

Reduced ability of newborns to produce CCL3 is associated with increased susceptibility to perinatal HIV-1 transmission

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ABSTRACT

Background: The role of CC chemokines CCL3, CCL4 and CCL5 in protection against mother-to-child transmission of HIV-1 is not well understood. We questioned whether foetal CC chemokine production was associated with protection against HIV-1 transmission due to viral exposure at delivery.

Methods: Using a nested case-control design we measured, by ELISA, spontaneous and mitogen-induced production of CCL3, CCL4, and CCL5 by mononuclear cells for 60 HIV-1 infected and 20 uninfected mother-infant pairs. 13 infants were infected intrapartum and 4 in utero. Immune activation markers were quantitated by ELISA in cord blood plasmas. The promoter and first intron of the two functional CCL3 genes (CCL3 and CCL3-L1) were sequenced. Copy numbers of CCL3 (occurs as 2 per diploid genome) and CCL3-L1 (variable in most populations) was determined by real-time PCR quantitation (cohort extended to give a total of 46 transmitting and 74 non-transmitting mother-child pairs).

Results: PHA-induced production of CCL3 ($p=0.002$) and CCL4 ($p=0.001$) by cord blood mononuclear cells was increased in infants born to HIV-1 infected mothers that remained uninfected when compared to control infants. However, infants who became infected intrapartum (IP) showed a deficiency in the production of CCL3 at birth. The production of CCL5 showed no relationship with HIV-1 infection outcome in the infant. Differences in production of CCL3 between exposed-uninfected (EU) and IP-infected infants could not be explained by differences in immune activation events prior to birth as measured by plasma levels of neopterin, beta-2-microglobulin and sL-selectin. However, a similar deficiency in mitogen-induced CCL3 production was evident in IP-transmitting mothers relative to non-transmitting mothers, suggesting that the underlying nature of this deficient response was genetically encoded. CCL3-L1 gene copy number was associated with CCL3 production (EU infants, $p=0.035$) and with vertical transmission ($p=0.019$). However, at equivalent CCL3-L1 gene copy numbers, IP infants relative to their EU counterparts had lower production of CCL3 suggesting that they may harbor some non-functional copies of this gene. Nucleotide changes that may influence CCL3 production were evident in CCL3 and CCL3-L1 genes upstream of exon 2.

Conclusion: Overall findings suggest that infants who display a deficient production phenotype of CCL3 are more likely to acquire HIV-1 infection during delivery, and that this reduced production is partly explained by reduced CCL3-L1 gene copy number.

BACKGROUND

The CC chemokines CCL3, CCL4 and CCL5 are the natural ligands for CCR5, a chemokine receptor utilized by R5 HIV-1 in addition to CD4 for entry into leukocytes. That the CC chemokines could block macrophage-tropic HIV-1 viruses from replicating *in vitro* raised the possibility that these molecules may contribute to protective immunity against HIV-1 *in vivo*. Supporting this hypothesis were prototype HIV-1 vaccine studies in rhesus macaques, showing production of CC chemokines by CD8 T-cells to be associated with protective immunity (Letner *et al.*, Nat Med 1996;2:767-75). Wasik *et al.* (J Immunol 1999;162:4355-64) demonstrated an over-expression of the CCL3, CCL4 and CCL5 in a small number of exposed-uninfected infants and suggested that these may mediate non-cytolytic inhibition of infection during perinatal HIV-1 transmission. Naturally-occurring host genetic variants of chemokine and chemokine receptor genes have further shown the important role of these molecules in altering the host immune response to HIV-1. Recently, variation in copy number of CC chemokine ligand 3-like 1 (CCL3-L1) genes was shown to be associated with host risk of HIV-1 infection and disease progression (Gonzalez *et al.*, Science 2005;307:1434-1440). However, no human prospective study has directly demonstrated that a deficient phenotype of limited production of one or more of the CC chemokines is associated with risk of acquiring HIV-1 infection.

This study set out to test prospectively if foetal CC chemokine production primed by HIV-1 *in utero* could protect against HIV-1 transmission with subsequent exposure at delivery.

METHODS

Patient samples: Cord blood samples were collected into EDTA vacutainers from infants born to HIV-1 infected women enrolled as part of a post-exposure prophylaxis (PEP) trial conducted at Chris Hani Baragwanath Hospital in Soweto, South Africa. In addition to cord blood collection, corresponding infant blood samples were also collected at birth, 6 weeks, and 3 months of age to determine infection outcome by HIV-1 DNA PCR. Blood samples were also collected from all corresponding mothers recruited to this study. From the 124 mother-child pairs for whom samples were collected, we selected for testing all infants who became infected with HIV-1 (13 were infected intrapartum [IP] and 4 were infected *in utero* [IU]); and a random sample of uninfected children born to HIV-1 infected mothers (43 exposed-uninfected EU) using a nested case-control design. A further 20 cord blood samples from infants born to HIV-1 uninfected mothers at the same site were collected to serve as negative controls. For the purpose of determining CCL3-L1 copy number and association with risk of HIV-1 infection, the cohort was extended to include stored samples from a current and prior study at the same site to yield a total of 46 transmitting and 74 non-transmitting mother-child pairs.

Chemokine production assays: PBMC isolated from mothers' samples and CBMC, resuspended at 3×10^6 cells/ml in RPMI containing 1% L-glutamine and 10% human serum, were unstimulated or stimulated with PHA at a final concentration of 12.5 µg/ml. Following 24 hours incubation at 37°C, culture supernatants were harvested and stored at -70°C. Supernatants were tested for CCL3, CCL4 and CCL5 production using Quantikine ELISA kits (R&D systems).

Real-time PCR for CCL3-L1 copy number determination: Real-time PCR was performed using an ABI PRISM 7500 according to the protocol supplied. For each sample, the β -globin, CCL3 and CCL3-L1 genes were amplified in duplicate, using approximately 20ng of genomic DNA per sample. CCL3 gene copy number was confirmed at 2 copies per diploid genome (pgd) for each sample, calculated using the Relative Quantification method and using β -globin (present at 2 copies pgd). CCL3 was then used as the endogenous control to calculate CCL3L1 copy number, again using the Relative Quantification method against a known copy control.

DNA sequencing of CCL3 and CCL3-L1. Genomic DNA was extracted from peripheral blood mononuclear cells using the Qiagen QIAamp DNA minikit. One hundred nanograms of genomic DNA was used in PCR amplification designed to co-amplify the region spanning the core promoter, exon 1 and most of intron 1 of both the CCL3 and CCL3-L1 genes. PCR was carried out using the Roche EXPAND High Fidelity System and amplicons were subsequently purified using the Qiagen QIAquick PCR Purification Kit and sequenced using four sequence-specific primers designed to selectively sequence either the forward or reverse of CCL3 and CCL3-L1 from the purified amplicon mixture. Sequencing reactions were set up using Big Dye Terminator Chemistry version 3.1 (Applied Biosystems) and run on the 3100 Genetic Analyser. Resulting sequences were assembled and analysed for the presence of single nucleotide polymorphisms (SNPs) using Sequencher software version 4.1.4.

RESULTS

Infant deficiency of CCL3 production is associated with HIV-1 acquisition

• PHA-induced release of CCL3 from CBMC was significantly elevated in the EU infants compared to the negative control group ($p=0.002$) (Figure 1B) suggesting that HIV-1 exposure *in utero* had primed elevated CCL3 production.

• IU-infected infants had the highest levels of spontaneous and PHA-induced production consistent with the effects of an established infection (Figure 1A and 1B).

• CBMC from the IP infants produced significantly less PHA-induced CCL3 than CBMC from the EU infants ($p=0.001$) and equivalent to that among the negative control group (Figure 1B) indicating that an infant deficiency of CCL3 production in the context of *in utero* viral exposure was associated with susceptibility to HIV-1 infection.

• CCL4 production from CBMC showed a similar pattern to that observed for CCL3 although differences between the groups were not as marked. In contrast, CCL5 production was very low and spontaneous production was inhibited in infants born to HIV-positive mothers. There was no suggestion that a deficiency in production of CCL5 was associated with acquisition of infection (data not shown).

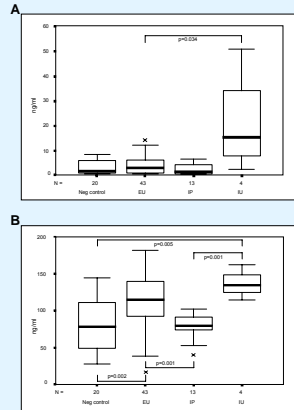


Figure 1. Spontaneous (A) and PHA-stimulated (B) release of CCL3 from *in vitro* PBMC cultures for infants born to HIV-1 uninfected mothers (Negative control) and for infants born to HIV-1 infected mothers who remained uninfected (EU), or were infected intrapartum (IP) or *in utero* (IU).

Immune activation events prior to birth do not account for differences in CCL3 production amongst EU and IP infants

• That a deficient infant CCL3 production was not due to a difference in *in utero* exposure between EU and IP infants was supported by similar levels of soluble immune activation markers (neopterin, β 2-microglobulin, and sL-selectin) in cord blood plasma of EU and IP infants (Figure 2) and by the fact that differences in CCL3 production between EU and IP infants were not attenuated after adjustment for maternal viral load or CD4 count.

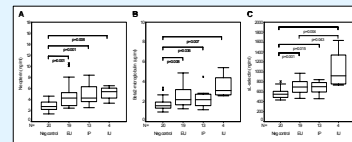


Figure 2. Levels of soluble immune activation markers in cord blood of infants born to HIV-uninfected and HIV-1 infected mothers. (A) Neopterin, (B) β 2-microglobulin, (C) sL-selectin.

Reduced production of CCL3 in mothers transmitting intrapartum

• Mothers transmitting intrapartum also presented with a deficient CCL3 production phenotype, suggesting that the underlying nature of deficient infant CCL3 production was genetic (Figure 3).

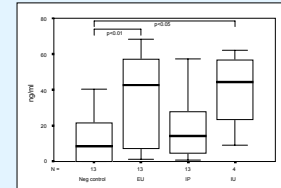


Figure 3. PHA-stimulated release of CCL3 from *in vitro* PBMC cultures for uninfected mothers (Neg control) and for HIV-1 infected mothers whose infants remained uninfected (EU), or were infected intrapartum (IP) or *in utero* (IU).

Reduced CCL3-L1 copy number in infants is associated with maternal-infant HIV-1 transmission

On the basis of our mother-child CCL3 production data we questioned which host genotypes might be responsible for the differences in induced production. In humans, CCL3 is encoded by two functional genes (CCL3/LD78a and CCL3-L1/LD78b) and a pseudogene LD78c. CCL3 occurs as two copies per diploid genome (pgd) while CCL3-L1 occurs in variable copy number in different individuals. For copy number determinations of CCL3 and CCL3-L1, we included additional mother-child pairs from the same hospital to make up a larger cohort of 74 non-transmitting and 46 transmitting mother-child pairs.

When comparing CCL3-L1 copy numbers amongst infants born to HIV-1 infected mothers (Figure 4), these were significantly reduced among those who became infected (median 4, range 1-10) relative to those who remained uninfected (median 5, range 1-8), confirming that the risk of acquiring HIV-1 is the result of susceptibility of the infant rather than simply due to a transmissibility factor of the mother.

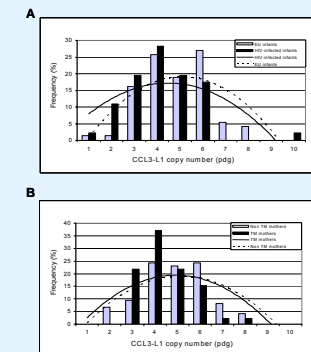


Figure 4. Distribution of CCL3-L1 copy number amongst HIV-1 infected mothers and their infants. (A) Proportions of uninfected and HIV-1 infected infants at each copy number of CCL3-L1. (B) Proportions of non-transmitting and transmitting mothers, matching the above infants, at each copy number of CCL3-L1. Trendlines were determined using a least squares polynomial equation.

Non-functional copies of CCL3-L1 contribute to the decreased production of CCL3 in IP infants

Having established independently an association between CCL3 production and HIV-1 transmission and between CCL3-L1 copy number and HIV-1 transmission, we questioned whether gene duplications of CCL3-L1 explained the difference in induced production of CCL3 between IP and EU groups of our study cohort. Gene copy number of CCL3-L1 did not account for the increased production in EU relative to IP group. At comparable CCL3-L1 gene copy numbers, EU infants' CBMC produced greater amounts of CCL3 than IP infants (Figure 5). This was most marked if CCL3-L1 copy number was high (≥ 4 copies) suggesting the existence of non-functional copies in IP infants.

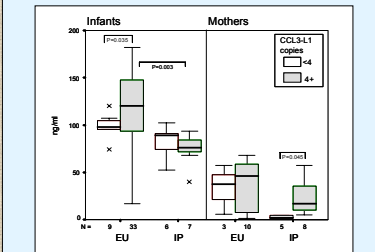


Figure 5. Maternal and infant PHA-induced CCL3 production stratified according to ≤ 4 and $4+$ copy numbers of CCL3-L1 genes exposed-uninfected (EU) and intrapartum infected (IP) infants and their mothers.

Identification of single nucleotide polymorphisms in CCL3 and CCL3-L1

The reduced abilities of mothers and infants in the IP group to produce CCL3 in response to mitogen, in part but not entirely due to reduced copy numbers of CCL3-L1, suggested that, other genetic variants may exist amongst the genes that code for this chemokine. The promoter regions, exon 1 and most of intron 1 of both CCL3 and CCL3-L1 genes were sequenced for the mothers who had CCL3 production levels determined (Figure 3) and their matched infants (i.e. a total of 86 sequences each for CCL3 (1240 bp) and CCL3-L1 (1550 bp)). These regions were selected based on former descriptions of associations with HIV/AIDS (Gonzalez *et al.*, Proc Natl Acad Sci USA 2001; 98: 5199-5204) and on likelihood of effects on gene transcription. Preliminary data revealed the presence of 4 single nucleotide polymorphisms (SNPs) in CCL3 and 3 in CCL3-L1 (data not shown).

CONCLUSIONS

Overall findings suggest that HIV-1 exposure *in utero* resulted in increased infant production of CCL3, but HIV-exposed infants who failed to respond with elevated CCL3 production were more vulnerable to acquiring HIV-1 infection at delivery. Mothers transmitting intrapartum also presented with a deficient CCL3 production phenotype suggesting that the underlying nature of deficient infant CCL3 production was genetic. Copy number of CCL3-L1 was lower in IP infants and their mothers than in EU infants consistent with an influence of copy number on induced protein production in culture, although copy number did not entirely account for the deficiencies in CCL3 production associated with risk of transmission, suggesting the presence of inactive copies of CCL3-L1 in IP infants. Future studies will be aimed at identifying the precise genetic determinants that define levels of CCL3 production so that genotypic assays can be developed that identify individuals at increased risk of HIV-1 infection or hastened disease progression.