Higher Placental Anti-Inflammatory IL-10 Cytokine Expression in HIV-1 Infected Women Receiving Longer Zidovudine Prophylaxis Associated with Nevirapine

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Abstract: Placental cytokine balance may be critical for the control of mother-to-child transmission (MTCT) of HIV. We assessed whether the type and duration of antiretrovirals used for prevention of HIV-1-MTCT modified the inflammatory cytokine profile. We investigated the levels of cytokine expression in the placentas of 61 HIV-1-infected women who received zidovudine (ZDV) plus single dose nevirapine (SD-NVP) or ZDV only for prevention of MTCT. Placentas of 38 HIV-1-uninfected women were included as controls. All placentas were obtained after vaginal delivery. Levels of mRNA and cytokine expression were quantified using real-time PCR and ELISA, respectively, in placental explants and 24-hour culture supernatants and analyzed in relation to the women's characteristics and the type and duration of antiretroviral prophylaxis. HIV-1-infected and uninfected women did not show any differences in the expression of placental cytokine secretion except for a trend toward lower TNF-α mRNA levels in HIV-1-infected women. Within the HIV-1-infected group, women who were exposed to a long duration of ZDV (>72 days) or received SD-NVP less than 5h prior to delivery, more frequently expressed detectable levels of IL-10 in their placentas (32% versus 7% ($p = 0.01$) and 32% versus 5% ($p = 0.02$), respectively). No infant was found to be HIV-1-infected. Our results showed a normalization of the placental cytokine balance in HIV-1-infected women receiving antiretroviral prophylaxis. Furthermore, the type and duration of antiretroviral prophylaxis have an impact on the placental anti-inflammatory IL-10 expression level, which may contribute to controlling HIV replication at the placental level, thus reducing MTCT of HIV-1.

Keywords: HIV-1, placenta, antiretroviral prophylaxis, zidovudine and nevirapine, IL-10 cytokine, mother-to-child transmission.

INTRODUCTION

The placenta constitutes a powerful natural barrier to HIV-1 transmission, presumably through its double layer of polarized cells, cytotrophoblasts and syncytiotrophoblasts. Indeed, primary cultures of purified human trophoblasts [1, 2] and trophoblast cell lines [3, 4] display limited permissiveness for HIV-1 infection by cell-free viruses and restricted HIV-1 replication. Cytokine balance in the placental environment is known to play an important role not only in the maintenance of pregnancy but also in favoring or inhibiting in-utero infection. For example, elevated levels of placental tumor necrosis factor-alpha (TNF-α) were associated with poor pregnancy outcome and higher risk of transmitting malaria to the infant [5-7]. HIV-1 can up-regulate placental interleukin (IL)-1β, IL-6, and TNF-α pro-inflammatory cytokines *in vitro* [8, 9]. These pro-inflammatory cytokines have been shown to activate HIV-1 proviral expression in trophoblast cells [10-12].

Antiretroviral prophylaxis during pregnancy and labor, and in neonates dramatically reduces perinatal HIV-1 transmission. In the PACTG 076/ANRS 024 trial, the use of zidovudine (ZDV) prophylaxis in non-breastfeeding women during pregnancy and labor and in neonates lowered the risk of transmission by two-thirds [13]. A single dose of nevirapine (SD-NVP) given to women at the onset of labor and to infants 48-72 hours after birth decreased the risk of transmission by almost half [14]. When SD-NVP was administered to women at the onset of labor in addition to ZDV prophylaxis starting from 28 weeks of gestation, mother-to-child HIV-1 transmission was reduced to less than 2% [15]. The effect of these antiretroviral drugs on decreasing maternal HIV viral load is insufficient to fully explain their efficacy for the reduction of perinatal HIV-1 transmission rates. For example, in the PACTG 076/ANRS 024 trial, the median viral load decrease associated with ZDV prophylaxis in HIV-1 infected pregnant

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women was 0.3 $\log_{10}$ copies/ml [16]. In the HIVNET006 study, a single dose of nevirapine given at the onset of labor resulted in a median viral load decrease of 1.3 $\log_{10}$ copies/ml at 1 week postpartum [17]. This suggests that there are additional mechanisms through which antiretrovirals prevent MTCT of HIV-1. Since both drugs easily cross the placenta, a pre- or post-exposure prophylactic effect in the neonate is very likely [15]. In addition, modification of the placental cytokine environment may contribute to reducing transmission.

Our objective was to determine whether the type and duration of antiretrovirals used for prevention of HIV-1 MTCT can modify the placental cytokine balance.

MATERIALS AND METHODS

Study Population

This prospective study was conducted in 4 hospitals in Chiang Mai and Lamphun provinces in northern Thailand. The protocol was approved by the Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University. All women participating in this study provided written informed consent. Women who had a vaginal delivery and did not give birth to twins were enrolled. HIV-1 infected women who delivered by caesarean section were excluded from the analysis.

Between April 2002 and September 2004, ninety-nine placentas, 61 from HIV-1 infected women and 38 from HIV-1 uninfected women, were collected. Fifty-nine of the HIV-1 infected women, enrolled at Health Promotion Center Region 10, Nakornping, and Lamphun hospitals, were participating in PHPT-2 [15] (ClinicalTrials.gov NCT00398684). They all received ZDV (300 mg twice daily) starting at 28 weeks' gestation, or as soon as possible thereafter. According to their randomization arm, women received either a single dose of NVP (200 mg) or a placebo at onset of labor. The remaining two HIV-1 infected women, enrolled at Maharaj Nakorn Chiang Mai hospital, received ZDV starting at 34 weeks' gestation.

The following data were collected from all women: age, gestational age at delivery, and mode of delivery. For HIV-1 infected women, the following additional data were collected: antiretroviral prophylaxis (type, timing, and duration), CD4 cell count (cell/mm^3) before 28 weeks of gestation and plasma HIV-1 RNA viral load at delivery ($\log_{10}$ copies/ml).

Diagnosis of HIV-1 infection in infants born to HIV-1 infected mothers was performed using DNA PCR on dried blood spots (Amplicor® HIV-1 DNA assay version 1.5, Roche Molecular Systems Inc., Branchburg, NJ, USA) as previously described [15]. For infants born to women enrolled at the Maharaj Nakorn Chiang Mai hospital, HIV-1 infection was assessed by HIV-1 serology (HIV Combi, Elecsys®, Roche, Germany and Anti-HIV-1/2 Plus, Enzygnost®, Dade Behring, Germany) at 18 months of age.

Placenta Processing

Within three hours post-delivery, placentas were processed to isolate placental chorionic villi as previously described [18]. Fragments (< 0.5 cm of thickness) of placental chorionic villi were immersed in 1.5 mL of RNA stabilization reagent (RNAlater™, Qiagen, Germany), stored immediately at 2-8°C for 24 hours, and then transferred to -70°C. Six grams of placental chorionic villi were cultured for 24 hours in 2 flasks (3 g/flask), each containing 20 mL of culture medium as previously described [18].

Quantification of Secreted Cytokines

Quantification of TNF-α, IL-8, IL-10, IL-15, IL-16, and regulated on activation normal T-cell-expressed and secreted (RANTES) in the placental culture supernatants was performed using a quantitative sandwich enzyme immunoassay (ELISA) technique with cytokine specific commercial kits as previously described [18]. Quantification of leukemia inhibitory factor (LIF) was performed at the laboratory of Immunology, CNRS UMR 5540, Université Bordeaux 2, France, using an in-house sandwich ELISA [19].

Quantification of Cytokine mRNA

Total RNA was extracted from thawed placental chorionic villi and reverse transcribed to cDNA using the Taqman® Reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Stromal cell-derived factor (SDF)-1, TNF-α, IL-8 and IL-10 mRNA levels were quantified using the Taqman real-time PCR assay as previously described [18]. The cytokine mRNA levels are expressed as multiples of the results obtained with reference cDNA, prepared from a term placental tissue of an HIV-1 uninfected woman, used as a calibrator (× cal).

Statistical Analysis

Characteristics of the 2 groups, HIV-1 infected and uninfected women, are described by n (%) for qualitative data and median (inter-quartile range; IQR) for quantitative data. Results of cytokine secretion and mRNA expression levels are presented as median and IQR for each group. Comparison of cytokine secretion and mRNA expression median levels between groups was performed through the non-parametric Mann-Whitney test. In addition, the proportion of women with detectable levels of cytokine secretion was compared between groups through the Chi-square test or the Fisher exact test, when necessary. Undetectable cytokine median levels were replaced by half of the value of the detectability threshold. No comparison of cytokine median levels was performed if less than 50% of women in one group had a detectable level.

Cytokine secretion or mRNA expression levels were compared in HIV-1 infected women according to the type, timing or duration of antiretroviral (ARV) prophylaxis. ZDV duration was divided into 2 categories according to the median, 72 hours. The time interval between NVP intake and delivery was also categorized according to the rounded median, 5 hours. Furthermore, this 5-hour cut-off value is relevant in terms of pharmacokinetics since it has been shown that the peak NVP plasma concentration is reached 3 to 7 hours after single dose intake during labor [17, 20]. Comparisons were performed through the Mann-Whitney test for quantitative results (levels of secretion or mRNA expression) and through the Chi-square test or the Fisher exact test, when necessary, for qualitative results (proportion of women with detectable secretion levels). Comparisons yielding a $p < 0.05$ were considered significant.

RESULTS

Characteristics of the Study Population

The demographic and gestational characteristics of the groups of HIV-1 infected and uninfected pregnant women are shown in Table **1**. The median age at delivery was not significantly different between the 2 groups (26 versus 28 years, $p = 0.56$), while the median gestational age at delivery was higher in the group of HIV-1 infected women (39.4 versus 38.6 weeks, $p = 0.05$). The antiretroviral prophylaxis received and biological characteristics are shown for the group of HIV-1 infected women. Eighteen percent of HIV-1 infected women received ZDV prophylaxis only.

Infant HIV-1 Status

The HIV-1 status could be determined for 60 of the 61 infants born to HIV-1 infected mothers - one was lost to follow up. Of the 60 infants with known HIV status, none were found to be HIV-1 infected.

No Difference in the Expression Levels of Cytokine Secretion Between HIV-1 Infected and Uninfected Women

Cytokines were quantified in placental culture supernatants of HIV-1 infected women and expression levels were compared to those of uninfected women (Table **2**). The proportion of samples with detectable expression levels of IL-10, TNF-α, and LIF as well as the median expression levels of LIF, IL-8, IL-15, IL-16, and RANTES were not significantly different between HIV-1 infected and uninfected women (Table **2**).

No Difference in the Expression Levels of Cytokine mRNA Between HIV-1 Infected and Uninfected Women

Expression levels of mRNA for the pro-inflammatory cytokines TNF-α and IL-8, the anti-inflammatory cytokine IL-10, and the chemokine SDF-1 were quantified in placental explants of HIV-1 infected and uninfected women (Table **3**). A trend toward lower TNF-α mRNA median expression levels was observed in placentas of HIV-1 infected women ($p = 0.06$) while the median expression levels of SDF-1, IL-8, and IL-10 mRNA of HIV-1 infected women were not significantly different from those of HIV-1 uninfected women.

Placenta Inflammatory Profile in Immunocompromised Women

Analysis of the relation between the maternal CD4 level and placental cytokine profile showed that women (n = 19) with CD4 count less than or equal to 250 cells/mm^3 expressed higher placental TNF-α mRNA (2.7 versus 1.1 × cal, $p = 0.007$) and IL-8 secretion levels (2,013 versus 1,517 pg/ml, $p = 0.02$) than women with CD4 count higher than 250 cells/mm^3 (n = 42).

Long Duration of ZDV Prophylaxis is Associated with Higher Placental IL-10 Secretion Level

We then assessed among HIV-1 infected women the effect of the duration of ZDV prophylaxis on the level of cytokine expression. TNF-α and IL-10 secretions were analyzed since TNF-α is a pro-inflammatory cytokine known to stimulate HIV-1 replication and IL-10 is an anti-inflammatory cytokine which inhibits the expression of TNF-α mRNA. The median expression level of TNF-α mRNA of women who had a long ZDV prophylaxis duration (> 72 days) was not significantly different from that of women who had a ZDV prophylaxis duration less than or equal to 72 days (1.8 versus 1.7 × cal, $p = 0.30$). In contrast, the proportion of supernatants with detectable levels of IL-10 was significantly higher in women who received ZDV prophylaxis for more than 72 days than in those who had a ZDV prophylaxis duration less than or equal to 72 days (32% versus 7%, $p = 0.01$) (Fig. **1**).

Table 1. Characteristics of the Study Population

Enrolment Sites	HIV-1 Infected Women (n = 61)	HIV-1 Uninfected women (n = 38)
Maharaj Nakorn Chiang Mai Hospital	2	12
Health Promotion Center Region 10	49	21
Lamphun Hopital	7	3
Nakornping Hospital	3	2
Characteristics:		
Age at delivery (years)	26 (22-31)	28 (23-30)
Gestational age at delivery (weeks)	39.4 (37.9–40.4)	38.6 (38.0-39.0)
Gestational age at ZDV prophylaxis initiation (weeks)	29 (28-39)	Not Relevant
Duration of ZDV prophylaxis (days)	72 (54–81)	Not Relevant
NVP plus ZDV prophylaxis	50 (82%)	Not Relevant
Time to delivery after NVP dosing (hours)	4.4 (1.9-8.2) (n = 46)	Not Relevant
CD4 cell count before 28 weeks gestation (cells/mm^3)	332 (241–494)	Not Relevant
HIV RNA load at delivery (log_{10} copies/ml)	4.1 (3.3–4.6) (n = 57)	Not Relevant

The data are expressed as median (interquartile range) or n.

Table 2. Cytokine Secretion Levels in Supernatants of 24h-Culture of Placental Explants from HIV-1 Infected and Uninfected Pregnant Women

Secretion of Cytokines and Chemokines	HIV-1 Infected Women (n = 61)	HIV-1 Uninfected Women (n = 38)	*p*
IL-10 detectable samples (%)	20	18	0.88[b]
TNF-α detectable samples (%)	48	32	0.12[b]
LIF detectable samples (%)	75	74	0.85[b]
LIF (UI/L)	55 (18-87)	50 (17-107)	0.99[c]
IL-8 (pg/ml)[a]	1787 (1086-2271)	1840 (1086-2702)	0.81[c]
IL-15 (pg/ml)[a]	11.0 (8.2-16.1)	10.8 (6.9-15.7)	0.48[c]
IL-16 (pg/ml)[a]	404 (253-585)	424 (249-512)	0.83[c]
RANTES (pg/ml)[a]	506 (296-726)	477 (359-795)	0.86[c]

The data are expressed as percentage of detectable samples and/or median (interquartile range).
[a] All samples had detectable level of cytokines.
[b] Chi-square test.
[c] Mann-Whitney test.

Table 3. Placental Cytokine mRNA Levels

Expression of Cytokine and Chemokine mRNA (× cal)	HIV-1 Infected women (n = 61)	HIV-1 Uninfected Women (n = 38)	*p*[a]
SDF-1	2.8 (1.6-6.7)	3.2 (1.9-6.4)	0.95
TNF-α	1.8 (0.8-4.9)	2.8 (1.7-4.5)	0.06
IL-8	2.4 (0.6-6.1)	3.1 (1.0-7.4)	0.37
IL-10	2.5 (1.2-6.2)	3.2 (1.8-5.3)	0.36

The data are expressed as median (interquartile range). The cytokine mRNA levels are expressed as multiples of the results obtained with a reference cDNA prepared from a term placental tissue of a HIV-1 uninfected woman and used as a calibrator (× cal).
[a] Mann-Whitney test.

Administration of NVP Less Than 5 Hours Prior to Delivery is Associated with Higher Placental IL-10 Secretion Level

The median expression level of TNF-α mRNA in women who received ZDV plus NVP was comparable to that of women who received ZDV only (1.6 versus 2.3 × cal, p = 0.24). Among women who received a single dose of NVP during labor, TNF-α mRNA expression levels were similar in those who received NVP at least 5 hours prior to delivery and those who received NVP less than 5 hours prior to delivery (1.1 versus 1.6 × cal, p = 0.19). Similarly, the proportion of placentas with detectable expression levels of IL-10 was not different between women who received ZDV plus NVP and those who received ZDV only (18% versus 27%, p = 0.48) (Fig. **2A**). However, among women who received a single dose of NVP during labor, detectable IL-10 secretion levels were found in higher proportion of those who received NVP less than 5 hours prior to delivery than in those who received NVP later (32% versus,5%, p = 0.02) (Fig. **2B**).

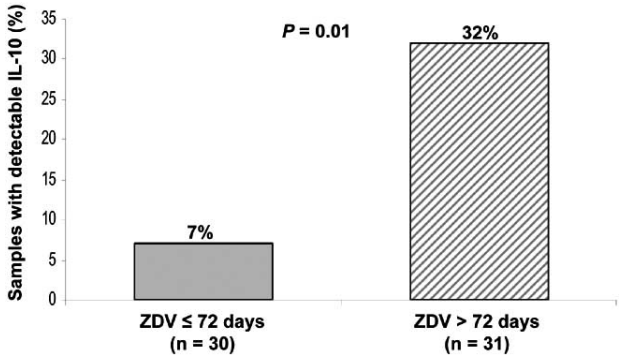

Fig. (1). Proportion of detectable IL-10 levels (≥ 5 pg/ml) in placental culture supernatants of HIV-1 infected women according to ZDV prophylaxis duration (72 days). IL-10 was evaluated by ELISA.

DISCUSSION

We report here, for the first time, an association between a higher expression level of the anti-inflammatory IL-10 cytokine in the placenta and either a longer ZDV prophylaxis duration or a shorter time to delivery after intake of NVP during labor.

HIV-1 infected patients usually exhibit high levels of inflammatory cytokines. The fact that no significant differences were observed between the median placental cytokine levels of HIV-1 infected and uninfected women suggests a possible effect of ARV in regulating cytokine expression at the placental level. However, this hypothesis could not be ascertained since all HIV-1 infected women in the study received ZDV or ZDV plus NVP. In the present study, placentas of HIV-1 infected women showed a trend toward lower median expression of TNF-α mRNA than placentas

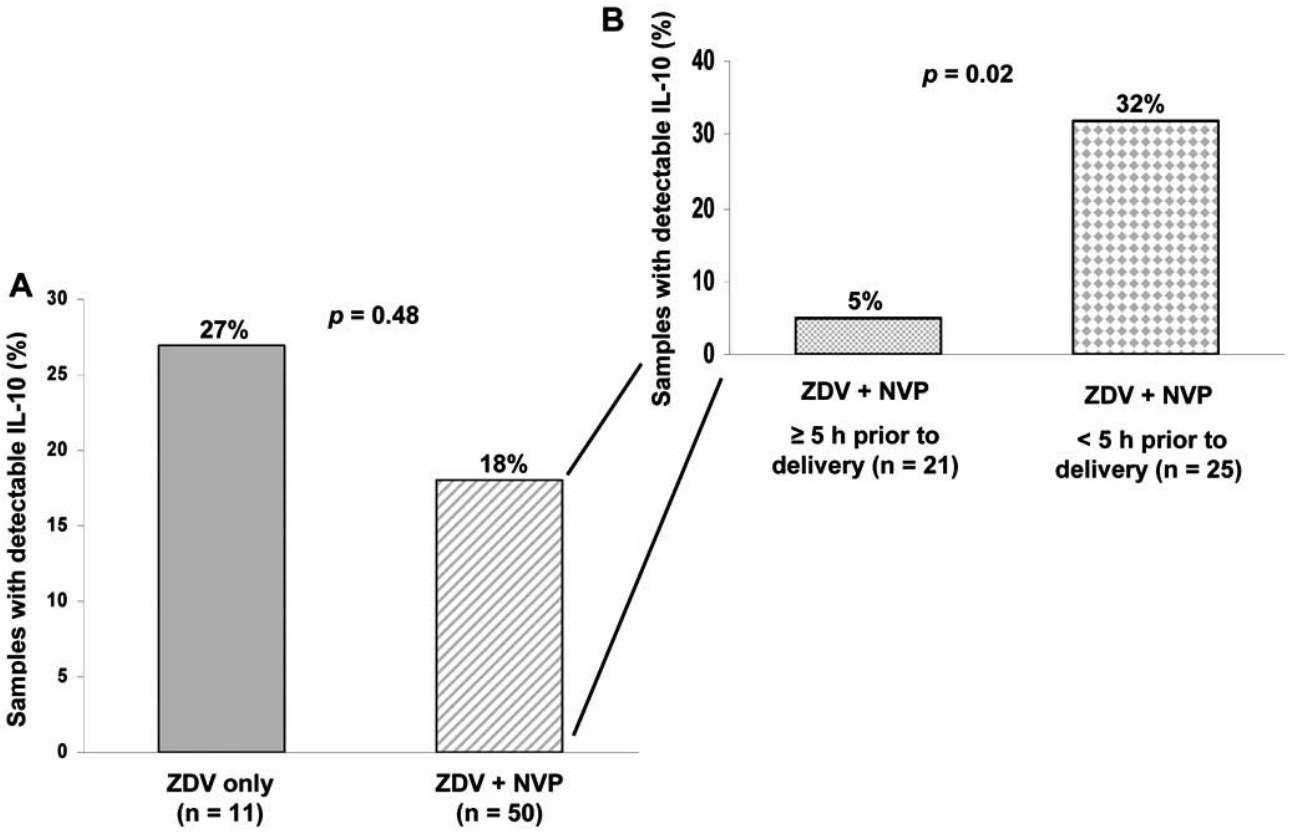

Fig. (2). Proportion of detectable IL-10 levels (≥ 5 pg/ml) in placental culture supernatants of HIV-1 infected women according to the ARV prophylaxis regimen. (**A**) Women received ZDV only or ZDV plus a single dose of NVP at onset of labor. (**B**) Women received NVP less than 5 hours or at least 5 hours prior to delivery. IL-10 was evaluated by ELISA. Time from NVP intake to delivery was not available for 4 HIV-1 infected women.

of HIV-1 uninfected women (Table **3**), suggesting that ZDV or ZDV plus NVP might decrease the expression of TNF-α mRNA. This hypothesis is consistent with our previous *in vitro* study showing a decrease of TNF-α transcripts when ZDV was added to normal placental histoculture [21]. Thus, ZDV or the combination of ZDV plus NVP may contribute to the normalization of the placental cytokine balance in HIV-1 infected women. This is also consistent with the Hygino *et al*. report [22] showing similar lymphoproliferation and IL-4, IL-10, IFN-γ and TNF-α levels in cord blood of neonates born to HIV-1 infected mothers who controlled their plasma HIV RNA and neonates born to HIV-1 uninfected mothers. Moreover, neonates born to HIV-1 infected mothers who received ZDV plus NVP during pregnancy had significantly higher IL-10 levels in their plasma than those born to untreated HIV-1 infected mothers.

In our study, a long ZDV prophylaxis duration (> 72 days) was associated with a higher proportion of detectable IL-10 secretion levels (p = 0.01). This proportion was also higher when NVP was given less than 5 hours prior to delivery (p = 0.02). These data suggest that ZDV itself could increase the production of IL-10 and this effect might be synergized by NVP. The effect of NVP on IL-10 secretion, which cannot be observed more than 5 hours after administration, is more difficult to interpret and could be related to a short duration of labor. Another possible explanation is that initial induction of IL-10 secretion during the first 5 hours after NVP intake could be followed by inactivation of IL-10 secretion through a negative feedback mechanism as a result of prolonged exposure to NVP [23-25]. However, this "anergic" state was not described by Schramm *et al*., who reported an increase in plasma activation markers such as neopterin, β_2-microblobulin, and soluble L-selectin in women exposed to SD-NVP [22]. Another hypothesis could be that the secreted IL-10 binds to its receptors and can no longer be detected in our system 5 hours after NVP intake. A recent paper also highlighted the impact of the duration of ARV treatment on the risk of HIV-1 MTCT in women with a low viral load and vaginal delivery [26].

Although an alteration of the TNF-α/IL-10 balance toward a pro-inflammatory cytokine profile has been associated with HIV-1 disease progression [27, 28], whether or not the increase of anti-inflammatory IL-10 cytokine, resulting from ZDV or ZDV plus NVP, may participate in the control of HIV-1 replication is still unclear. The fact that immunocompromised women expressed higher placental TNF-α mRNA and IL-8 secretion levels likely reflects their more advanced disease stage. The sample size was too limited to analyze these results according to the type and duration of the ARV prophylaxis. In comparing placental cytokine profiles from transmitting versus non-transmitting mothers, Behbahani *et al*. reported an imbalance of placental cytokine

distribution towards lower levels of IL-2 and higher levels of IL-4 and IL-10 cytokines in the placenta of HIV-1 non transmitting mothers [29]. In the present study, it was not possible to assess whether or not placental cytokine levels were associated with perinatal HIV-1 transmission since no infant was infected by HIV-1.

The exact molecular mechanisms by which ZDV and NVP control HIV-1 transmission through placental cytokine balance are not known. A possible mechanism could be that ZDV and NVP exert their activity through the transcriptional NF-kB pathway to down-regulate TNF-α gene expression, thus diminishing HIV replication [30].

In conclusion, this study demonstrates that longer zidovudine prophylaxis and shorter time to delivery after NVP dosing are associated with a higher placental anti-inflammatory IL-10 expression. This effect may contribute to reducing MTCT of HIV-1 through a reduction in HIV replication at the placental level. Therefore, the effect of ARV drugs on placental cytokine profiles should be considered in developing further strategies to prevent HIV-1 MTCT.

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