

NATURAL KILLER CELLS AT THE FOETAL-MATERNAL INTERFACE OF HIV-1 INFECTED PREGNANT WOMEN

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Abstract

Objectives: Several lines of evidence suggest that Natural Killer (NK) cells have an important role in antiviral defence. Thus, the impairment of NK cell function in the course of HIV infection contributes to a decreased resistance against HIV. Therefore, the interaction between the presence of NK cells and p24 at the placental interface of HIV-1 infected mothers was investigated.

Methods: Evidence of human immunodeficiency virus (HIV) replication was sought in fifty five human placentas obtained from pregnancies complicated by maternal HIV-1 infection. The placentas were examined for p24 antibodies and NK cells (CD56+) using immunohistochemical staining techniques. Viral RNA and CD4+ cell counts were measured in plasma samples obtained from all mothers and babies immediately after delivery.

Results: Presence of p24 antigens was found in 94.6% of placental samples. Natural Killer cell activity was observed in 98.3% of samples. There was no statistically significant correlation between number of NK cells and vertical transmission ($p = 0.145$). However, the risk for vertical transmission was increased 3.4 times more if NK cell values were low [OR= 3.424 (95% CI 0.65-17.89)].

Conclusion: The presence of p24 in placental tissue was not influenced by maternal viral load. Low NK cell values increased the risk for vertical transmission.

Keywords: NK cells, vertical transmission, p24 antigens, placenta, *in utero*

Introduction

The placenta provides a potential barrier between maternal and foetal circulation. The human placenta is of the villous haemochorial type and consists of foetal villi bathed directly in circulating maternal blood^[1]. The villi outermost layer consists of syncytiotrophoblasts, which forms a multinucleated epithelium maintained by an underlying population of mononuclear cytotrophoblastic cells. The mesenchymal cells of the villous core are separated by a trophoblastic cell basement membrane layer. The villous core houses a significant number of macrophages (Hofbauer cells, some of which express CD4 molecules) and foetal capillaries^[1]. Trophoblastic cells constitute the external layer of chorionic villi and are in direct contact with maternal blood. These cells are primary target for maternal blood-borne infections. However, the placental barrier is not complete, and there is evidence that bidirectional traffic of cells, including leucocytes, may occur in human pregnancy^[2]. Infection of human placental tissue cells with HIV-1 requires direct contact with infected leucocytes^[3]. The mechanisms regulating the transfer of HIV into placental cells are varied and integrated.

Infection of the foetus occurs at various trimesters. The majority of *in-utero* infections occur during the final trimester of pregnancy. The infection rate in the second trimester is reported at 2-5%^[4]. Transmission during the first trimester appears to be rare^[5]. Therefore, understanding the mechanisms or routes involved in intrauterine transmission of HIV during pregnancy and parturition is important for planning intervention strategies.

Human NK cells are a major population of lymphocytes and are innate effectors critical to early host defense. Expressed as subsets CD56+ and/or CD16+, NK cells recognise and mediate spontaneous killing of a wide variety of target cells including viral and bacterial infected cells^[6,7]. Based on this information it follows that the loss of NKT cells could have a profound negative impact on normal immune functioning. This study was undertaken to investigate the relationship, if any, between the presence of NK cells (CD 56+) and p24 antigens at the placental interface in HIV-1 infected mothers and their association with risk factors in mother-to-child-transmission (MTCT).

Patients and Methods

Collection, Transport and Storage of Human Placental Samples

Placental samples from infected HIV-1 mothers were collected from fifty five patients post delivery. Ethical approval for the study was obtained from the Ethics Committee (University of KwaZulu-Natal) along with informed consent from all participating women. The placental specimens were immediately placed into 10% formal saline used as preservative and were transported on ice, to the laboratory for testing. A portion of the tissue sample was removed, processed and embedded in wax. The paraffin blocks were carefully handled and sectioned

in order to avoid cross-contamination. Sections were prepared from the wax embedded samples and placed onto Poly-L-Lysin slides (Sigma, USA) for immunohistochemistry staining.

Quantitation of HIV-1 RNA (Roché Amplicor Version 1.5, Germany) were performed on plasma and CD4+ cell counts (Becton Dickinson, USA) on blood samples, obtained from all mother- baby pairs in the study.

Laboratory Methods

Serial sections of paraffin wax embedded placental tissue, 4-5 μ m thick were placed onto Poly-L-Lysin coated slides (Sigma, USA) and each examined by immunoperoxidase immunohistochemistry using monoclonal mouse Anti-Human Immunodeficiency Virus, p24 (DAKO, Denmark, Code No. M0857) and primary antibodies to CD56+ (Zymed, USA, Clone 123C3) for the presence of HIV antigens and NK cells (CD56+) respectively. Duplicate sections of all specimens were immunolabeled. Commercially available CD56+ and p24 positive controls (DAKO) were used to verify test specificity and sensitivity. Negative controls were performed for p24 antigens by omission of the primary antibody. All positive cases showed more than one immuno-stained cell.

CD56+ cells negative controls were performed by using normal mouse immunoglobulin (IgG) control antibodies at the same concentration as the primary antibodies provided in the kit as per manufacturer's protocol. Duplicate placental sections from HIV negative women were also stained for CD56+. This was done to assess NK cell activity in placentas of HIV negative women. Controls and randomly selected placental sections were evaluated by a pathologist for quality assurance.

To maintain consistency, p24 antigen and CD56+ estimation was done according to number of stained cells/30 hpf. Infected placental cells were assigned categories ranging from absent (0 cell/30hpf), category A (20-30 cells/30hpf), category B (10-19 cells/30hpf) and category C (≤ 4 cells/30hpf). Sections were regarded as negative in the absence of immunoreactive p24 antigen in distinct cells. Natural Killer (CD56+) cells stained dark brown with monoclonal antibodies. An estimation of the total number of neutrophils and lymphocytes was performed on all tissue sections to gauge the overall inflammatory response in the placenta. A basic Haematoxylin and Eosin staining technique was used for the assessment of neutrophils and lymphocytes (inflammatory cells). Reagents were prepared prior to staining of slides. Viral load and CD4+ count procedures were performed according to manufacturer's protocol.

Haematoxylin and Eosin Staining Method

Tissue sections mounted onto Poly-L-Lysin coated slides (Sigma, USA) were placed in water. Thereafter, the sections were dewaxed in xylene and transferred

to alcohol. The slides were washed in water and stained in Haematoxylin for 5 minutes. The stained slides were washed in running water and blued in lithium carbonate. Counter stain of eosin was applied to all sections for 1 minute. Sections were dehydrated, cleared and mounted in DPX (mounting medium). The nuclei stained blue and the cytoplasm stained pinkish red.

Statistical Methods

Both parametric and nonparametric statistical analyses were used to assess the relationship between variables. Linear regression was used to determine the significance of inter-related interactions and the risk ratio. Confounding was assessed by the backward elimination method. Variables which did not alter the risk ratio were eliminated. Variables such as maternal viral load and gender of babies which changed the risk ratio were maintained and extensively evaluated. The entire analysis was conducted using SPSS statistical software.

Results

Detection of HIV-1 antigens and CD56+ Cells in Placental Tissue Using Immuno-histochemistry Staining

Immunohistochemistry of infected placental cells produced strong ring like granular cytoplasmic staining of HIV- positive cells only when anti- p24 was used. HIV negative control cells (DAKO) showed no staining with or without anti-p24. Both syncytiotrophoblasts and cytotrophoblasts displayed staining to p24 antigens. Detection of p24 antigens was noted in 52 (94.6%) placentas. The median value was 10 cells/30hpf.

The presence of CD56+ cells were observed using primary antibodies (Zymed, Clone 123C3) specific for CD 56+ cells. A similar grading system as used for p24 antigen was utilized to quantify the presence of CD56+ cells in placental tissue. The presence of NK cells was observed in 54 (98.2%) placental tissue sections. The median cell count was 10 cells/30hpf.

No significant association was observed between CD56+ cells and the presence of p24 antigens ($p = 0.438$). Overall, low NK (CD56+) cell values were observed with high p24 antigen values and vice versa. There was evidence of NK cell activity in majority of infected placentas. Assessment of CD56+ cells in placental tissue of HIV negative women was performed to gauge a general overview of NK activity in uninfected placentas. The number of CD56+ cell counts in placentas of HIV negative women ranged from 8 to 10 cells/30hpf.

HIV-1 Detection in Placentas Compared with Maternal Viraemia and CD4+ Cell Counts

Plasma HIV-1 RNA levels of all infected mothers were analysed in conjunction with their CD4+ counts and p24 antigen in the placenta. Parametric statistical analysis between maternal viral load ($p = 0.448$), CD4+ cell counts ($p = 0.660$) and the presence of placental p24 antigens was not significant. High p24 antigens with low or high viral RNA copies were evenly distributed. There was a small variance of 1.1% in p24 antigens (Figure 1A). Similarly, high p24 antigen values were observed with both high and low CD4+ cell values. The R Sq linear value 0.002 (0.2% variance) was negligible for any definitive conclusion (Figure 1B). The presence of p24 antigens in placental tissue of HIV-1 infected mothers is not influenced by their viral load or CD4+ cell count. It was also noted that viral load of the babies born to the 3 (5.4%) mothers whose placentas did not demonstrate the presence of p24 antigens, was < 400 copies/ml.

Relationship between Placental Natural Killer Cells, Maternal CD4+ Cell Counts and Babies Viral RNA Copies

The level of placental NK cell activity was examined in mothers with uninfected and infected babies. The median placental NK cell counts for the mothers with uninfected and infected were 10/30hpf (IQR 5-20/hpf) and 4/30 hpf (10-20/30hpf) respectively. No statistical differences in NK cell activity were observed between both groups of mothers ($p = 0.083$). This could be due to a small sample size which, leads to lower

statistical power. However, it was noted that there was a lower median NK cell value in placentas of mothers with infected babies in comparison to mothers with uninfected babies.

A logistic regression equation was performed to evaluate NK immune response at the placental interface and the protection it confers on babies against HIV-1 infection (Table 1). The variables which were statistically accepted were the log viral load of mothers, gender of babies and NK cell values. The protective effect of NK cells was evaluated in category B (≤ 4 NK cells/30hpf) and category C (10-19 NK cells/30hpf) using category A (20-30 cells/30hpf) as the baseline value. Category A was chosen, in the equation, as the baseline for evaluation, on the basis that having a low NK cell value is associated with the risk of infection when compared with a higher NK cell presence. Therefore, the highest category with the lowest risk was chosen as baseline. There was no statistically significant association in both categories B ($p = 0.145$; 95%CI 0.655 – 17.891) and C ($p = 0.940$; 95%CI 0.138 - 6.237). Data analysis revealed that the risk of vertical transmission was 3.4 times more in placentas which fell in category B (<4 NK cells/30hpf). Further regression analysis revealed that with every 1 log increase in maternal viral load the risk of transmission was increased approximately 3.3 times more. Maternal log viral load emerged as a significant predictor of viral transmission ($p = 0.047$). No statistically significant association was established between the presence of NK cells and gender ($p = 0.069$). However, even after adjustment for placental NK cells and maternal viral load, the male to female risk of acquiring the infection increased by 3.7 in favour of female babies.

Babies CD4+ cell counts and placental p24 counts were added to the logistic regression model to assess the risk of babies acquiring the infection. Babies CD4+ cell counts ($p = 0.582$; 95%CI 0.140 to 1.00) and the presence of placental p24 antigen ($p = 0.964$; 95%CI 0.099 to 1.094) were not predictors to viral transmission. According to the odds ratio, babies CD4+ counts were affected by every 1 log increase in mother's viral load (Table 1).

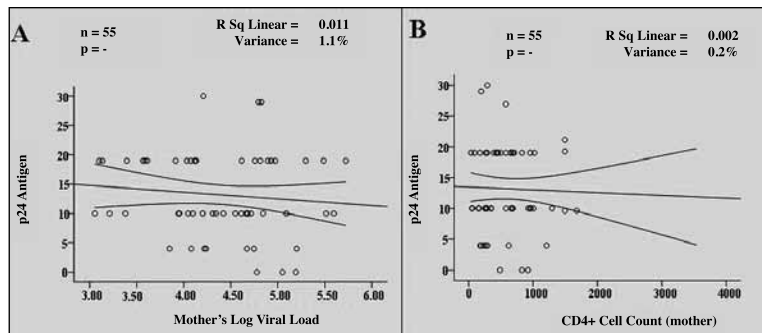
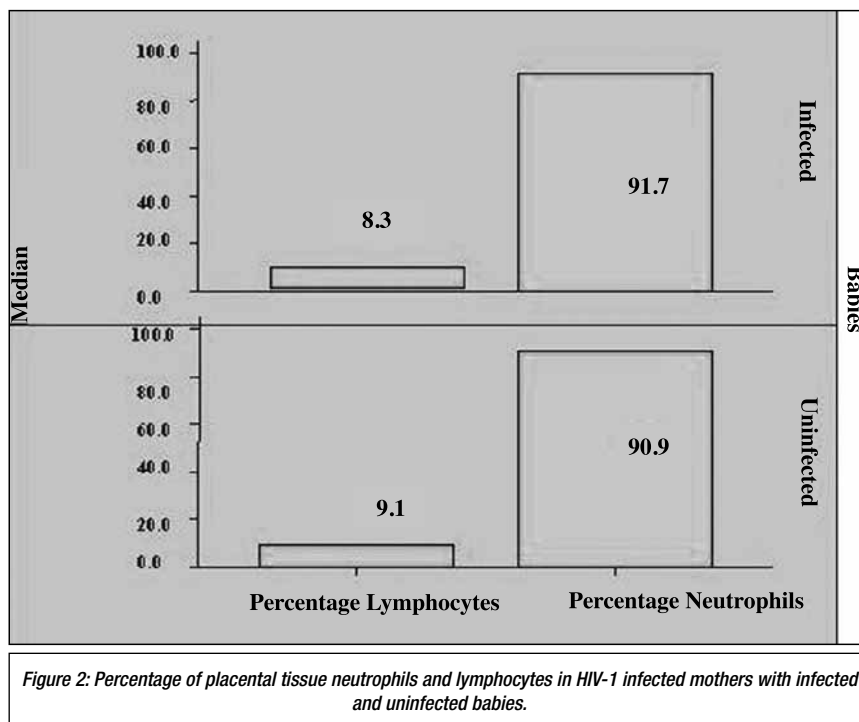


Figure 1: Scatter plot of mothers log viral load (A) and CD4+ cell counts (B) compared to the presence of p24 antigens in placental tissue of mothers.

Table 1: Logistic Regression to Determine the Predictive Value of NK Response in the Placenta and Risk of Infection in Babies. N=55

	Wald	df	Sig.	Odds Ratio	95.0% C.I. for EXP(B)	
					Lower	Upper
Log Viral Load of Mothers	3.943	1	0.047	3.278	1.016	10.578
Gender of Babies	3.309	1	0.069	3.701	0.904	15.159
CD4+ cells (babies)	0.303	1	0.582	1.000	0.140	
NK Overall (20-30 cells/30hpf) - Category A	3.386	2	0.184	-	-	-
NK (<4 cells/30hpf vs* 20-30 cells/30hpf) - Category B	2.129	1	0.145	3.424	0.655	17.891
NK (10-19 cells/30hpf vs* 20-30 cells/30hpf) - Category C	0.006	1	0.940	0.929	0.138	6.237
Constant	6.587	1	0.010	0.001	-	-

*vs denotes versus



Overall Presence of Inflammatory Cells in Placental Tissue

Distribution of neutrophils and lymphocytes (inflammatory cells) was assessed in all placental cells. Circulating inflammatory cells were differentiated from tissue inflammatory cells so as to adequately assess only the level of tissue immune response to infection.

The cellular response in placental tissue of mothers with uninfected and infected babies was investigated. The difference between placental inflammatory response of mothers with infected and uninfected babies was minimal. A median of 90.9% for neutrophils and 9.1% for lymphocytes was observed in placental tissue of mothers with uninfected babies. The median neutrophil and lymphocyte percentage in placental tissue of mothers with infected babies was 91.7% and 8.3% respectively (Figure 2). Viral transmission to the baby was not related to the presence or absence of neutrophils or lymphocytes. Generally the presence of leucocytes suggests an immunological response and the importance of these cells for foetal survival.

Discussion

The presence of p24 in placentas was analysed in relation to immunological and virological factors and its implication in vertical transmission. Investigation into the mechanisms regulating the transmission of placental HIV has inherent difficulties. Quantitation of viral and cellular responses in placental tissue is a challenge because there are insufficient separation techniques to differentiate trophoblastic cells from nontrophoblastic cells. Further difficulties are encountered in separating maternal cells from foetal cells at the placental interface. Therefore, a staining technique was chosen which would allow some visual indication of identification. An immunohistochemical technique was used for measuring the level of viral invasion. Although the stain is specific for p24 antigen it is unable to differentiate between cells containing viral antigens and those with viral particles, a limitation of evaluation using immunohistochemistry. However, observation of either viral antigens or viral particles indicates viral presence.

Placentas were placed into two categories. Those with the presence of p24 antigens (infected) and those without p24 antigens (uninfected). Altogether, 94.6% of placentas in this study bore evidence of HIV-1 infection. Previous studies, using immunohistochemistry techniques retrieved evidence of HIV infection in 14 of 39 (36%), 5 of 11 (45.5%) term placentas and 10 of 23 therapeutic abortions^[8, 9]. These studies suggested that the transmission routes were a haematogenous route from maternal intervillous space to villous stromal cells or bidirectional flow between chorion laeve and amniotic fluid^[8, 9]. The

authors further suggested that transmission may occur during early gestation by direct contact between basal deciduas and budding trophoblastic cells or entry into foetal circulation via the small veins from the capsular deciduas.

Some studies examined placental tissue obtained from HIV-1 infected mothers and concluded that because of the absence of p24 antigens, the placenta forms an efficient barrier to viral transmission^[10]. However, cohorts in these studies were undergoing antiretroviral therapy during their pregnancy. Mothers in this study were antiretroviral naive during pregnancy which may account for the elevated detection of p24 antigens in 94.6% of placental samples. Other studies indicated that cells can be infected by HIV when coming into contact with infected leucocytes^[11, 12, 13, 14]. The substantial differences in detection levels may also be due to study populations from diverse geographical regions. Therefore, it is important to further interrogate contributing factors to HIV activity in placental tissue in different population groups.

Presence of p24 antigens in placental tissue was evaluated against the number of placental NK cells, maternal viral load and CD4+ cell counts. The analysis revealed that the presence of p24 antigens in placental tissue was not influenced by maternal viral load or CD4+ cell counts. These findings are in line with previous studies which reported that there was no relation between placental infections and, either CD4+ counts or plasma viral loads. Some authors have commented that an inversely proportional relationship exists between the presence of p24 and maternal viraemia and has suggested that the inverse relationship may indicate differences in tropism between HIV-1 in placenta and plasma^[9].

NK cells comprise between 6-21% of the total lymphocyte population^[15]. Comprehension of pathways which mediate NK cells activity and their implication in viral responses remain a challenge^[16]. Amplified responses of the NK (CD56+) cell at sites of viral replication have been found to produce and release numerous chemokines which are effective against certain pathogens^[16]. In this study plasma HIV-1 RNA levels in infected mothers did not appear to influence NK response in the placentas.

Studies have documented the significant role of NK cells as active participants in immune response to pathogens and have also reported that patients' deficient in NK cells and infants, where NK cell compartments does not fully appear are immunocompromised^[17, 18]. Here, the presence of NK cells is reported in 98.2% of placental samples. Evaluation of CD56+ in peripheral blood was not established in this study which limits any conclusive comments on the interaction of CD56+ between blood and placenta. Other studies show that increased numbers of circulating peripheral blood CD56+ cells results in greater

presence of CD56+ cells at the placental interface. Based on these findings we can make the assumption that placentas which demonstrated low CD56+ counts could possibly be due to low circulating peripheral blood CD56+^[18, 19]. Larger studies are required to validate these findings.

Analysis of the functional activity of NK cells at the placental barrier induced by p24 antigen stimulation showed that there were lower median NK cell values in placentas of mothers with infected babies as compared with the uninfected cluster. Although not statistically significant, low placental NK cells were associated with the risk of infection when compared with higher NK cell presence. The risk of vertical transmission was increased 3.4 times more in placentas which had lower NK cell values. The association between placental NK cells and vertical transmission has not been conclusively established in other studies. In lieu of the limited information available for comparison with other studies, comments about the interaction and role of NK cells in vertical transmission can only be made from data in this study. Further studies are required to test the validity of our observations.

Logistic regression analysis revealed that the risk of viral transmission increased in babies with lowered CD4+ cell counts. According to the odds ratio, babies CD4+ counts were affected by every 1 log increase in mother's viral load. However, CD4+ count and presence of p24 antigens did not emerge as predictors to vertical transmission. Given the lack of direct linkage between the detection of p24 in placental tissue and viral RNA copies in infected babies' plasma, other cell mediated immunological responses require investigating. Also, we cannot exclude the possibility that undetectable levels of viral copies in babies in contact with infected placentas will not at a later date emerge seropositive. Overall, maternal viral load emerged as a strong predictor for risk of infection from infected mothers to their infants. Data analysis indicated that female babies were 3.7 times more likely to acquire the infection than males. This finding is supported by another study which has reported that girls were at a higher risk of early (*in utero*) HIV infection than boys^[20]. These findings remain an interesting avenue of research for future studies.

Trophoblasts have been proposed to play a critical role in modulating virus spread to the foetus^[21]. To eliminate complications in assessment due to non-trophoblastic cells, circulating inflammatory cells infected with the virus was not included in the evaluation. It has been suggested that p24 antigens in non-trophoblastic cells may be a reflection that the maternal blood in the placenta contains infected CD4+ cells.

Overall there was no difference in the total number of inflammatory cells in placental tissue of mothers with babies considered infected *in utero* and those uninfected at birth. Viral transmission to the babies was not associated with the presence or absence of placental neutrophils or lymphocytes. Generally the presence of leucocytes suggests an immunological response and is important of cells for foetal survival^[21].

In concluding the investigation we found that 15 (27.3%) of 55 babies were considered infected *in utero*. It appears that maternal immune competence in clearing or preventing p24 antigens from the placenta appears to be intact in 40 (72.7%) mothers whose babies were born with undetectable levels of viral RNA copies at birth. We need to consider this result as a tentative estimation, since the babies were not available for retesting. Therefore, it is difficult to speculate on how many of the babies will eventually succumbed to the virus. A limitation of the study which restricts comments on the final depth and range of immunological responses in vertical transmission.

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