of PNGS in the V1V5 region of the pseudo-typed viruses and the levels of CA-p24 captured by DC-SIGN (Fig. 4C and 4D). We found a significant correlation between viruses from TM and NTM (r^2=0.2, P = 0.03) (Fig. 4C) but no correlation in the clade C viruses suggesting another selection for these glycans opposed to DC-SIGN binding (Fig.4D). In conclusion we found no evidence of enhanced capture or efficiency of transfer to TZM-bl cells for the viruses undergoing MTCT in our cohort.

No difference in sensitivity of Env pseudo-typed viruses from TM, NTM or children to potent NAbs

Using the TZM-bl neutralization assay we tested the sensitivity profile of our selected pseudo-typed viruses to a panel of well characterized human NAbs; IgG1b12 (b12) directed to the CD4 binding site, glycan dependent 2G12 and the

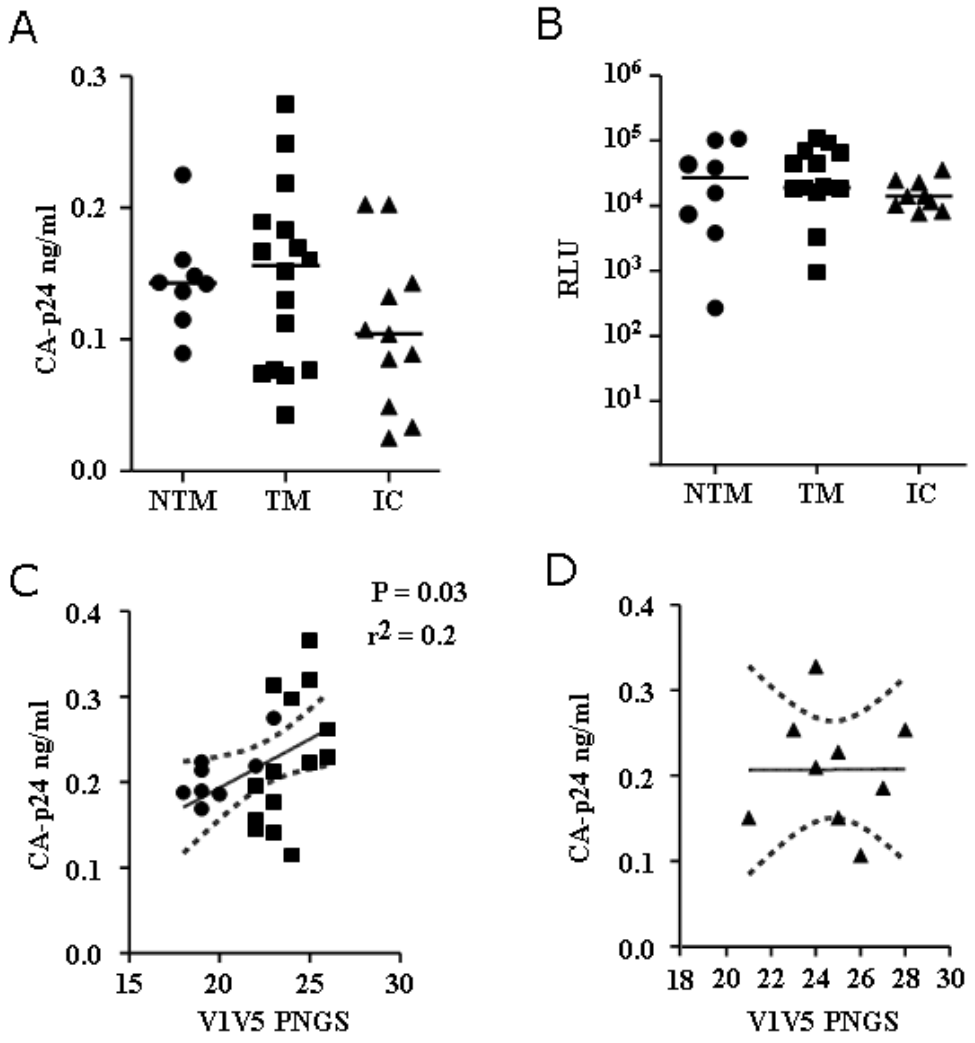

Figure 4. DC-SIGN mediated capture and transfer of Env pseudo-typed viruses from NTM, TM and infected children (IC)
(**A**) Quantity of Env pseudo-typed virus captured by Raji-DC-SIGN cells as determined by CA-p24. (**B**) Extent of infection of TZM-bl cells by captured Env pseudo-typed viruses expressed in relative luciferase units (RLU). (**C** and **D**) Linear regression analysis of the V1-V5 number of PNGS and virus capture by Raji-DC-SIGN cells, in (**C**) NTM and TM and (**D**) in IC. NTM are represented by circles, TM by squares, and IC by triangles.

gp41 Membrane Proximal External Repeat (MPER) NAbs 2F5 and 4E10 (Table 2). We found that all pseudo-typed *env* clones were sensitive to neutralization by 4E10. The IgG1b12 epitope was present in the viruses of five transmission pairs (90/91, 130/131, 250/251, 300/301 and 370/371), the viruses of two of them (300/301, 370/371) were resistant to neutralization by b12. Two of the 3 clones of mother 100 and the clone of child 301 were resistant to 2G12 in spite of possession of al the 5 Putative N-Glycosylaton Sites (PNGS) associated with neutralization. The clones of TM 90 and 300 were resistant to 2G12 whilst one of the two clones of child 91 and the clone of child 301 showed sensitivity to neutralization. The subtype C variants of transmission pair 130/131 and NTM 270, with an A→Q mutation at the C terminal of the 2F5 epitope, provided resistance to this NAb. A negative correlation was found between the length of variable loop V4 and the IC_{50} values with 4E10 (r^2=0.152, P = 0.032) and the IC_{50} values of 2F5 (r^2=0.13, P = 0.0009) (data not shown). Overall we found no

significant differences in neutralization sensitivity between the gp160s of NTM, TM and children with the NAbs tested.

Table 2 Neutralization of Env pseudo-typed viruses by panel of NAbs

Subject	Subtype		Transmission route	IC50 ug/ml			
				2G12	b12	2F5	4E10
90P02	A	TM	IU	>30	3.88	14.41	4.33
90P05	A	TM	IU	>30	4.05	13.21	5.30
91P26	A	IC	IU	>30	2.17	11.57	2.82
91P342	A	IC	IU	0.52	15.29	5.43	2.48
100P37	A	TM	IU	>30	>30	5.54	0.61
100P21	A	TM	IU	>30	>30	8.16	1.42
100P32	A	TM	IU	>30	>30	0.47	0.09
101P19	A	IC	IU	>30	3.87	1.71	1.00
130P17	C	TM	PP or BF	>30	2.64	>30	1.62
130P16	C	TM	PP or BF	>30	4.83	>30	1.56
131P10	C	IC	PP or BF	>30	0.37	>30	4.94
250P10	A/C	TM	IU	3.26	1.93	2.52	1.41
250P11	A/C	TM	IU	0.89	4.60	1.41	2.05
251P22	A/C	IC	IU	0.04	2.68	2.78	1.06
290P04	A	TM	IU	0.66	0.93	0.39	0.67
290P01	A	TM	IU	2.30	0.50	0.21	0.56
291P187	A	IC	IU	0.08	1.16	0.60	0.32
300P29	A	TM	PP or BF	>30	>30	4.03	3.68
300P31	A	TM	PP or BF	>30	>30	3.50	0.42
301P91	A	IC	PP or BF	2.14	>30	0.20	0.23
370P23	A	TM	PP or BF	>30	>30	3.03	1.65
370P19	A	TM	PP or BF	>30	>30	0.61	0.28
371P131	A	IC	PP or BF	>30	>30	0.58	0.27
NTM							
160P40	A	NTM	NA	>30	>30	2.92	2.88
160P38	A	NTM	NA	>30	>30	1.52	1.44
200P20	A	NTM	NA	>30	0.11	0.71	0.51
200P22	A	NTM	NA	>30	0.02	1.84	0.98
270P03	C	NTM	NA	>30	1.86	>30	3.79
270P07	C	NTM	NA	>30	1.25	>30	2.03
380P02	A	NTM	NA	0.56	0.03	0.42	0.24
380P04	A	NTM	NA	1.36	0.04	0.62	0.87

White: IC50>30
Light gray: IC50 >10<30
Dark gray: IC50 <10

TM: Transmitting Mother
IC: Infected Child
NTM: Non-transmitting Mother
NA: Not Applicable
PN: Peri Partum
BF: Breastfeeding

Strong autologous neutralization by plasma from TM and infected children

We next tested the autologous neutralization by plasma from mothers collected one week *pre-partum* (before nevirapine was given and termed "screen") and between 16 and 18 weeks *post-partum* (termed "late") as well as corresponding plasma from both infected and uninfected children. For these assays we utilized the corresponding mothers pseudo-typed (Fig. 5A) and JRFL pseudo-typed (Fig. 5B) virus strains. Plasma of week 16 from one IU infected child (101) and one uninfected child (161) were missing. There were no differences observed between TM and NTM in the concentration of overall IgG in plasma's (data not shown). There was also no correlation observed between viral load and the

autologous neutralization by plasma's (P = 0.78, r^2=0.004) (data not shown). We found that plasma from TM was significantly better in neutralizing autologous gp160 than the NTM (P = 0.002 for screen plasma and P = 0.006 for late plasma) and that plasma from infected children neutralized better than plasma from uninfected children (P = 0.02). The JRFL gp160 was also better neutralized by TM than by NTM (P = 0.07 for screen plasma and P = 0.02 for late plasma) but the 50% inhibitory dose (ID_{50}) was 0.5 to 0.9log lower than in the autologous neutralization experiments. In conclusion, autologous plasma from both TM and infected children neutralized gp160s from the corresponding mothers more efficiently than plasma from the NTM and the uninfected infants.

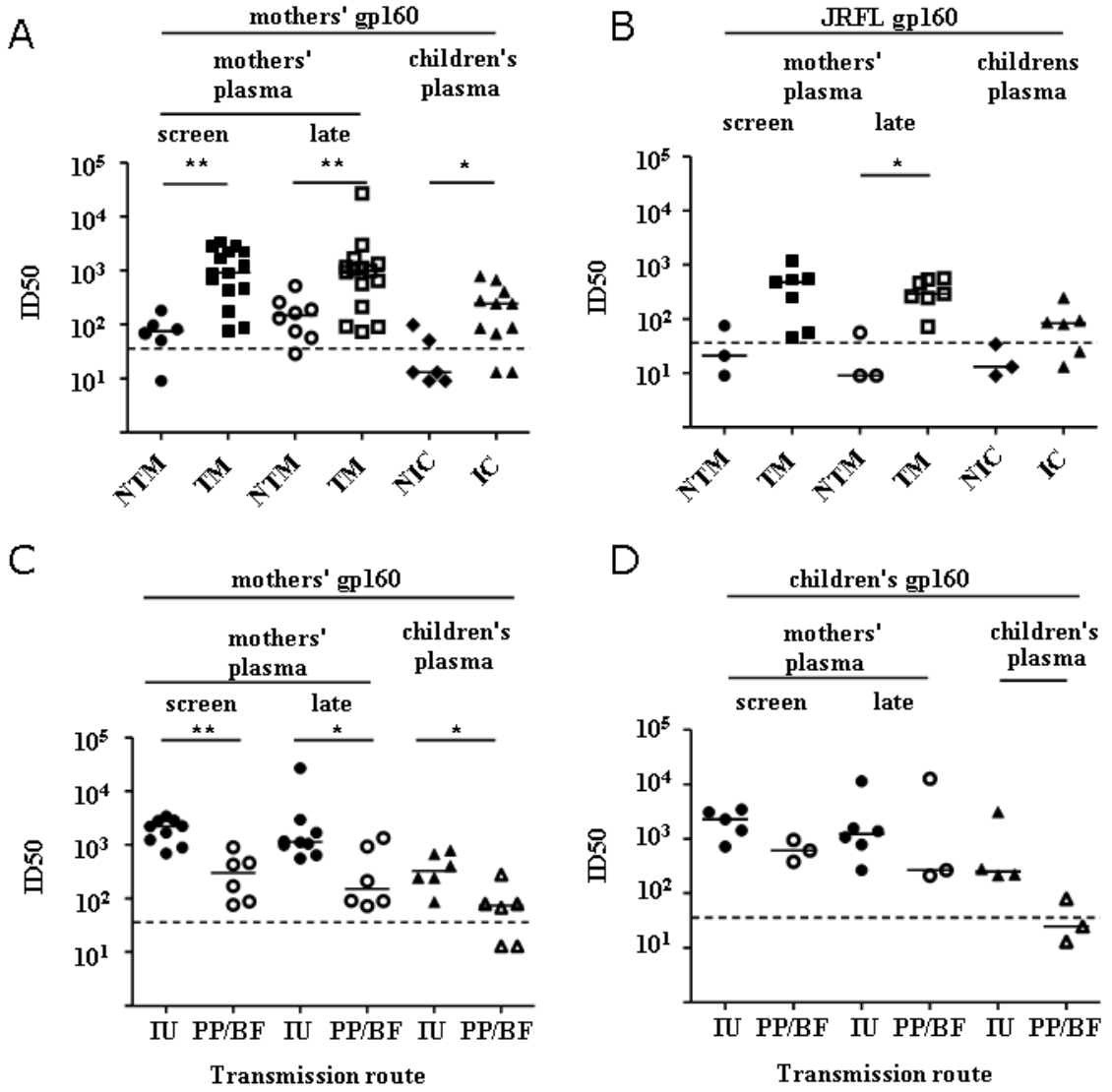

Figure 5. Plasma neutralization of NTM, TM, infected children (IC) and non-IC
(**A**) Neutralization of Env pseudo-typed viruses carrying NTM and TM gp160. (**B**) Neutralization of Env pseudo-typed viruses carrying the JRFL gp160. Neutralization from NTM is represented by dots, from TM by squares, from NIC by diamonds and from IC by triangles. (**C**) Autologous neutralization of Env pseudo-typed viruses carrying TM gp160/gp41, divided into IU and PP/BF and (**D**) neutralization of children's Env pseudo-typed viruses. Neutralization by plasma from IU TM is represented by closed dots, from PP/BF TM by open dots, from IU IC by closed triangles and from BF IC by open triangles. The dashed line indicates the lowest plasma dilution tested (1→48). *Probability that the groups are similar is <0.05, **Probability that the groups are similar is < 0.01.

Neutralization of plasma more evident in IU transmissions than in PP/BF transmissions

To further investigate differences in neutralization activity between the different transmission routes, we analyzed separately the viruses of the mothers who transmitted IU and those who transmitted PP or BF. We tested neutralization using autologous screen plasma and late plasma as well as plasma from the corresponding child (Fig. 5C). We found significantly better neutralization of the gp160s of the IU TM than of the PP/BF TM by screen plasma (P = 0.002), by late plasma (P = 0.03) and by children's plasma (P = 0.02). We also analyzed the neutralization of the IU and PP/BF infected children's *env* clones by screen plasma and late plasma of the corresponding mothers (Fig. 5C) and by autologous plasma (Fig. 5D). Screen plasma from the PP/BF transmitting mothers tended to neutralize the gp160s of children less than plasma from the IU TM (P = 0.07), but no difference in neutralization was found with the late plasma's. Autologous gp160s of the corresponding child were better neutralized by screen plasma from PP/BF TM than from NTM (P = 0.06 and P = 0.02) (data not shown). The IU infected children's plasma tended to neutralize autologous gp160s better than the PP/BF infected children (P = 0.06). At the lowest plasma dilution tested (1:36) 2/3 PP/BF transmitted viruses could not be inhibited to 50% while 1/3 was 50% inhibited at a dilution of 1:80, indicating possible immune escape of these viruses. In conclusion we found that viruses of IU transmission pairs were better neutralized than those of PP/BF transmissions.

No difference in IgG binding to subtype B gp140 envelope trimer between plasma's from TM and NTM

To rule out that differences in total IgG confused our results, we measured the concentration of IgG in the plasma's of mothers and children. We found no difference between TM and NTM but the HIV-1 infected children demonstrated higher levels of IgG in their plasma than the uninfected children (12,6 g/l and 5.7 g/l, P = 0.02) (data not shown). We also investigated the levels of IgG that binds to gp140 envelope trimer in an ELISA assay. To enhance the statistical power of the experiment we included 14 additional plasma samples from NTM from our cohort. We found no differences between TM and NTM. Similar results were obtained with a subtype A gp140 trimer. We also investigated the gp140 binding properties of plasma of the infected children extended with 21 uninfected children from our cohort and again no differences were found (data not shown).

Multi-variate analysis of *env* sequences

In an earlier report we analyzed the gp120 *env* sequences for TM, NTM and infected children and identified two PNGS (N234 and N339) which associated with viruses that preferentially underwent MTCT [20]. We performed a multi variable analysis to test correlations of the presence of one or both PNGS with infectivity, CD4 interaction, CCR5 interaction, DC-SIGN interaction and neutralization sensitivity. We could not identify significant associations with alterations within these sites and the phenotypes tested (data not shown).

DISCUSSION

We studied the viral and immunologic correlates of the HIV-1 subtype A or C or A/C recombinant gp160 envelope proteins from seven mother/child pairs whose children were infected either during gestation or soon after birth and from four NTM with viral loads and CD4 counts in the same range. We found no viral correlates that could have facilitated HIV-1 MTCT, but TM demonstrated higher plasma neutralization capacity than NTM. IU TM neutralized their autologous Env pseudo-typed viruses and those of their infants to significantly higher levels than mothers that transmitted HIV-1 either PP or via BF. Furthermore, plasma from infants infected IU were able to neutralize their autologous Env pseudo-typed viruses as well as those from their mothers to higher levels in comparison to those infected PP/BF, who showed no autologous neutralization capacity.

It has been hypothesized that HIV-1 variants with a replication fitness advantage are those more likely to be transmitted or dominate and selectively outgrow upon infection. Although replication capacity cannot be directly extrapolated from infectivity, a study using single-cycle viruses from subtype C infected MTCT pairs has reported that such viruses from children have a higher fitness than those generated from the mothers and that this is restricted to the V1V5 region of the envelope [15]. Earlier studies found no difference in infectivity between mothers and children's gp160s tested in a single-cycle assay on TZM-bl cells [21,23]. We found here that viruses derived from children were less infectious for TZM-bl cells than those derived from their mothers. This may reflect differences in time of sampling. We studied viruses soon after transmission whereas other studies were conducted with viruses amplified later allowing time for viruses to adapt and gain fitness. Additionally, the discrepancies observed may be partially explained by differences in the cell lines used for pseudo-virus production and the envelope/backbone ratio used in generating the pseudo-typed viruses [50,51].

The vast majority of HIV-1 infections are initiated by a single or a limited number of viral variants, irrespective of transmission route or HIV-1 subtype, indicating a severe bottleneck to transmission [17,52]. X4 viruses are rarely transmitted. The variants undergoing both horizontal as well as MTCT are mainly R5 [4,5,53]. Whether this results from selection of R5 viruses, the early replication outgrowth of R5 over X4 strains or the limited presence of X4 viruses at the site of exposure is still unknown [54-56]. A higher affinity for HIV-1 binding to CD4 or the CCR5 coreceptor may provide a level of selection, however, in concordance with earlier publications studying horizontal as well as vertical transmission cases we did not find differences between transmitted and not transmitted variants in ability to interact with either CD4 or CCR5 [13,14,16].

HIV-1 MTCT requires virus to cross a mucosal barrier, be it via the placenta or the oral/gastrointestinal tract. Mucosal infection can be heightened through the interaction of HIV-1 with an array of C-type lectins, including DC-SIGN, expressed on DCs lying below the mucosal epithelium [41-43]. DC-SIGN allows for the capture of virus and its heightened transfer to $CD4^+$ lymphocytes via *trans-infection*. We found no difference in the efficiency of DC-SIGN capture and transfer of virus to TZM-bl cells between NTM, TM and children. A positive correlation, however, was found between the number of PNGS with DC-SIGN capture when analyzing *env* clones from TM as well as NTM but not for viruses from children. This suggests that viruses transferred to children are not selected based on their capacity for binding DC-SIGN. It should be noted that the pseudo-typed viruses studied were produced within the human C33A cell line and

therefore likely possess the relevant post-translational glycosylation modifications relevant to the human *in vivo* situation [50,57]. Furthermore, other C-type lectins expressed on DCs can capture HIV-1, however here we specifically focused on DC-SIGN since genetic polymorphisms within this molecule have been associated with risk of transmission [44]. It remains to be assessed whether other molecules expressed on DCs can associate with heightened viral capture and transfer and thereby contribute to MTCT.

HIV-1 subtype B R5 Env variants derived from acutely infected individuals via horizontal transmission have been shown to possess NAb inhibitory profiles similar to those derived from chronically infected individuals [58-61]. However, other studies analyzing viruses undergoing sexual transmission indicate that these variants encode gp120s with a compact structure and a reduced number of PNGS that are more sensitive to the effects of NAbs [17,59]. Sensitivity to NAbs may therefore not be a disadvantage in horizontal transmission where the recipient is Ab naïve. In MTCT the situation is different where the child inherits HIV-1 Abs from the mother. It has recently been shown that NAbs can provide selection for a gp120/gp41 envelope with the capacity to induce more potent and broadly neutralizing Abs [62]. Here we found no differences between the viruses from mothers and children for inhibition with the broadly NAbs b12, 2G12, 2F5 and 4E10, suggesting no such selection regarding envelope structure.

Reports studying mother child pairs have shown better neutralization by NTM than by TM suggesting a protective role by Abs [24,25]. Here we found no such effect and actually identified a stronger neutralization profile of autologous viruses within the TM opposed to NTM group. In accordance, we found no neutralization of JRFL with plasma from NTM whilst we did observe neutralization of the same virus with plasma from NTM. One limitation to our study, and inherent to all such studies, is that the exact timings of transmission are not known and where viral adaptations can occur. When we compare the TM to NTM neutralizations of either autologous virus or JRFL the same effect is seen for both early and late plasma from mothers and where the neutralization of viruses from children, all amplified from a time point between plasma sampling of the mother, show similar results. These findings collectively indicate that the observed effect of heightened neutralization is unlikely to be due to differences in timings of transmissions and the subsequent development of escape or reduced neutralization sensitivities. Another limitation to our study is the low number of mother child pairs where HIV-1 transmission occurred and especially when considering sub-groupings. However, it is clear that autologous NAb responses, in either the mother or infant, do not protect against MTCT, and may in fact be detrimental when considering IU transmissions. We highlight that additional studies of larger cohorts need to be undertaken to confirm such conclusions, but our results indicate that autologous NAb responses are not protective.

In both PP and BF transmission HIV-1 has to cross the oral or intestinal mucosal layer. *Ex vivo* experiments have shown that HIV-1 infected macrophages and to a lesser extent HIV-1 lymphocytes are able to migrate across the oral and intestinal epithelium [40]. For IU transmission the virus has to transgress the trophobastic layer that separates the placenta from the fetal blood and the amniotic fluid. Experiments were performed with the BeWo polarized trophoblast cell line that mimics the barrier between maternal and fetal blood and between placenta and amniotic fluid [35]. As shown by these experiments PBMCs, lymphocytes and macrophages are able to fuse to the apical side of the trophoblasts, resulting in release of infectious virus at the basal

lateral side of the monolayer in parallel with infection through the mucosa of the fetal digestive tract [35].

During gestation a considerable amount of maternal IgG is transferred to the fetus and additionally IgG is transferred to the newborn child by breastfeeding. This process is mediated by the FcRn receptor expressed on trophoblasts and intestinal mucosa which binds maternal IgG followed by transcytosis and release at the basal side of the mucosa into the fetal blood, into the amnion fluid or the digestive tract of the child [35,63]. This mechanism may provide a means whereby HIV-1 in IgG/HIV-1 complexes can bind FcRn and be transported to the fetus or the newborn child. This process will be favored by higher concentrations of Env binding IgG. Although others have reported stronger binding (gp160, V3 and gp41 directed) IgG Abs in TM opposed to NTM we found higher levels of NAbs favor MTCT [37,38]. The exact conformation of the HIV-1/IgG complex that is able to bind in the proper manner to FcRn and thereby be transcytosed may have restrictions, steric or otherwise. High affinity between IgG and the virus particle may therefore be significant for both neutralizing activity as well as for FcRn transcytosis of the virus-IgG complex. Ab dependent enhancement of HIV-1 binding and infection of certain cell types has been previously demonstrated *in vitro* and may indeed explain for our observed findings [45,64-66].

We demonstrate here that in the cohort we investigated the neutralizing function of maternal Abs, rather than preventing, associates with a higher risk of IU MTCT. In future studies it would therefore be prudent to consider the different routes of exposure when comparing immunologic factors associated with MTCT, in both the mother and the infant. These results indicate that different mechanisms may support viral transmission via the variant routes of exposure. More so, our results suggest caution when considering using NAbs for passive immunization to prevent IU MTCT and in inducing high levels of NAbs in pregnant vaccine recipients. Larger studies need to be performed to support these findings and especially to characterize the specificities and properties of such induced Abs and to understand which responses can be beneficial opposed to being detrimental.

MATERIALS AND METHODS

Study population and ethics statement

The study was approved by an Independent Ethics Committees (IEC) in the Netherlands, the STEG-METC (ref no R01-089). In the absence of an operational IEC in Rwanda at the time, the Ministry of Health's Treatment and Research AIDS Center (TRAC), the Rwandan National Malaria program (PNLP) and the "Cellule de recherché" at the CHK acknowledged the approval of the Dutch IEC. All women provided written informed consent for both themselves and their children. Plasma samples were utilized from HIV-1 positive mothers (n=30) and their infants from Rwanda [20]. All women received single dose nevirapine at onset of labor. Maternal plasma was collected prior to delivery (screen), at labor (early) and week 16 and 18 (late) after delivery. Child's plasma was collected at delivery (early) and 16 weeks (late) of age. All children were breastfed during the 16 week study period.

HIV-1 RNA extraction and amplification

HIV-1 RNA was isolated from plasma samples according to the method of Boom *et al* [67]. Viral RNA was converted to cDNA as previously described [6] with an input of 6,000 copies RNA per amplification. We selected this copy number to limit recombination events that can arise during PCR amplification and to minimize the risk associated with such an approach. Reverse transcription of RNA to single-stranded cDNA was performed using the SuperScript III protocol according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA) with a modification. RNA, Betaine (1 M) deoxynucleoside triphosphates (0.5 mM each), and 0.25 µM primer OFM19 (5'-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3'; nucleotides [nt] 9604 to 9632 of the HXB2 sequence) were incubated for 5 min at 65°C to denature secondary structure of the RNA. First-strand cDNA synthesis was carried out in 20 µl reaction mixtures with First-Strand Buffer containing 5 mM dithiothreitol, 2 U/µl of an RNase inhibitor (RNaseOUT), and 10 U/µl Super- Script III. The reaction mixture was incubated at 55°C for 65 min and inactivated by being heated to 65°C for 15 min. The resulting cDNA was used immediately for PCR.

Gene amplification

Full-length *rev/env* genes (including parts of the first exon of the *tat* gene; the entire *vpu*, *rev*, and *env* genes; and parts of the *nef* gene) were amplified by nested PCR from plasma-derived viral cDNA as previously described with minor changes [6]. Briefly, 20 µl of bulk cDNA (containing 100 to 1,000 viral templates) was subjected to first-round PCR in a volume of 50 µl. PCR was performed by using an Expand High Fidelity PCR system (Roche Diagnostic Corporation, Indianapolis, IN) in 5µl Expand PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, and 0.2 µM of Vif1 (5'-GGGTTTATTACAGGGACAGCAGAG-3'; nt 4900 to 4923) and OFM19 primers. The following cycling conditions were used: 93°C for 2 min followed by 10 cycles of 93°C for 10 s, 58°C for 30 s, and 68°C for 5 min, and followed by 20 cycles of 93°C for 10 s, 58°C for 30 s, and 68°C for 5 min extended with 20 s with each cycle, with a final extension of 68°C for 7 min. Second-round PCR was performed by using 5 µl of the first-round PCR product and primers EnvA (5' GGCTTAGGCATCTCCTATGGCAGGAAGAA-3'; nt 5954 to 5982) and EnvN (5'-CTGCCAATCAGGGAAGTAGCCTTGTGT-3'; nt 9145 to 9171) under the same conditions used for the first-round PCR.

Construction of envelope clones

The final PCR products of the predicted size (±3.2 kb) were ligated into the pcDNA3.1.V5-His TOPO TA vector according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA) and transformed into Stbl4 cells by electroporation (Invitrogen Life Technologies, Carlsbad, CA). Reaction mixtures were plated on LB-Amp (ampicillin; 100µg/ml) plates and cultured 24 h at 30°C. Colonies were screened with a colony PCR using primers T7 (5'-TAA TAC GAC TCA CTA TAG GG A-3') and FGS021 (5'-CTT TCA TTG CCA CTG TCT TCT GCT-3'). Clones containing the right size and orientation were grown in LB-Amp (ampicillin; 100 µg/ml) and plasmid was isolated with the Bioké NucleoSpin Kit (Machery-Nagel, Düren, Germany). The plasmids were screened by generating Env pseudo-typed virus and tested for infectivity as described below and the infectious clones were selected.

Cells and transfections

C33A cells and TZM-bl cells (obtained through the NIH ARRRP from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc., Durham, NC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Breda, The Netherlands), each supplemented with MEM nonessential amino acids and 10% foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml) as previously described (58). One day prior to transfection $2.5x10^6$ C33A cells were seeded in a 25 cm² culture flask. After 24 h the cells were transfected using the CaPi method. In short: HEPES-buffer (50 mM HEPES pH7.1, 250 mM NaCl, 1.5 mM Na_2HPO_4) was added to 9 µg of the envelope containing plasmids and 3.9 µg of plasmid pSG3Δ*env* in a volume of 293 µl, mixed well and 40 µl of 2M $CaCl_2$ was added and again mixed well. After 45 min incubation at rt the mixture was added to the C33A cells. As a positive control JRFL envelope was used and pSG3Δ*env* alone for the negative control. After 18 h the medium was refreshed and the pseudo-typed virus containing supernatant was harvested after 48 h of incubation. Virus was stored at -80°C. The virus concentration was quantified by measuring its CA-p24 antigen content by ELISA.

Sequencing and phylogenetic analysis

The infectivity competent *env* clones were sequenced using the ABI PRISM Big Dye Terminator Kit (Applied Biosystems, Forster City, CA, USA). Sequences were assembled using the CodonCode Aligner program (CodonCode Corporation, Dedham, USA). Alignments were performed taking the translation codons into account and the sequences were translated into amino acids using the BioEdit program (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA). Phylogenetic analysis of the envelopes was performed with the Neighbor-Joining (N-J) method of MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007). Positions containing an alignment gap were included for pair wise sequence analysis**.** The number PNGS was determined using the program available at the HIV Sequence Database (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). The gp160 envelope sequences have been submitted to GenBank under accession numbers JX508855 - JX508885.

Single-cycle infections and inhibition assays

One day prior to infection, $17x10^3$ TZM-bl cells were added to a 96-well plate and cultured overnight. Infections were performed in 200 µl with 1 pg CA-p24 virus input and medium supplemented with 10µg/ml DEAE dextran. Two days post-infection the medium was removed, the cells were washed once with PBS and lyzed in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the Luciferase Assay kit (Promega) and a Glomax luminometer according to the manufacturer's instructions (Turner BioSystems, Sunnyvale, CA). All infections were performed in duplicate and repeated at least once. Cells infected with negative control pseudo-typed virus were used to correct for background luciferase activity. The infectivity of each mutant without inhibitor was defined as 100%. Non-linear regression curves were determined and IC_{50} or ID_{50} values were calculated using Inhibitor versus Response variant of the Non Linear Regression function of GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com) and a significance level of $P < 0.05$ was used for all the analyses.

The program Factor Correction was used to remove multiplicative between-session variation [68]. For determination of the infectivity of the pseudo-typed viruses, three step serial dilutions of virus were used. In inhibition experiments an amount of virus equal to 1pg of p24/well was used. In experiments with maraviroc or OKT-4 (eBioscience, San Diego, CA) TZM-bl cells were incubated with 3 step dilutions of inhibitor for 30 min at 37° before the pseudo-typed virus was added. For inhibition with sCD4-183 pseudo-typed virus was incubated with 3 step dilutions for 30 min at 37° before infection of the cells. The maximum concentration of sCD4, maraviroc and OKT4 was 10 μg/ml, 10 ng/ml and 30 ng/ml respectively.

Virus capture and transfer by Raji-DC SIGN cells

For the DC-SIGN capture and transfer experiments 50 μl of Raji or Raji-DC-SIGN cells ($20x10^6$ cells/ml) were mixed with 150 μl of pseudo-typed virus equal to 50 ng/ml CA-p24 and incubated for 2 h at 37°, washed 4x with ice cold PBS and resuspended in 90 μl of culture medium. 10 μl of this suspension was used in the CA-p24 ELISA to quantify the amount of virus captured by DC-SIGN, corrected for non-specific binding to the Raji cells. 15 μl of the suspension was used to infect TZM-bl cells. After 48 h the TZM-bl cells were lyzed and luciferase activity was measured as described above.

HIV-1 neutralization

Assays measuring HIV-1 inhibition using NAbs b12, 2F5, 4E10 and 2G12, maternal or children's plasma were performed as previously described [69], using virus input equal to 1 pg CA-p24 of pseudo-typed virus/well. Before infection the virus was incubated with 3 step dilutions of NAb or plasma for 30 min at 37°. The maximum Ab concentration used in neutralization assays was 10 μg/ml. The lowest dilution of plasma was 1:36. All plasma samples were heat inactivated at 56°C for 30 min before use. Plasma from a seronegative donor was used as a negative control and showed no neutralization activity at 1:36 dilution.

Determination of IgG concentrations

IgG concentrations in plasma were measured using the Cobas C502 (Roche, Roche Diagnostics, Darmstadt, Germany) according to the manufacturers instructions.

gp140 trimer binding ELISA

The ELISA was performed as earlier described [70,71]. Microlon 96 well plates (Greiner Bio-One, The Netherlands) were coated overnight with D7324 Ab 100x diluted in 0.1 M $NaHCO_3$, pH 8.6 and washed 2x with TBS (150 mM NaCl, 10 mM TRIS, pH 7.5). SOSIP.R6-IZ-D7324 trimer diluted in TBS/10% FCS was added and incubated for 2 h at RT, and washed 2x with TBS followed by 1 hr incubation with 2% skimmed milk in TBS. Serial dilutions of plasma in TBS/2% milk/20% sheep serum were added and incubated for 2 h followed by 5 washes with TBS/0.05% Tween-20. Goat- anti-human Fc-HRP (Jackson Immuno Research, England) 1:1000 diluted in TBS/2% milk/20% sheep serum was added and incubated for 30 min. followed by 5 washes with TBS/Tween. All reactions were

performed in 100 µl/well. Colorimetric detection was performed by adding 50 µl of 1%TMB (Sigma-Aldrich, the Netherlands), 0.01% H_2O_2 , 1 M Sodium Acetate, 0.1 M citric acid and the reaction was stopped by adding 50 µl 0.8 M H_2SO_4/well when appropriate and absorption at 450 nm was measured.

Statistical Analysis

To avoid confounding of the analysis caused by the use of more than one data point derived from each of the members in the groups of NTM, TM and infected children we performed the Kruskall-Wallis test to determine within and between individual differences in our results on infectivity, CD4 interaction, CCR5 interaction, DC-SIGN interaction and neutralization sensitivity and with a Bonferroni correction for multiple testing. No significant differences were found between the individuals within each group and therefore the Mann-Whitney test was used to analyze the differences between the groups. Analysis of the association of the presence of one or both PNGS N234 and N339 with infectivity, CD4 interaction, CCR5 interaction, DC-SIGN interaction and neutralization sensitivity was done with the multivariate logistic regression analysis using PASW 17.0. hypotheses testing, all variables were introduced through forward stepwise procedure. A significance level of $P < 0.05$ was used for all the analyses.

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Chapter 7

GENERAL DISCUSSION

Transmission of HIV-1 from mother to child accounts for a significant proportion of infections in those countries where ART is still limited. For example, in 2009 over 370,000 children less than 15 years of age were infected with HIV-1, with the vast majority being infected via MTCT (www.unaidstoday.org). In the absence of ART approximately a third of mothers will transmit HIV-1 to their children during pregnancy, labour or through breastfeeding, and at approximately equal ratios [1,2]. The better understanding of the host viral interactions leading to transmission will be essential if new and novel intervention strategies are to be developed which can greatly restrict HIV-1 MTCT.

i) HIV-1 variants undergoing transmission and disease progression

Numerous hypotheses have been proposed to explain why disease progression within HIV-1 infected individuals is so variable. Host genetic factors have been associated with variant disease course, such as co-receptor availability or HLA allelic variation [3,4]. A few studies have associated virus characteristics with slower progression such as a defective *nef* gene or decreased HIV-1 replication capacity [5,6]. Nevertheless, few studies have investigated genotypic differences of viruses circulating in individuals with variable progression profiles. The vast sequence diversity within HIV-1 gp120 renders such studies difficult with no consensus findings in the results reported. There has been a distinct lack of clarity in defining identifiable patterns of amino acid sequence changes being associated with distinct clinical categories of patients [3].

The majority of research linking viral variation with HIV-1 transmission and disease progression has focused predominantly on the gp120 envelope gene. One of the key characteristics of HIV-1 infection is the preferential transmission of viruses utilizing the CCR5 coreceptor (R5) and the infrequent transmission of CXCR4-using (X4) or dual-tropic viruses (R5/X4) [7-9]. We have also provided an indication that CQ treatment aimed at preventing MTCT via breastfeeding may in fact heighten HIV-1 viral loads in treated mothers and therefore should not be considered for treatment purposes aimed at lowering HIV-1 MTCT. This restriction has been attributed to a number of factors including cell-type of infection and a better immune control of X4 viruses. It has also been well documented that a switch from R5 to X4 viruses during disease is linked with increased HIV-1 RNA concentrations, reduced $CD4^+$ cell counts and faster disease progression [10]. Which precise mechanisms control this switch in co-receptor activity is still poorly understood, but many studies have addressed this by comparing HIV-1 gp120 envelope sequences. The most obvious genetic alterations occur in the variable regions of the envelope, which alter predominantly overall amino acid charges and PNGS [11-14]. In all likelihood the diversity within the gp120 variable regions are contributing to co-receptor activity and NAb escape, both of which likely provide for selection during transmission and subsequent viral evolution during disease progression. Alterations in PNGS numbers and positions have been shown to greatly influence the extent to which Abs can neutralize HIV-1 and prevent infection [15-17].

Virus selection during MTCT may be influenced by the binding affinity of the gp120 envelope glycoprotein to a number of cell receptors. A higher affinity for the CD4 receptor or the CCR5 co-receptor may be of advantage for the infecting virus particle. A low net charge within the V3 variable loop of gp120 and the presence of PNGS on position 301 of the V3 loop being the variants preferentially transmitted and associated with higher CCR5 affinity [18-22]. It has been well described that the length of the V1V2 region can vary during the course of HIV-1

infection influencing the ability of the virus to infect CCR5 or CXCR4 expressing cells or alter the potential for induced Ab responses to neutralize HIV-1 [13,15,23]. Studies of adult HIV-1 transmission pairs in Africa have shown that viruses undergoing horizontal transmission possess Env genotypes with shorter variable loops and fewer numbers of PNGS which can associate with the development of anti-HIV-1 Ab responses [24]. Correlations between variable loop length and number of putative PNGS have been reported for MTCT. In some studies fewer Env PNGS are found in the transmitted viral variants whilst other studies do not find differences in total number but have found the position of the PNGS to associate with risk of transmission [25-28].

It has been postulated that HIV-1 transmission can be enhanced by binding of the gp120 glycan moiety to the C-type lectin DC-SIGN, which can be expressed to high levels on immature DCs and facilitate infection of $CD4^+$ T lymphocytes either locally or in distal lymph nodes [29-31]. DC-SIGN expressed by a subset of B-cells in the tonsils and blood may also contribute to the infection of $CD4^+$ lymphocytes [32]. If this mode of transmission plays a role in MTCT, selection of the transmitting virus may be driven by more efficient binding of the virus to DC-SIGN. The same envelope alterations which have been shown to modulate coreceptor usage of HIV-1 have also been shown to modulate how efficiently virus interacts with DC-SIGN, thereby influencing which viruses evolve during disease progression and which can alter coreceptor usage patterns or the extent to which viruses are inhibited by such factors as CC-chemokines and NAbs [33]

The role of DC-SIGN in supporting HIV-1 transmission via breastfeeding has been recently highlighted by the finding that certain polymorphisms within the gene coding for this C-type lectin can associate with the risk of HIV-1 infection [34]. This association would further support the hypothesis that factors present in breast milk which possess the capacity to bind DC-SIGN can modulate transmission risk via breastfeeding. BSSL and MUC1, both present in human milk, have been shown to potently bind DC-SIGN and prevent viral capture and transfer to CD4 cells [35-37]. BSSL is highly polymorphic at both the genomic and protein level and is composed of multiple 11 amino acid repeat domains at the C-terminus of the molecule. Alterations within the number of repeats have been shown to associate with the strength of milk samples from different mothers to block binding to DC-SIGN and inhibiting HIV-1 capture and transfer to $CD4^+$ lymphocytes [38]. It will be informative to identify whether alterations within such host factors can associate with risk of HIV-1 transmission via the breastfeeding route. Interestingly, alterations within the BSSL gene have also been associated with the course of disease progression in HIV-1 infected individuals [39]. In addition, other bodily fluids have been shown to contain compounds with the capacity to bind DC-SIGN and in seminal plasma MUC6 and Clusterin have both been reported to possess anti-HIV-1 activity, suggesting a common phenomenon [40,41]. DC-SIGN is not the only C-type lectin with the capacity to bind HIV-1 and others may also facilitate infection via the different MTCT routes [42]. A better understanding of the molecular interactions between the gp120 envelope and these molecules will provide pointers as to which regions of the molecule need to be targeted to prevent transmission. Interventions can be envisaged where pregnant women are treated with small molecule inhibitors which can prevent viral receptor interactions or where vaccination can induce Abs to inhibit infection.

ii) Novel therapies for preventing HIV-1 transmission

ART is currently prescribed to prevent HIV-1 MTCT and can be given to the mother when aiming to prevent IU or PP transmissions and to the mother or infants when considering inhibiting transmissions via breastfeeding. The benefits, however, may be outweighed by numerous disadvantages such as toxicity, resistance and pricing. The anti-malarial drugs CQ HCQ are cheap and widely available and these are both weak bases that affect acid vesicles leading to the dysfunction of several enzymes such as those involved in protein post-translational modification. In HIV-1 infected individuals CQ treatment may therefore lead to impaired glycosylation of the HIV-1 envelope protein [16,43-46]. This could decrease the capacity by which HIV-1 can undergo viral capture and transfer to $CD4^+$ T lymphocytes via cells expressing DC-SIGN, modulate NAb epitopes (as seen for 2G12) as well as reduce the glycose shield protecting against Abs binding gp120 [47]. Furthermore, CQ can interfere with virus replication in $CD4^+$ T lymphocytes and this inhibition is likely conferred at the cellular level [48]. The altered endosomal pH as induced with CQ treatment can also reduce IL-6 production, followed by down-regulation of HIV-1 production in chronically infected $CD4^+$ T lymphocytes and monocyte cell lines [46,49]. CQ also inhibits Tat mediated transactivation of HIV-1 transcription [50]. For these reasons it has been postulated that the treatment of HIV-1 infected individuals with CQ or HCQ may result in lower viral loads as well as a reduction in their capacity to transmit HIV-1. Within the context of our own studies we identified that CQ indeed had the effect of reducing HIV-1 infectivity and restricting DC-SIGN mediated HIV-1 capture and transfer to CD4+ T lymphocytes *in vitro* [48]. However, within the context of the CHARGE study we identified that CQ treatment in HIV-1 positive breastfeeding mothers had the effect of significantly increasing viral loads, chapter 4. These results bring into question the use of CQ in preventing MTCT and indeed the use of the drug in general in regions of HIV-1 prevalence.

iii) Vaccines for preventing HIV-1 transmission

To-date the field of HIV-1 vaccine design has been full of disappointment. A phase I vaccine trial inducing humoral immune responses through vaccinating with the monomeric HIV-1 envelope gp120 protein induced no detectable protective effect [51]. The vaccine was deemed to have failed through inducing Ab responses that could not protect against the broad range of primary isolates and variant subtypes which circulate within the population, but which could inhibit the more neutralization-sensitive lab-adapted strains of HIV-1. Subsequently, the STEP trial that induced strong $CD8^+$ CTL responses was prematurely halted when it was shown that participants within the vaccine arm of the study were more likely to become HIV-1 infected than those individuals within the placebo group [52]. It was also demonstrated that individuals with preexisting immunity against the vector, Ad5, and uncircumcised men were at higher risk of becoming HIV-1 infected. A similar finding was recently identified in the HVTN505 study where a trend towards more infections in the vaccine versus placebo arm was found, again with the study being prematurely halted [NIH]. In this study Ad5 positive and uncircumcised men were excluded from participating, indicating these two parameters were not contributing to the heightened transmission. In both these studies no differences in viral loads were found in vaccine breakthroughs indicating that the induced CTL responses were also not able to limit viral replication. One interesting difference between the studies is that the HVTN505 trial contained gp120 Env immunogens with most participants inducing binding Ab responses. It is highly likely that the mechanism

providing for heightened transmission can be different between the two trials. To-date the only HIV-1 vaccine trial deemed to report any degree of success is RV144 [53]. This was a trial conducted in Thailand where both CTL and Ab responses were induced. The protective effect after 12 months was 31%. However, the effect was lost in the subsequent period and the statistical significance reported was marginal. The interesting finding of this study is that the protective effect observed has been reported to stem from induced binding of Abs to the V2 region of gp120 which does not associate with classical virus neutralization [54]. Collectively, these results indicate that a successful HIV-1 vaccine will have to induce both B and T cell immune responses, however, the nature and magnitude of these responses remains unknown. Furthermore, all vaccines tested to-date have focused on preventing HIV-1 sexual transmission with no trials conducted aimed at curtailing MTCT.

Ab driven gp120 modifications

The HIV gp120/41 Env glycoprotein is the main molecule against which NAb responses are mounted in HIV-1 infected individuals and which can be induced early in infection [16,55-58]. Autologous NAbs are more likely to be found early in infection and with heterologous NAbs induced later in disease, although no real clinical benefit has been associated with the induction of such NAbs and with most individuals escaping their predicted beneficial effects. It has been well documented that the early autologous responses induce escape mutations within the gp120/gp41 envelope protein [16,58]. These escape mutations have been extensively studied in patients and have been shown to encompass many alterations, including the V1V2 length and the number of PNGS in variable regions. Although not influencing disease course through controlling viral load effectively, the alterations induced are strikingly similar to those described in directing coreceptor activity and the switch from the R5 to X4 phenotype as described earlier. One of the most interesting alterations concerning the switch in coreceptor activity is the loss of PNGS in the V3 region of the virus [13]. This is significant since the loss of this specific glycosylation event has been implicated in exposing an array of neutralizing epitopes that are present within the gp120 V3 region. It has also been described that a specific glycosylation site within the V4 region of gp120 when removed had an influence on enhancing the neutralizing potential of HIV-1 with soluble CD4 (sCD4) as well as the b12 NAb, suggesting that this is a glycosylation site masking the CD4bs [59]. An effective NAb response may therefore select for the maintenance of this specific site, and thereby maintaining CCR5 usage and preventing coreceptor switching from occurring. A number of studies have been performed in non-human primates indicating that X4 variants can be controlled by SIV immune responses and that the R5 variants preferentially grow out early in infection and once the immune responses diminished then the virus modulated coreceptor activity [60-62]. The literature clearly supports the concept that induced NAb responses can drive the gp120/gp41 envelope modifications observed during disease progression and determine the resultant viral phenotypes.

Reports studying mother child pairs, where maternal NAbs are passed from the mothers to the infants, have shown better neutralization by NTM than by TM suggesting a protective role by Abs [63,64]. In this thesis we found no such effect and actually identified a stronger neutralization profile of autologous viruses within TM opposed to NTM (chapter 6). We also found no neutralization of JRFL with plasma from NTM whilst we did observe neutralization of the same

virus with plasma from TM. We are aware that there are limitations here in that the exact timing of transmissions is not known, nor where gp120/gp41 adaptations may occur and result in viruses of differing neutralizing phenotypes emerge. When we compared the TM to NTM neutralizations of either autologous virus or JRFL, the same effect was seen for both early and late plasma from mothers and where the neutralization of viruses from children, all amplified from a time point between plasma sampling of the mother, show similar results. These findings collectively indicate that the observed effect of heightened neutralization is unlikely to be due to differences in timings of transmission and the subsequent development of escape or reduced neutralization sensitivities.

One of the most intriguing aspects of our results is the finding that heightened HIV-1 autologous NAb responses associate preferentially with IU transmissions and are not seen for either PP or BF transmissions. This would advocate for a hypothesis describing an Ab mediated enhancement of HIV-1 transmission across the placental barrier. In both PP and BF transmission HIV-1 has to cross the oral or intestinal mucosal layer. *Ex vivo* experiments have shown that HIV-1 infected macrophages and to a lesser extent HIV-1 lymphocytes are able to migrate across the oral and intestinal epithelium [65]. For IU transmission the virus has to transgress the trophoblastic layer that separates the placenta from the fetal blood and the amniotic fluid. Experiments were performed with the BeWo polarized trophoblast cell line that mimics the barrier between maternal and fetal blood and between placenta and amniotic fluid [66]. As shown by these experiments, PBMCs, lymphocytes and macrophages are able to fuse to the apical side of the trophoblasts, resulting in release of infectious virus at the basal lateral side of the monolayer in parallel with infection through the mucosa of the fetal digestive tract [66].

During gestation a considerable amount of maternal IgG is transferred to the fetus and additionally IgG is transferred to the newborn child by breastfeeding. This process is mediated by the FcRn receptor expressed on trophoblasts and intestinal mucosa which binds maternal IgG followed by transcytosis and release at the basal side of the mucosa into the fetal blood, into the amnion fluid or the digestive tract of the child [66]. This mechanism may provide a means whereby HIV-1 in IgG/HIV-1 complexes can bind FcRn and be transported to the fetus or the newborn child. This process will be favored by higher concentrations of Env binding IgG. Although others have reported stronger binding (gp160, V3 and gp41 directed) IgG Abs in TM opposed to NTM, we found that higher levels of NAbs favor MTCT [67,68]. The exact conformation of the HIV-1/IgG complex that is able to bind in the proper manner to FcRn and thereby be transcytosed may have restrictions, steric or otherwise. High affinity between IgG and the virus particle may therefore be significant for both neutralizing activity as well as for FcRn transcytosis of the virus-IgG complex. Ab-dependent enhancement of HIV-1 binding and infection of certain cell types has been previously demonstrated *in vitro* and may indeed explain our observed findings [69-72]. It has also been shown that viruses coated with NAbs can be more efficiently captured by DC-SIGN, cleared of their Abs, and subsequently infect $CD4^+$ T lymphocytes. It was also demonstrated that binding was mediated via DC-SIGN and the observed enhancement provided by the HIV-1/IgG complexes binding to the Fc receptor. If DC-SIGN, or other C-type lectins with the capacity to bind gp120/gp41, are expressed at the placental trophoblast intersection then a mechanism enhancing transmission can be envisaged.

The concept of Abs enhancing HIV-1 replication is not new and many reports have previously described such effects [69-71,73-76]. However, the concept of Abs being able to increase HIV-1 MTCT, specifically IU, is new and extremely worrisome. These results have major implications for any HIV-1 prevention strategy aimed at inducing strong NAb responses in women or in administering passive immunizations with NAbs aimed at preventing IU transmissions in pregnant women. It can be argued that trials in preventing MTCT are the ideal setting to test the effectiveness of an HIV-1 vaccine, given that the timing where induced immune responses need to be at their peak and the virus against which the response needs to be effective are both known. If, indeed our results are borne out, such vaccine strategies may be highly detrimental in pregnant women.

FUTURE PERSPECTIVE

We have indicated that CQ treatment aimed at preventing MTCT via breastfeeding may in fact heighten HIV-1 viral loads in treated mothers and therefore should not be considered for treatment purposes aimed at lowering HIV-1 transmission. In addition, we have shown that mothers transmitting HIV-1 *in utero* to their infants have higher autologous neutralizing response than mothers that did not transmit, indicating Ab mediated enhancement of infection. Understanding better the mechanisms underlying both these findings will undoubtedly lead to new intervention strategies aimed at preventing MTCT.

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Addendum

ABBREVIATIONS

Ab	-	antibody
AIDS	-	acquired immunodeficiency syndrome
ART	-	antiretroviral therapy
BSSL	-	bile salt stimulated lipase
CA	-	capsid
CA-p24	-	capsid-protein 24
CQ	-	chloroquine
DC	-	dendritic cell
DC-SIGN	-	DC-specific ICAM-3 grabbing non-integrin
DNA	-	deoxyribonucleic acid
ELISA	-	enzyme-linked immunosorbent assay
Env	-	envelope
Fc	-	heavy chain of IgG
FCS	-	fetal calf serum
FIV	-	feline immunodeficiency virus
gp41	-	glycoprotein 41
gp120	-	glycoprotein 120
HCQ	-	hydroxylchloroquine
HIV-1/2	-	human immunodeficiency virus type 1/2
HM	-	human milk
iDC	-	immature dendritic cell
Ig	-	immunoglobulin
IL	-	interleukin
mAb	-	monoclonal antibody
MTCT	-	mother to child transmission
NAb	-	neutralizing antibody
NC	-	nucleocapsid
PBS	-	phosphate-buffered saline
PCR	-	polymerase chain reaction
PNGS	-	potential N-linked glycosylation sites
R5	-	HIV-1 which uses CCR5 as a co-receptor
R5X4	-	HIV-1 which can use both CCR5 and CXCR4 as a coreceptor
RLU	-	relative light units
RNA	-	ribonucleic acid
RT	-	reverse transcriptase
RT-PCR	-	reverse transcription PCR
VL	-	viral load
WHO	-	world health organization
X4	-	HIV-1 which uses CXCR4 as a coreceptor

SUMMARY

Many viral as well as host factors can be associated with the risk of HIV-1 mother to child transmission (MTCT). The better understanding of such factors as well as the molecular interactions involved will ultimately lead to the development of new products with the capacity to inhibit transmission. In this thesis we aimed to better define the viral gp120 envelope characteristics, both genotypic and phenotypic, associated with HIV-1 transmission and more so via the MTCT route. We also aimed to address whether the anti-malarial drug chloroquine (CQ) can be used to modulate HIV-1 gp120 envelope characteristics associated with HIV-1 infection and whether this drug could be effective at lowering HIV-1 loads in breastfeeding mothers.

In **chapter 2** we describe two families in which both parents and two children had been infected with HIV-1 for nearly 10 years, but who demonstrated variable parameters of disease progression. We analyzed the gp120 envelope sequence and compared individuals that progressed to those that did not in order to decipher evolutionary alterations that are associated with disease progression when individuals are infected with genetically related virus strains. The analysis of the V3-domain positive charge demonstrated an association between higher charges and disease progression. The ratio between the amino acid length and the number of potential N-linked glycosylation sites was also shown to be associated with disease progression with the healthier family members having a lower ratio. In conclusion, in individuals initially infected with genetically linked virus strains the V3-positive charges and N-linked glycosylation patterns are associated with HIV-1 disease progression and can follow varied evolutionary paths.

In **chapter 3** we describe the effects that CQ has on the *in vitro* infection and replication profile of HIV-1. It has been reported that CQ can decrease HIV-1 viral loads *in vivo* and can accumulate in breast milk of lactating women. We found that viruses generated in the presence of CQ are not hampered for infectivity and/or replication capacity. We did determine that viruses generated in the presence of CQ demonstrated a reduced capacity for DC-SIGN mediated *trans*-infection of $CD4^+$ T-cells. We analyzed the gp120 envelope protein sequences of viruses cultured in the presence or absence of CQ and observed a loss of two PNGS involved in binding of the monoclonal NAb 2G12 and in DC SIGN binding in viruses cultured with CQ. These results suggest that CQ may be a useful drug aimed at reducing HIV-1 MTCT via breastfeeding.

In **chapter 4** we describe the effect of CQ when given to breastfeeding mothers over a 16 week period from time of delivery. We observed a 2.5 fold higher concentration of CQ in breast milk compared with blood plasma but these higher concentrations of CQ did not result in a decline of the viral load in the milk of the treated mothers. In contrast to previous reports, we found a significant increase in HIV-1 plasma viral loads in the CQ treated versus placebo mothers. When analyzing predicted gp120 Env protein sequences no

variant characteristics were found that could explain the increased viral loads.

In **chapter 5** we studied the genotypic properties of the HIV-1 gp120 Env proteins from 30 mothers infected with HIV-1 subtype A or C viruses of whom 7 were known to have infected their children either during gestation or soon after birth. We analyzed and compared the sequences of the V1-V5 region of the gp120 envelopes from transmitting mothers (TM), non-transmitting mothers (NTM) and their infected children. No differences were found with respect to the lengths and number of potential N-glycosylation sites (PNGS) in the V1-V5 region, but we identified that viruses with a PNGS at positions AA234 and AA339 were preferentially transmitted and that viruses with PNGS-N295 showed a disadvantage for transmission. We also found that the frequency of PNGS-N339 in the viruses of TM and infected children from other cohorts was significantly higher than in viruses undergoing sexual transmission. These results provide evidence that the presence of the PNGS-N339 site in the gp120 envelope confers an advantage to HIV-1 when considering MTCT.

In **chapter 6** we describe the viral and immunologic correlates of the HIV-1 gp160/gp41 Env proteins of the 7 mother/child pairs and 4 NTM from the cohort studied in chapter 4. We found no differences in HIV-1 infectivity or interaction with DC-SIGN that could have facilitated HIV-1 MTCT but we did find that TM showed significantly higher plasma neutralization capacity than NTM. TM who infected *in utero* were found to neutralize their autologous Env's, and those of the corresponding children, to significantly higher levels than mothers that transmitted the virus *peri-partum* or through breastfeeding. Furthermore, plasma from children infected *in utero* were able to neutralize their autologous Env's as well as those from their mothers to higher levels in comparison to those infected *peri-partum* or by breastfeeding. These findings have consequences for the development of active or passive vaccination strategies aimed at preventing MTCT *in utero*.

SAMENVATTING

De overdracht van HIV-1 van moeder naar kind (ook wel transmissie genoemd, Mother-To-Child-Transmission, MTCT) is afhankelijk van eigenschappen van zowel het virus als de gastheer. Een beter begrip van de factoren en de moleculaire interacties die betrokken zijn bij de transmissie van het virus, zouden uiteindelijk kunnen leiden tot het ontwikkelen van vaccins en medicijnen die transmissie kunnen verkomen. Bij het onderzoek in dit proefschrift hadden we tot doel om meer te weten te komen over de eigenschappen van het HIV-1 envelop eiwit en de reactie van de gastheer daarop, die er voor zorgen dat het virus al dan niet overgedragen kan worden. Het envelop eiwit, of gp160, zit aan de buitenkant van het virus. Een deel ervan, het gp120, steekt naar buiten uit. Het zorgt er voor dat HIV-1 de CD4 positieve T-cel binnen kan komen, maar is ook het doelwit van het immuunsysteem van de gastheer die (neutraliserende) antistoffen kan maken die kunnen verhinderen dat HIV-1 de cel binnendringt. Dit onderzoek naar de genetische code en functie van de HIV-1 envelop is in het bijzonder gericht op de overdracht van HIV-1 van moeder naar kind. Bij moeders die borstvoeding geven is onderzocht of het anti-malaria middel Chloroquine (CQ) in staat is om de eigenschappen van het HIV-1 envelop gp120 zodanig te veranderen dat de kans op transmissie vermindert en de hoeveelheden virus in de borstvoeding verlaagd worden.

In **hoofdstuk 2** beschrijven we twee families waarvan beide ouders en twee kinderen al 10 jaar HIV-1 bij zich dragen. Het ziekteverloop bij de verschillende leden van de families blijkt echter heel verschillend te zijn. Van familieleden die ondanks de HIV-1 infectie gezond zijn gebleven en van diegenen die AIDS hebben ontwikkeld hebben we geanalyseerd wat van het HIV-1 gp120 de genetische code is, die ook wel basenvolgorde of sequentie genoemd wordt. Het doel hiervan was om te onderzoeken wat er bij deze genetische zeer verwante virussen toe leidt dat het ene virus evolueert tot een ziekmakend HIV, terwijl een ander virus dat niet doet. Het blijkt dat bij een variabel gedeelte van de envelop, het V3 domein, een hogere lading samengaat met het ontstaan van ziekte. Een lading van (een gedeelte van) een eiwit komt doordat de bouwstenen (de aminozuren) van een eiwit een negatieve, een neutrale, of een positieve lading hebben. De optelsom van de ladingen bepaalt dan wat de netto lading van in dit geval V3 is. Behalve de lading van het V3 blijkt bij de virussen uit deze families ook de verhouding tussen de grootte van de envelop en het aantal potentiële N-glycosylering plaatsen samen te gaan met het ontwikkelen van ziekte. Potiëntele N-glycosylering plaatsen (PNGS) zijn plaatsen op een eiwit zoals de HIV-1 envelop, die gedefinieerd worden door een bepaalde, vastliggende genetisch code, waaraan suikers gekoppeld kunnen worden. Die suikers kunnen er dan voor zorgen dat de envelop net wat andere eigenschappen krijgt. Zo kan bijvoorbeeld het immuunsysteem anders reageren op een envelop met een bepaald patroon van suikers dan op een envelop met een ander patroon. We hebben hier gevonden dat hoe meer potentiële plaatsen voor suikers er op een envelop zitten, hoe minder snel iemand ziek wordt. We hebben dus gevonden dat mensen die met nauwelijks verschillende virussen besmet

worden toch een heel verschillend ziekteverloop kunnen vertonen. Een virus kan zich in elk individu anders ontwikkelen, waarbij een virus dat meer ziekmakend is, een hoger lading op V3 heeft en relatief meer plaatsen waarop suikers (kunnen) zitten.

In **hoofdstuk 3** beschrijven we de effecten die Cloroquine (CQ) heeft op de infectie en vermeerdering van HIV-1 *in vitro* (in het laboratorium). Uit andere, eerdere studies is de conclusie getrokken dat CQ de hoeveelheid HIV-1 in het bloed kan verminderen en dat CQ zich kan ophopen in de moedermelk van vrouwen die borstvoeding geven. Dit zou kunnen beteken dat CQ als antiviraal middel gebruikt zou kunnen worden bij vrouwen die borstvoeding geven. Daarom hebben we onderzocht of virus dat in de aanwezigheid van CQ is opgegroeid anders cellen infecteert of zich anders vermeerdert, maar we hebben daar geen aanwijzingen voor gevonden. We hebben wel gevonden dat virus dat in de aanwezigheid van CQ gevormd wordt, minder goed in staat is om aan DC-SIGN, een molecuul dat op dendritische cellen zit, te binden en via een mechanisme dat *trans*-infectie wordt genoemd overgedragen te worden aan CD4-positieve T-cellen. De dendritische cel is een cel van het immuunsysteem die zich meestal onder de huid, of andere oppervlakken bevindt zoals in de long en het maag-darmkanaal. De dendritische cel breekt normaal gesproken een virus af en maakt er kleine stukjes van die aan andere cellen van het immuunsysteem worden gepresenteerd. HIV-1 kan dit proces overleven en vervolgens de CD4-positieve T-cel infecteren waarin HIV-1 zich kan vermeerderen. Dit proces wordt *trans*-infectie genoemd. We hebben gevonden dat het kweken van virus in de aanwezigheid van CQ samengaat met het verlies van twee potentiële N-glycosylerings plaatsen. Deze plaatsen zijn betrokken bij de binding aan DC-SIGN maar ook bij binding aan de monoclonale antistof 2G12. Deze monoclonale antistof bindt aan suikers op de envelop en is in staat om te voorkomen dat HIV-1 de T-cel binnengaat. Al met al hebben deze experimenten in het laboratorium ons de indruk gegeven dat CQ een bruikbaar middel zou kunnen zijn om de overdracht van HIV-1 via borstvoeding te voorkomen.

In **hoofdstuk 4** wordt beschreven wat er gebeurt als CQ na de bevalling 16 weken lang daadwerkelijk wordt gebruikt door moeders die borstvoeding geven. We hebben gevonden dat de concentraties van CQ in moedermelk 2,5 maal zo hoog zijn dan in het bloed. We hebben niet gevonden dat deze hogere concentratie van CQ in de borstvoeding leidt tot een lagere hoeveelheid virus in de melk. In tegenstelling tot wat in andere studies is gevonden, hebben we in het bloed juist een duidelijk verhoogde hoeveelheid virus gevonden bij de met CQ behandelde moeders maar niet in onbehandelde moeders. We hebben dit niet kunnen verklaren door de genetische code van het gp120 envelop eiwit te onderzoeken. In de envelop hebben we geen karakteristieke veranderingen gevonden die de toename van de hoeveelheid virus in het bloed zouden kunnen verklaren.

In **hoofdstuk 5** hebben we de groep van 30 HIV-1 geïnfecteerde moeders uit hoofdstuk 4 voor en na de bevalling bestudeerd. Zeven van hun 30 kinderen bleken tijdens de zwangerschap, tijdens of kort na de geboorte met HIV-1 geïnfecteerd te zijn. Het subtype van de virussen is A, C, of in één moeder en kind paar een kruising tussen de twee. We hebben de genetische code bepaald van het grootste en belangrijkste gedeelte (van V1 tot V5) van het envelop gp120 eiwit, zowel van moeders die het virus niet aan hun kinderen hebben overgedragen (Niet Transmitterende Moeders, NTM) als van moeders die HIV-1 wel aan hun kinderen hebben overgedragen (Transmitterende Moeders, TM) en hun besmette kinderen. Tussen het envelop gp120 eiwit van de NTM en de TM hebben we geen verschillen gevonden in de lengte van het eiwit of het aantal potentiële N-geglycolyseerde plaatsen (PGNS). Wel bleek de plek in de envelop waar de PGNS aanwezig is te verschillen. Virus dat overgedragen is blijkt bij voorkeur een PNGS te hebben op de posities N234 en N339. We hebben de gegevens onderzocht die in de literatuur beschikbaar zijn over andere HIV-1 geïnfecteerden die hun virus hebben overgedragen. Het blijkt dat N339 duidelijk meer aanwezig is in de envelop van moeders die het virus aan hun kinderen overdragen, maar niet bij sexueel overgedragen HIV-1. Dit alles wijst er op dat HIV-1, dat een envelop gp120 eiwit heeft met een PNGS op positie N339, relatief gemakkelijk van moeder naar kind wordt overgedragen.

In **hoofdstuk 6** hebben we het hele envelop eiwit bestudeerd van de 7 moeders en hun geïnfecteerde kinderen en van 4 niet transmitterende moeders. Behalve naar de eigenschappen van de envelop hebben we ook gekeken naar de antistofreactie die ze in de moeders en kinderen opwekken. Tussen het hele envelop eiwit van de niet transmitterende en transmitterende moeders (NTM en TM) hebben we geen verschil gevonden wat betreft virologische eigenschappen zoals infectiviteit en binding aan DC-SIGN die belangrijk zouden kunnen zijn bij de overdracht van HIV-1 van moeder naar kind. We hebben wel gevonden dat ten opzichte van NTM de transmitterende moeders (TM) duidelijk grotere hoeveelheden neutraliserende antistoffen maken tegen hun eigen envelop en die van hun kinderen. Dat is heel onverwacht. Voordat we deze experimenten deden, veronderstelden we dat veel neutraliserende antistoffen de overdracht van HIV-1 van moeder naar kind juist zouden tegengaan. TM die tijdens de zwangerschap hun kinderen besmetten, maakten de grootste hoeveelheden neutraliserende antistoffen, groter dan moeders die hun kinderen tijdens de bevalling of tijdens het geven van borstvoeding besmetten. De tijdens de zwangerschap geïnfecteerde kinderen hadden meer neutraliserende antistoffen tegen hun eigen envelop en die van hun moeder dan kinderen die tijdens de bevalling of door borstvoeding zijn geïnfecteerd. De uitkomsten van deze experimenten zijn niet zonder gevolgen voor de te ontwikkelen vaccins of preventief toe te dienen (monclonale) antistoffen, die tot doel hebben om te voorkomen dat kinderen door hun moeder met HIV-1 geïnfecteerd worden.

CURRICULUM VITAE van ELISABETH BAAN (ELLY)

Elly Baan werd geboren op 27 april 1946 te Amsterdam. Zij bezocht de lagere school in Amsterdam en Amstelveen van 1952 tot 1958 gevolgd door MULO-B opleiding te Amstelveen van 1958 tot 1962. Zonder dat ze ooit een laboratorium van binnen gezien had was haar grootste wens deze magische wereld te leren kennen. Daarom solliciteerde zij bij het Chemisch Laboratorium van Werkspoor Amsterdam en werkte daar van 1962 tot 1964 aan de analyse van metalen. In deze periode volgde zij de avondopleiding voor leerling-analisten te Amsterdam. Omdat haar belangstelling zich in andere richtingen begon te ontwikkelen, solliciteerde zij in 1982 bij het Biochemisch laboratorium van de Universiteit van Amsterdam (hoofd Prof. E.C. Slater) waar zij tot 1969 als analiste Prof. P. Borst assisteerde bij het onderzoek naar de biogenese van mitochondriën. Van 1969 tot 1976 werkte zij aan het Biochemisch Laboratorium aan de Vrije Universiteit van Amsterdam (hoofd Prof. R.J. Planta) aan het onderzoek naar de biogenese van gist ribosomen. In deze periode volgde zij de VWO avondopleiding te Amsterdam. In de periode van 1976 tot 1980 genoot Elly fulltime van het moederschap. Toen in 1980 de tijd daarvoor rijp was werd zij aangenomen bij het Laboratorium voor Medische Microbiologie, afdeling Experimentele Virologie van de Universiteit van Amsterdam (hoofd Prof. J. van der Noordaa). Hier werkte zij aanvankelijk aan de oncogenetische eigenschappen van BKV, later aan onderzoek naar de relatie tussen cervixcarcinoom en HPV. In deze periode volgde zij de HLO avondopleiding in Delft. In 1990 werd zij aangenomen bij de afdeling Humane Retrovirologie (hoofd Prof. J. Goudsmit) waar zij werkte aan het sequencen van het HIV-1 genoom geïsoleerd uit patiëntenmateriaal. Tot 2011, het jaar dat zij gepensioneerd werd, bleef zij werkzaam bij het Laboratorium voor Medische Microbiologie, waar de afdeling Retrovirologie inmiddels weer omgedoopt werd tot Experimentele Virologie en waar Prof. B. Berkhout hoofd werd. Zo rond het jaar 2007 ontstond de grote wens van Elly om haar carrière als research analist af te sluiten met een zelfstandig onderzoek dat zou leiden tot een proefschrift en het behalen van de doctorsgraad. Tot haar grote blijdschap werd haar droom werkelijkheid dankzij de instemming en steun van haar promotor en de beide co-promotores.

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PhD PORTFOLIO

Name PhDstudent:	Elisabeth Baan
PhD Period:	2007-2012
Name PhD supervisors:	Prof. dr. B. Berkhout dr. W.A. Paxton and dr. G. Pollakis

PhD TRAINING

General courses

Basic Coarse: The AMC World of Science	2007	1
Infectious Diseases	2007	1
Introduction to Bioinformatics	2008	1
Practical Biostatistics	2009	1

Seminars and workshops

Masterclass by R. A. Koup	2012	0.2
Weekly lab seminar	2007-12	5
Weekly PhD student meeting	2007-12	5
Ruysch Lectures (AMC seminar)	2007-12	1
Refereer avond (departmental seminar)	2007-12	1

Conferences

Keystone Symposium HIV Evolution, Genomics and Pathogenesis, Whistler, March 2011	2011	1
Netherlands Conference on HIV Pathogenesis, Prevention and Treatment, Amsterdam, NCHIV, November 2012	2012	0.25

Poster presentations

Keystone Symposium HIV Evolution Genomics and Pathogenesis, 2011 "Characteristics of viral envelopes involved in mother to child transmission of HIV-1"	2011	0.5
NCHIV 2012 conference "Increased autologous neutralizing activity associated with HIV-1 mother to child transmission"	2012	0.5

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