


Peer reviewed ORIGINAL ARTICLE

INTERACTION BETWEEN NK CELLS AND HLA-G1 AT THE PLACENTAL INTERFACE OF HIV-1 INFECTED PREGNANT WOMEN: ADDITIONAL RISK FACTORS OR PHYSIOLOGICAL ASSOCIATION?

S Moodley (PhD)
Department of Biomedical Sciences, Mangosuthu University of Technology, South Africa

Grants: Support for the study was received from The National Research Funding- Thuthuka Programme and Mangosuthu University of Technology Research Grant.

Corresponding author: Shamala Moodley | email: shamala@mut.ac.za | tel: +27 (0)31 907 7450 | fax: +27 (0)31 907 7451

ABSTRACT

Background: Human Leucocyte Antigen-G (HLA-G) molecules are involved in the inhibition of cell-mediated immune responses and could promote the propagation of HIV-1 infection across the placental interface thus increasing the risk of vertical transmission. Therefore, the objective of this study was to assess whether the Major Histocompatibility Complex (MHC) - coded molecule HLA-G inhibits Natural Killer (NK) cell activity thereby, assisting viral penetration across the placental barrier in HIV-1 positive pregnant women.

Study Design & Methods: Natural Killer (CD56+) cell activity and placental HLA-G1 expression was assessed using immunohistochemistry and real-time polymerase chain reaction (RT-PCR) techniques, respectively. Studies were performed on a total of fifty five placental samples obtained from HIV-1 infected mothers at birth.

Results: Low numbers of NK cells increased risk of vertical transmission [OR = 3.424 (95%CI 0.65-17.89)]. The risk of babies becoming infected increased by 1.3 with every 1 unit increase in HLA-G1 expression. A positive correlation was observed between mothers’ log viral load and transmission of infection to the baby (p = 0.047; 95%CI 1.029-11.499).

Conclusion: Low NK cell activity at the placental interface increased the risk of vertical transmission. Maternal viral load remained a strong predictor of viral transmission.

KEYWORDS
Natural Killer cells (CD56+), Human Leucocyte Antigen-G1, vertical transmission, viral load, up regulation.

INTRODUCTION

Natural Killer (NK) cells are a population of low-density, large granular lymphocytes, which mainly develop and differentiate in bone marrow and then enter into the circulation. These cells comprise approximately 5-20% of peripheral blood lymphocytes and are involved in the innate immune response against certain microbial, viral and parasitic infections [1, 2]. In response to proinflammatory stimuli, which may be induced by a viral infection, NK cells migrate to various tissues and organs of the body. In the mucosal decidual tissues of the maternal uterus, NK cells are the most abundant class of lymphocyte,
representing up to 95% of all lymphocytes \(^3\), \(^4\).

Acting as important regulators of antiviral immune responses, NK cells secrete a multitude of soluble mediators which directly interacts with other immune cells. NK cells express an array of inhibitory and activating receptors and co-receptors, which bind to their cognate ligands expressed on the surface of target cells. These ligands include classical and nonclassical MHC class I antigens, MHC-like proteins, and a variety of other self- and virus-derived molecules \(^5\).

In this context, the placenta, a foetally derived organ expressing both paternal and maternal MHC class antigens, should induce a maternal alloimmune reaction, which in turn should lead to foetal rejection. However, the trophoblastic cells are usually not destroyed \(6\).

At the implantation site, the pregnant uterine mucosa is infiltrated by large granular lymphocytes CD56+, a subset of NK cells. The population of trophoblastic cells that invade into the maternal decidua express the class I antigen, HLA-G which partly regulates NK cells \(6\).

The expression of HLA-G is normally restricted to the placenta during pregnancy specifically to those trophoblasts at the maternal-foetal interface. However, most evidence suggests that it protects the foetus from maternal uterine NK cell lysis \(7\). It is known that, once HLA-G is expressed, it becomes the major NK inhibitory ligand \(8\).

MHC class I molecules are membrane-bound glycoproteins and have been known to be key factors in immunosurveillance against pathogens and tumour appearance \(9\). HLA-G exhibits unique structural characteristics: as a result of alternative splicing of a single primary transcript, various membrane bound and soluble isoforms, including short variants, are produced \(10\). The four membrane-bound HLA-G includes HLA-G1, G2, G3 and G4. The three soluble isoforms are HLA-G5, G6 and G7. Only the membrane bound HLA-G1 and the soluble HLA-G5 isoforms are associated with β₂ microglobulin, the binding site for CDB\(^\text{+}\). These isoforms act in diverse inflammatory conditions protecting tissue against NK and T cell infiltration. Inhibition of these inflammatory cells creates an anti-inflammatory cytokine environment \(10\), \(11\), \(12\). Destruction of T and NK cells occurs when HLA-G binds to the inhibitory receptor KIR3DL4 expressed on NK cells and ILT receptors on both T and NK cells \(13\). The immunosuppressive activity of HLA-G in non-viral inflammatory conditions extends to viral infections. It has been reported that HLA-G expression in cells is also up-regulated following infection with human cytomegalovirus (CMV) and HIV \(14\), \(15\), \(16\). The suggestion is that viruses might use HLA-G to subvert adverse immune responses. The aim of this study was to assess whether suppression of NK cell activity by HLA-G expression assist human immunodeficiency viral penetration across the placental barrier in HIV-1 positive pregnant mothers. HLA-G1 protein which is encoded by the full-length mRNA associated with β₂-microglobulin was used to assess HLA-G1 expression.

**METHODS**

**Participant Recruitment**

The patients attending the antenatal clinic (ANC) for general monthly antenatal examination were recruited with consent, into this study. All participating patients were counselled before establishing the HIV status. Peripheral blood samples were collected from participants at 28 weeks of pregnancy by the attending gynaecologist and sent to the laboratory for HIV testing. The patients’ serological status was determined by enzyme-linked immunosorbent assay (ELISA) (Determine, Abbott, USA). Patients who were HIV positive at the initial screening were counselled and written informed consent was obtained from all participating mothers. Ethical approval for the study was obtained from the Ethics Committee (University of KwaZulu-Natal).

**Sample Collection and Storage**

Blood samples were collected into 2 EDTA vacutainer tubes from the mothers (2-5 mls) at delivery and from babies (± 1 ml) immediately after birth. Samples were transported from the site of collection at room temperature (20-25°C) to the laboratory to determine viral load. Safety measures were taken during transport of samples. Placental samples were collected at the time of delivery for ribose nucleic acid (RNA) extraction, and placed into 10% formal saline. The tissue samples were thereafter transported on ice, to the laboratory for testing. On arrival at the laboratory portions of the placenta were cut, processed and embedded in paraffin wax. Sections were prepared from the wax embedded samples and placed onto Poly-L-Lysin slides (Sigma, USA) for immunohistochemical staining. The paraffin blocks were carefully handled and sectioned in order to avoid cross-contamination. The remaining samples were stored at -70°C for RNA extraction and complementary deoxyribonucleic acid (cDNA) synthesis.

**Viral Load**

Viral load was determined on all mother-baby pair plasma samples, according to manufacturer’s protocol (Roche Amplicor Version 1.5, Germany). The rate of mother-to-child transmission (MTCT) was based on the HIV test result on infants samples collected at birth. All babies who had a viral load of >400 copies/ml at birth were considered infected in utero \(17\). Those babies with viral RNA copies <400 copies/ml were considered uninfected pending further viral load investigations.

**Immunohistochemical Staining for CD56\(^\text{+}\) cells in Placental Tissue**

Duplicate formalin fixed wax embedded samples, 4-5 μm thick were placed onto Poly-L-Lysin coated slides (Sigma, USA) and incubated overnight at 40°C. Briefly, sample sections were treated in a microwave oven at 750 Watts for 5 minutes, cooled for 20 minutes at room temperature and washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (DAKO, Glostrup, Denmark) for 5 minutes and thereafter washed three times in PBS. Sections were incubated for 1 hour with primary antibodies to CD56\(^\text{+}\); a marker for uterine NK cells (Zymed, USA, Clone 123C3) diluted 1:300. This was followed by sequential 30 minutes incubations with a biotinylated link antibody and peroxidase labelled streptavidin. The sections were counterstained with Mayer’s Haematoxylin and mounted with distilled water.

**Sample Collection and Storage**

Blood samples were collected into 2 EDTA vacutainer tubes from the mothers (2-5 mls) at delivery and from babies (± 1 ml) immediately after birth. Samples were transported from the site of collection at room temperature (20-25°C) to the laboratory to determine viral load. Safety measures were taken during transport of samples. Placental samples were collected at the time of delivery for ribose nucleic acid (RNA) extraction, and placed into 10% formal saline. The tissue samples were thereafter transported on ice, to the laboratory for testing. On arrival at the laboratory portions of the placenta were cut, processed and embedded in paraffin wax. Sections were prepared from the wax embedded samples and placed onto Poly-L-Lysin slides (Sigma, USA) for immunohistochemical staining. The paraffin blocks were carefully handled and sectioned in order to avoid cross-contamination. The remaining samples were stored at -70°C for RNA extraction and complementary deoxyribonucleic acid (cDNA) synthesis.

**Viral Load**

Viral load was determined on all mother-baby pair plasma samples, according to manufacturer's protocol (Roche Amplicor Version 1.5, Germany). The rate of mother-to-child transmission (MTCT) was based on the HIV test result on infants samples collected at birth. All babies who had a viral load of >400 copies/ml at birth were considered infected in utero \(17\). Those babies with viral RNA copies <400 copies/ml were considered uninfected pending further viral load investigations.

**Immunohistochemical Staining for CD56\(^\text{+}\) cells in Placental Tissue**

Duplicate formalin fixed wax embedded samples, 4-5 μm thick were placed onto Poly-L-Lysin coated slides (Sigma, USA) and incubated overnight at 40°C. Briefly, sample sections were treated in a microwave oven at 750 Watts for 5 minutes, cooled for 20 minutes at room temperature and washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (DAKO, Glostrup, Denmark) for 30 minutes and thereafter washed three times in PBS. Sections were incubated for 60 minutes with primary antibodies to CD56\(^\text{+}\); a marker for uterine NK cells (Zymed, USA, Clone 123C3) diluted 1:300. This was followed by sequential 30 minutes incubations with a biotinylated link antibody and peroxidase labelled streptavidin. The sections were counterstained with Mayer’s Haematoxylin and mounted with distyrene plasticiser and xylene (DPX). Positive controls, using commercially available CD56\(^\text{+}\) tissue slides were included in each staining batch. Slides with normal mouse immunoglobulin (IgG) control antibodies at the same concentration as the primary antibodies provided in the kit as per manufacturer’s protocol, were used as negative controls. Duplicate placental sections from HIV negative women were also stained for NK (CD56\(^\text{+}\)). This was done to assess NK cell activity in placentas of HIV negative women.
The presence of NK (CD56⁺) cells was visualised as dark brown cells.

Real-Time PCR

Samples were homogenized using a modified Trizol method, followed by a total RNA extraction using the Arum protocol (BIORAD, USA. Cat. No. 732-6820) [18]. RNA concentration was determined by spectrophotometer NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE) For HL-G expression studies, 500 ng of total RNA were reverse transcribed using iScript cDNA (The BIORAD iScript cDNA,Cat.No.1708891). The procedure was performed according to manufacturer’s protocol. The obtained cDNA served as a template for quantitative Real Time PCR. Quantification of the HLA-G1 gene was performed using forward and reverse primers. The primers, manufactured by Inqaba (SA) were designed to amplify and detect a 98-bp segment of the non classical class I HLA-G1 gene. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as internal control to normalize data. Fast Start SYBR GREEN I (Roche Diagnostics, Germany) and the LightCycler Master mix were transferred into the sample carousel of the LightCycler Instrument and cycled.

The real-time (RT) reaction was performed using one cycle at 95°C for 10 minutes followed by 40 cycles of polymerase chain reaction (PCR) which included: denaturation at 95°C for 10 seconds, 60°C for 50 seconds, annealing at 72°C for 16 seconds and extension at 85°C for 5 seconds with a single fluorescence measurement. All temperature transition rates were programmed at 20°C/s. After amplification was complete a final melting curve was recorded at 95°C for 30 seconds and 80°C for 15 seconds (20°C/s). Slow heating of the sample was accomplished at 95°C with a ramp rate of 0.1 C/s. Fluorescence was measured continuously during the slow temperature ramp to monitor the dissociation of the SYBR Green I. This was followed by a final cooling step to 40°C. Melting point (Tm) calculations were performed using the Roche Molecular Biochemical LightCycler Relative Quantification Software, Version 3.5. The quality of the RNA for cDNA synthesis was determined using agarose gel electrophoresis.

Statistical Methods

Some variables were unequally distributed; therefore non parametric tests were used. Correlations were assessed using Pearson’s correlation test. The Chi Square test was used for assessing probabilities between variables and categories. Linear regression was used to determine the significance of inter-related interactions and risk ratios. 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. SPSS statistical software was used for all descriptive analyses in the study.

RESULTS

Assessment of Placental Natural Killer Cells (CD56⁺) against Babies Viral RNA Copies

NK cell activity was examined in placentas of mothers with babies born uninfected and infected at birth. Median placental NK cell counts in mothers with uninfected and infected babies were 10/30/hpf with an interquartile range [IQR] of 5-20/hpf and 4/30 hpf (IQR 10-20/hpf) respectively. No significant correlation was observed at the 95% confidence level (p = 0.083). However, it was noted that there was a lower median placental NK cell value in mothers with infected babies in comparison to mothers with uninfected babies.

A logistic regression equation was performed to evaluate placental NK immune response and the protection it confers on babies against HIV-1 infection. Data analysis revealed that the risk of vertical transmission was 3.4 times more in placentas with low NK cell values.

Further regression analysis revealed that with every 1 log increase in maternal viral load the risk of transmission was increased approximately 3.4 times more. Maternal log viral load emerged as a significant predictor of viral transmission (p = 0.045) (Table 1).

Placental Expression of Human Leucocyte Antigen-G1 and its Interaction with NK Cells in HIV-1 Infected Pregnant Women

HLA-G1 expression using real-time PCR was calculated as a ratio using HLA-G/GAPDH values. The results were reported as fold changes using a threshold value of 1. Normal ranges have not been previously established for placental HLA-G1 expression. Therefore, comments on references ranges are restricted. The expression of HLA-G1 in HIV-1 infected placental tissue showed no significant correlation when compared to the presence of NK cells (p = 0.841). (Fig.1)

Relationship between Human Leucocyte Antigen-G Expression and Maternal Viraemia in Mother-to-Child Transmission of HIV Infections

A threshold value of one was used to report HLA-G1 fold changes. Placental HLA-G1 fold increases was observed in 37 (67.2%) women, whilst 18 (32.7%) demonstrated placental values < 1fold. A positive correlation between maternal viral load and placental HLA-G1 expression was observed (p = 0.038) (Fig. 2).

According to logistic regression analyses, increased maternal viral loads increase the risk of vertical transmission (p = 0.045; 95%CI 1.029-11.499). No significant correlation was noted.

<table>
<thead>
<tr>
<th>Table 1: Logistic Regression to Determine the Predictive Value of Maternal Viraemia, HLA-G1 Expression and Babies Gender. N=55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Log Viral Load of Mothers</td>
</tr>
<tr>
<td>NK cells</td>
</tr>
<tr>
<td>HLA-G1</td>
</tr>
<tr>
<td>Gender of Babies</td>
</tr>
<tr>
<td>Constant</td>
</tr>
</tbody>
</table>

Variables entered: log viral load of mother, NK cells, HLA-G level of expression and gender of babies. * Denotes Odds Ratio values.
with HLA-G1 and vertical transmission. \( p = 0.066; 95\% CI 0.983 - 1.712 \). However, the odds ratio indicated that the risk of infection increased by 1.3 with every 1 unit increase in HLA-G1 expression. Furthermore, data reflected that female babies are 3.7 times more likely to become infected than males (Table 1).

**Influence of HLA-G1 on Babies Viral Load**

In the following analyses the infants were grouped according to their viral load at birth. Babies with viral load of >400 copies/ml at birth were designated to the infected group and those born with viral RNA copies <400 copies/ml were designated to the uninfected group at birth.

The vertical transmission rate on plasma samples tested at birth was 27.3\%. Correlation of placental HLA-G1 expression and plasma viral load of babies in both the infected and uninfected groups fall on the level of significance \( p = 0.05 \). The mean HLA-G1 fold increase in placentas of mothers with infected babies was 2.17 (median of 2.22) and the mean of mothers with uninfected babies was 0.55 (median 1.17). An absolute difference in median HLA-G1 values between the placentas of mothers with infected babies was approximately 1 which is considered statistically significant.

Evidence of differences in HLA-G1 expression in mothers with infected female babies and infected male babies was sought (Fig.3). The median HLA-G1 ratio was higher in mothers with infected male babies (2.3) than in those with uninfected male babies (1.3). A statistically significant association was observed \( p = 0.013 \). The female babies showed no difference in values \( p = 0.963 \). Overall, HLA-G1 emerged as a factor for infection in males.

**Regulation of HLA-G in Placentas of HIV-1 Infected Women**

HLA-G1 expression was up-regulated 3.95 times more in placentas of mothers with infected babies (Fig.4). The following calculation was used to calculate up-regulation: mean value of HLA-G1 expression (2.17) in mothers with infected babies (>400 viral RNA copies/ml) divided by the mean value of HLA-G1 (0.55) in mothers with uninfected babies (<400 copies/ml).
DISCUSSION

Natural Killer cells constitute an important component of the innate immune system. Now recognised as important cells with effector and regulatory functions, NK cells have been known to kill virus infected cells \[19, 20\]. Significant advances have been made in understanding how NK cells function and regulate innate and adaptive immune responses \[21\]. Using this knowledge as a platform, scientists have investigated the role of these cells in HIV and other viral infections. Furthermore, studies have demonstrated that HLA-G, the major MHC class I molecule expressed at the cell surface of extravillous trophoblast cells, is likely to exert inhibition of viral peptide presentation to NK (CD56+) cells in uterine infection \[22\].

This study was undertaken to assess the level of NK cell activity in the presence of placental HLA-G1 expression in HIV-1 infected placentas. Furthermore, the study provides information which raises a debate as to whether the presence of HLA-G1 increases the risk of MTCT.

In this study we observed lower median NK cell values in placentas of HIV-1 infected mothers with infected babies as compared with uninfected babies. Although no statistical significance was noted, the risk of vertical transmission was 3.4 times more in placentas which had lower NK cell values. This suggests that the presence of NK cells may lead to an increase in placental capacity to ward off viral penetration. However, in lieu of the limited information available for comparison with other studies we can only comment on the presence of NK (CD56+) cell activity from data in this study. Further studies are required to validate our observations and enhance our understanding of the role of NK cells in controlling viral transmission across the placental interface.

Further data analysis revealed that the viral load of infected mothers did not appear to influence NK (CD56+) cell response at the placental interface. Statistical limitations could be related to small sample size.

Our transcriptional analysis of HLA-G1 has reassuringly identified HLA-G1 in all placental samples. The interaction between placental NK (CD56+) cells and HLA-G1 was investigated. Evidence suggests that HLA-G protects foetal cells from lysis by maternal uterine NK cells, which are found in large numbers around invading trophoblastic cells. Analysis of the interaction between NK cell activity and HLA-G1 expression at the placental interface showed no significant correlation. Studies conducted by some researchers indicated that HIV-1 infected patients showed a significant decrease and a selective depletion of in NK cell subsets \[19, 24, 25\]. Therefore, if circulating NK cells are depleted by HIV, the NK response in the mother's immune system can range from adequate to suppressed. In this study NK activity in peripheral circulating blood was not established. However, based on the findings of previous studies an assumption can be made that since all the mothers in this study were HIV-1 infected, circulating NK cell levels will be low, leading to low NK activity at the placental interface. Another mechanism which inhibits the activity of immunocompetent NK cells is placental HLA-G expression. This is further suggestive that HLA-G may assist viral particles to evade the immune system \[26\].

It is widely accepted that maternal viral load is a strong predictor of vertical transmission \[25\]. This study has also demonstrated a positive correlation between maternal viral load and vertical transmission. Some authors have commented that HLA-G expression occurs in a complex manner by several cytokines. It has also been reported that HLA-G expression has been up regulated with cytomegaly virus and HIV \[27, 28, 29\].

Data consolidated by Lafon et al (2005) indicated that up regulation of HLA-G expression impeded host antiviral responses based on NK and T cells \[30\]. In agreement with these earlier comments this study reports an up-regulation of HLA-G1 in HIV-1 infected placental tissue. The odds ratio established that the risk of infection increased by 1.3 with every 1 fold increase in HLA-G1 expression. Although statistically significant, evidence of clinical significance between the two variables need to be established with larger studies.

To the best of our knowledge we are the first to explore the role of HLA-G1 in vertical transmission. The study found that median HLA-G1 values were higher in mothers with babies infected at birth than mothers with uninfected infants. We report here an absolute difference of 1 in the median HLA-G1 values between the placentas of mothers with infected babies thus establishing statistical significance.

According to logistic regression analysis females were 3.7 times more likely to acquire the infection. However, when gender susceptibility to HLA-G1 expression was explored graphically, a statistically significant association was observed between infected and uninfected male and female babies and HLA-G1 expression. No difference in HLA-G1 expression was observed between the infected and uninfected female babies. HLA-G1 emerged as a significant risk factor in males than in females. In view of these discrepancies a separate analysis needs to be conducted on male and female babies to establish the risk of infection. Other factors besides the presence of HLA-G1 could have increased the 3.7 odds ratio in favour of females. A recent study conducted by Biggar et al (2006) reported that girls were at a higher risk of in utero HIV infection than boys. The author proposed that minor histocompatibility reactions between maternal lymphocytes and Y chromosome-n derived antigens reduce the risk of HIV transmission in boys \[31\]. The magnitude of these differences is still to be tested. We found that HLA-G1 was up-regulated 3.95 times more in placental tissue of mothers with infected babies than in mothers with uninfected babies. It raises the debate whether the expression of HLA-G1 at the placental barrier of HIV-1 infected women represents implication guilty by association or a symbiotic physiological interaction.

CONCLUSION

It was found that 27.3% of babies were considered infected in utero. Maternal immune competence in clearing or preventing p24 antigens from the placenta appears to be intact in 72.7% of mothers whose babies were born with undetectable levels of viral RNA copies at birth. This result is 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 succumb to the virus. This is a limitation of the study which restricts comments on the final depth and range of immunological responses in vertical transmission. Therefore, it creates a platform for more comprehensive future studies using larger sample size and stratification of study participants with different ethnic backgrounds.

ACKNOWLEDGEMENTS

I thank Professors S Carpenter and R Bobat for insightful discussions, Mangosuthu University of Technology Research Directorate and the NRF for their support. I would also like to thank the patients and clinician Dr ME Essa who made this study possible.
REFERENCES