Reduced ability of newborns to produce CCL3 is associated with increased susceptibility to perinatal human immunodeficiency virus 1 transmission

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The role of CC chemokines in protection against mother-to-child human immunodeficiency virus type 1 (HIV-1) transmission is not well understood. It was observed that mitogen-induced production of CCL3 and CCL4 by cord-blood mononuclear cells was increased among infants born to HIV-positive compared with HIV-negative mothers, and that a deficiency in production of CCL3 was associated with increased susceptibility to intrapartum HIV-1 infection. CCL3-L1 gene copy number was associated with CCL3 production and with vertical transmission. However, at equivalent CCL3-L1 gene copy numbers, infants who acquired HIV-1 infection relative to their exposed but uninfected counterparts had lower production of CCL3, suggesting that they may harbour some non-functional copies of this gene. Nucleotide changes that may influence CCL3 production were evident in the CCL3 and CCL3-L1 genes upstream of exon 2. Our findings suggest that infants who display a deficient-production phenotype of CCL3 are at increased risk of acquiring HIV-1, indicating that this chemokine in particular plays an essential role in protective immunity.

INTRODUCTION

The CC chemokines CCL3, CCL4 and CCL5 are the natural ligands for CCR5 (Cocchi et al., 1995), a chemokine receptor utilized by R5 human immunodeficiency virus type 1 (HIV-1) in addition to CD4 for entry into leukocytes (Deng et al., 1996; Dragic et al., 1996). Aside from their role in chemotaxis, the CC chemokines are also involved in regulation of cell-mediated immunity (Matsukawa et al., 2000). The fact that the CC chemokines could block macrophage-tropic HIV-1 viruses from replicating in vitro (Cocchi et al., 1995) 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 (Heeney et al., 1998; Lehner et al., 1996). Wasik et al. (1999) demonstrated an overexpression of 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.

Antiviral chemokines produced by activated leukocytes in HIV-1 exposed–uninfected individuals may play a role in establishing relative resistance to HIV-1 infection, as observed in persons who remain uninfected despite multiple high-risk sexual exposures (Paxton et al., 1996; Zagury et al., 1998).

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 (reviewed by O’Brien & Nelson, 2004). 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., 2005). However, no human prospective study has demonstrated directly that a deficient phenotype of limited production of one or more of the CC chemokines is associated with risk of acquiring HIV-1 infection.

The context of mother-to-child transmission of HIV-1 provides an ideal model in which to test potential mechanisms...
associated with host protective immunity to HIV-1. Measuring immune responses at birth among uninfected infants allows the distinction to be made between those infants who escape infection and those who succumb to infection. We questioned whether fetal CC chemokine production was associated with protection against HIV-1 transmission due to viral exposure at delivery and observed that infants with deficiencies in production of CCL3 were more likely to acquire HIV-1 infection. CCL3-L1 gene copy number only partially explains this phenotype, suggesting that infants who acquire infection may harbour some non-functional copies of this gene. Our findings support the hypothesis that this chemokine in particular plays an important role in protective immunity among HIV-1 exposed–uninfected infants.

**METHODS**

**Patient samples.** Cord-blood samples were collected into EDTA vacutainers by cordocentesis from infants born to 124 HIV-1-infected women enrolled as part of a post-exposure prophylaxis (PEP) trial conducted at Chris Hani Baragwanath Hospital in Soweto, South Africa (Gray et al., 2005). Women who had either received no prenatal care or who received prenatal care at community clinics where HIV testing and access to antiretroviral drugs were unavailable were eligible for this trial and were tested for HIV after delivery. Their infants were randomized to receive either nevirapine or zidovudine as prophylaxis, started within 24 h of birth. 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. All 6-week samples were tested first and, if found to be negative, it was assumed that the infant was uninfected. The 6-week samples that tested positive were further analysed by testing the birth sample to distinguish between intrauterine and intrapartum infection. Confirmation of intrapartum infection was obtained by testing the 3-month samples of infants who were HIV-1 DNA PCR-positive at 6 weeks, but negative at birth. From the 124 mother–child pairs from whom samples were collected, we selected for testing all infants who became infected with HIV-1 [13 were infected intrapartum (IP) and four were infected in utero (IU); one infected infant was excluded because sample preparation was inadequate for this analysis] and a random sample of uninfected children born to HIV-1-infected mothers [43 exposed–uninfected (EU)] by using a nested case–control design. The clinical characteristics of the HIV-1-infected mothers and their infants are shown in Table 1. 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.

Peripheral-blood samples were also collected from all mothers recruited to this study. 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 (Kuhn et al., 2001b) to yield a total of 46 transmitting and 74 non-transmitting mother–child pairs. Written informed consent was obtained from all study participants and the study was approved by the Institutional Review Boards of the investigators.

**Isolation of cord-blood mononuclear cells (CBMCs).** CBMCs and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Histopaque Ficoll (Sigma). Contaminating erythrocytes were lysed by using a solution of 0·15 M NH₄Cl, 10 mM KHCO₃ and 1 mM sodium/EDTA (pH 7·0). After isolation, the number of viable cells was determined by trypsin blue exclusion and the cells were resuspended at 3 × 10⁶ cells ml⁻¹ in RPMI medium containing 1 % l-glutamine for use in the chemokine-production assay.

**Chemokine-production assays.** PBMCs isolated from mothers’ blood samples and CBMCs, resuspended at 3 × 10⁶ cells ml⁻¹ in RPMI medium containing 1 % l-glutamine, were unstimulated or stimulated with phytohaemagglutinin (PHA) at a final concentration of 12·5 μg ml⁻¹. Human serum (10 %) was then added to each well. Following 24 h incubation at 37°C in a moist, 5 % CO₂ atmosphere, culture supernatants were harvested and stored at −70°C. Supernatants were tested for CCL3, CCL4 and CCL5 production by using Quantikine ELISA kits (R&D Systems).

**Quantification of CCL3, CCL4 and CCL5 in plasma.** Peripheral levels of CCL3, CCL4 and CCL5 in the HIV-1-infected and uninfected women in this study, and in the cord blood of infants born to these women, were quantified by using Quantikine ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Quantification of soluble immune-activation markers in cord-blood plasma.** β₂-Microglobulin and sL-selectin levels were determined by using Quantikine ELISA assays (R&D Systems) as described by the manufacturer. Neopterin levels were quantified by

| Table 1. Clinical characteristics of the HIV-1-infected mothers and their infants

Results are expressed as means ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Exposed–uninfected (EU)</th>
<th>Intrapartum-infected (IP)</th>
<th>Intrauterine-infected (IU)</th>
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<tbody>
<tr>
<td>n</td>
<td>43</td>
<td>13</td>
<td>4</td>
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<tr>
<td>Maternal characteristics</td>
<td></td>
<td></td>
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<tr>
<td>CD4 T-cell count</td>
<td>573 ± 42</td>
<td>430 ± 57</td>
<td>504 ± 269</td>
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<tr>
<td>CD4 : CD8 ratio</td>
<td>0·65 ± 0·06</td>
<td>0·44 ± 0·07</td>
<td>0·66 ± 0·41</td>
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<tr>
<td>HIV-1 RNA copies ml⁻¹ (log₁₀)</td>
<td>3·87 ± 0·16*</td>
<td>4·69 ± 0·19</td>
<td>4·99 ± 0·46</td>
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<tr>
<td>Age (years)</td>
<td>25·6 ± 0·8</td>
<td>27·6 ± 1·6</td>
<td>25·8 ± 3·4</td>
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<tr>
<td>Parity</td>
<td>1·95 ± 0·15</td>
<td>2·42 ± 0·38</td>
<td>1·75 ± 0·25</td>
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<tr>
<td>Post-exposure prophylaxis (PEP) regimen</td>
<td>28 zidovudine;</td>
<td>Five zidovudine;</td>
<td>Two zidovudine;</td>
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<tr>
<td></td>
<td>15 nevirapine</td>
<td>eight nevirapine</td>
<td>two nevirapine</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>2948 ± 64</td>
<td>2896 ± 139</td>
<td>2750 ± 162</td>
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*The EU group has a significantly lower viral load than the IP (P=0·012) and IU (P=0·031) groups.
using an Immunotech ELISA system (Beckman Coulter). The minimum detectable dose of β2-microglobulin is <0.2 μg ml⁻¹, for sL-selectin <0.3 ng ml⁻¹ and for neopterin 0.2 ng ml⁻¹.

**HIV-1 quantification.** HIV-1 RNA levels (expressed as log₁₀ units) were quantified by using the Roche Amplicor RNA Monitor assay (Roche Diagnostics Systems, Inc.) with a lower detection limit of 400 HIV-1 RNA copies ml⁻¹.

**DNA sequencing of CCL3 and CCL3-L1.** Genomic DNA was extracted from PBMCs by using a Qiagen QIAamp DNA minikit. One hundred nanograms of genomic DNA was used in a 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. The upstream primer (5'-CTCCACAGCAGTACGGCC-3') was designed to bind to a consensus region flanking the Alu element, which is present only in CCL3-L1, and the downstream primer (5'-CCGGATCATGCTGAGAAGA-3') was designed to bind to a consensus region in intron 1 that is approximately 50 bp upstream from the start of exon 2. The primers thereby amplified two fragments, a 1550 bp CCL3-L1-specific amplicon and a 1240 bp CCL3-specific amplicon. PCR was carried out by using the Expand High Fidelity system (Roche). Amplicons were subsequently purified by using a Qiagen QIAquick PCR Purification kit and sequenced by using Applied Biosystems) and run on a 3100 Genetic Analyser (Applied Biosystems) according to the protocol supplied. For each sample, the sequence either the forward or reverse sequence of CCL3 and CCL3-L1 from the purified amplicon mixture. Sequencing reactions were set up by using Big Dye Terminator chemistry version 3.1 (Applied Biosystems) and run on a 3100 Genetic Analyser (Applied Biosystems). Resulting sequences were assembled and analysed for the presence of single-nucleotide polymorphisms (SNPs) by using the SEQUEANCHER software version 4.1.4 (Gene Codes Corporation), by alignment with published sequences (Nakao et al., 1990) [GenBank accession nos D90144 (CCL3) and D90145 (CCL3-L1)].

**Real-time PCR for CCL3-L1 copy-number determination.** The following primers and probes were synthesized (UCT): β-globin gene upstream, 5'-GGCAACCTATAGGTGAAGGC-3'; β-globin gene downstream, 5'-GGTGACGCAGCGCATCATA-3'; β-globin gene probe, 5'-CATGGCAAGAATTGTGCTGC-3'; CCL3-L1 gene upstream, 5'-TCTCCACAGCAGTCTAAACAGA-3'; CCL3 and CCL3-L1 genes downstream, 5'-CTGGACACCACCTCTCAGTC-3'; CCL3-L1 gene probe, 5'-AGGGCCAGTTCATTCTGAAG-3' (Townson et al., 2002). In addition, CCL3 gene upstream 5'-TCTCCACAGCAGTCTAAACAGA-3' and CCL3 gene probe 5'-AAGGCGGCAGGTTCATTCTGAAG-3' were designed and synthesized. All probes were labelled with 5'-6-carboxyfluorescein (FAM) and a 3' 6-carboxytetramethylrhodamine (TAMRA) quencher.

Real-time PCR was performed by using an ABI Prism 7500 (Applied Biosystems) according to the protocol supplied. For each sample, the β-globin, CCL3 and CCL3-L1 genes were amplified in duplicate, using approximately 20 ng genomic DNA per sample. CCL3 gene copy number was confirmed at two copies per diploid genome (p.d.g.) for each sample, calculated by using the relative-quantification method (as per the protocol supplied) and using β-globin (present at two copies p.d.g.) as the endogenous control. CCL3 was then used as the endogenous control to calculate CCL3-L1 copy number, again using the relative-quantification method against a known-copy control. Samples giving a result of a single CCL3-L1 gene copy p.d.g. were confirmed by sequencing to ensure homozygosity.

**Statistical analysis.** Levels of the chemokines and CCL3-L1 copy numbers were compared pairwise across the groups by using the non-parametric Mann–Whitney U test. Correlations were calculated by using the Spearman’s Rank correlation coefficient. Multivariate analysis was conducted by using logistic-regression models. The statistical analyses were performed by using SPSS software (version 11.0; SPSS Inc.). All statistical tests were two-tailed and significance was considered to be *P*<0.05.

**RESULTS**

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

By using a nested case–control design, we measured the production of CC chemokines by CBMCs for 60 infants born to HIV-1-infected mothers and for 20 infants born to HIV-1-uninfected mothers (negative-control group). The infants born to the HIV-1-infected mothers were selected to include a random sample of 43 who remained uninfected [exposed–uninfected (EU) group], 13 who were infected intrapartum (IP group) and four who were infected *in utero* (IU group) (Table 1). HIV-1-infected mothers were only identified as HIV-positive after birth; all children were given post-exposure prophylaxis with either nevirapine or zidovudine (Gray et al., 2005).

PHA-induced release of CCL3 from CBMCs was elevated significantly in the EU infants compared with the negative-control group (*P*=0.002) (Fig. 1b), suggesting that HIV-1 exposure *in utero* had primed elevated CCL3 production. Not surprisingly, IU-infected infants had the highest levels of spontaneous and PHA-induced production, consistent with the effects of an established infection (Fig. 1a, b). Most striking, however, was the finding that CBMCs from the IP infants produced significantly less PHA-induced CCL3 than CBMCs from the EU infants (*P*=0.001) and equivalent to that among the negative-control group (Fig. 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 CBMCs showed a pattern similar to that observed for CCL3 (Fig. 1d, e), although levels were generally lower and the differences between the groups were not as marked. In contrast, CCL5 production (Fig. 1g, h) 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.

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

We next tested whether the lower production of CCL3 in the IP infants might be the result of inadequate priming prior to birth. Levels of the soluble immune-activation markers neopterin (indicative of activation of monocytes and macrophages), β2-microglobulin (antigen-presenting cell and T-cell activation) and sL-selectin (shed from activated lymphocytes, monocytes and polymorphonuclear cells) were raised in plasma of infants born to HIV-1-infected
mothers relative to negative controls (Fig. 2). However, there were no notable differences between EU and IP infants; thus, immune-activation events prior to birth did not account for their different CCL3 production.

**Increased production of CCL3/CCL4 by mothers’ PBMCs is associated with maternal HIV infection**

To examine whether there was a similar deficiency in CCL3 production among mothers of IP-infected infants, we measured the production of CC chemokines by maternal PBMCs. PHA-stimulated production of CCL3 ($P = 0.015$) and CCL4 ($P < 0.001$) was significantly higher among HIV-infected mothers (EU, IP, IU combined) than among uninfected mothers, and PHA-stimulated production of CCL5 tended towards elevation ($P = 0.06$) (Fig. 3).

PHA-stimulated production of CCL3, as in the IP infants, tended to be lower among mothers transmitting intrapartum, but this did not reach statistical significance.

**Elevated peripheral levels of CCL3 in maternal plasma are associated with non-transmission during labour and delivery**

Levels of CCL3, CCL4 and CCL5 were also quantified in the plasma of the HIV-1-infected women and the uninfected controls (Fig. 3c, f, i) and in cord-blood plasma of their infants (Fig. 1c, f, i). CCL3 levels in HIV-1-infected mothers who transmitted intrapartum were significantly lower ($P = 0.036$) than levels among infected mothers who did not transmit (EU). IU mothers tended to have elevated peripheral levels of CCL3 (Fig. 3c), reflecting the unstimulated

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**Fig. 1.** Spontaneous and PHA-stimulated release of CCL3, CCL4 and CCL5 from *in vitro* CBMC cultures and cord-blood plasma levels of CCL3, CCL4 and CCL5 for infants born to HIV-1-uninfected mothers (neg control) and for infants born to HIV-1-infected mothers who remained HIV-1-uninfected (EU, exposed–uninfected) or were infected intrapartum (IP) or *in utero* (IU). Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars) and outliers (∗). Sample numbers and significant differences between groups are indicated.
production by PBMCs observed in Fig. 3(a). Cord-blood plasma levels did not distinguish between EU and IP.

**Relationship between CCL3 and transmission is not explained by mothers’ viral load or CD4 T-cell count**

No associations were found between the ability of infant CBMCs to produce CCL3 and maternal HIV-1 viral load or CD4 T-cell count. Adjusting statistically for maternal viral load and CD4 count, the association between decreased levels of CBMC PHA-stimulated CCL3 and increased risk of intrapartum infection remained significant ($P = 0.039$).

**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/LD78α and CCL3-L1/LD78β) and a pseudogene, LD78γ (Menten et al., 2002). CCL3 occurs as two copies p.d.g., whereas CCL3-L1 occurs in variable copy number in different individuals. Increased CCL3-L1 gene copy number has been correlated with increased CCL3 production in lipopolysaccharide-stimulated monocytes (Townson et al., 2002), making copy number of this particular gene a likely candidate for differential CCL3 production in vivo. In a recent study, Argentinian children with CCL3-L1 copy numbers lower than their population median had a higher risk of acquiring HIV-1 vertically (Gonzalez et al., 2005).

For copy-number determinations of CCL3 and CCL3-L1, we included additional mother–child pairs from the same hospital (Kuhn et al., 2001b) to make up a larger cohort of 74 non-transmitting and 46 transmitting mother–child pairs. The CCL3 gene was consistently found to be present at two copies p.d.g. in all samples tested. Despite a trend towards reduced copy numbers of CCL3-L1 amongst transmitting mothers (median 4, range 3–8) when compared with non-transmitting mothers (median 5, range 2–8), this was not statistically significant. However, when comparing CCL3-L1 copy numbers amongst infants born to HIV-1-infected mothers (Fig. 4), these were reduced significantly among those who became infected (median 4, range 1–10) relative to those who remained uninfected (median 5, range 1–8) ($P = 0.019$), 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. There was no relationship between maternal viral load or CD4 counts and their infants’ copy number of CCL3-L1.

**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 the IP group. At comparable CCL3-L1 gene copy numbers, EU infants’ CBMCs produced greater amounts of CCL3 than did those of IP infants (Fig. 5). This was most marked if CCL3-L1 copy number was high (at least four copies), suggesting the existence of non-functional copies in IP infants.

**Identification of SNPs 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 encode this chemokine. The promoter regions, exon 1 and most of intron 1 of both the CCL3 and CCL3-L1 genes (Fig. 6a, b) were sequenced for the mothers who had CCL3 production levels determined (Fig. 3a–c) 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., 2001) and on likelihood of effects on gene transcription. Data revealed the presence of four SNPs in CCL3 and three in CCL3-L1.

Within the CCL3 gene (Fig. 6a), it was evident that the SNPs described previously as occurring together in intron 1 at positions p + 199 and p + 545 (p + 113 and p + 459 on the chimpanzee CCL3 gene, respectively) (Gonzalez et al., 2001) were, in all instances, associated with a newly identified SNP (C→T) in the promoter region at p − 86. This particular three-SNP haplotype was encountered more frequently in the IP infants (5/13) and their mothers (5/13) than amongst EU infants (3/13) or their HIV-1-infected mothers (2/13), but, given the small numbers, was not statistically significant. The SNP at p + 702 (G→C) occurred rarely, independently of the three-SNP haplotype.

No variations in nucleotide sequence were noted in the promoter region of CCL3-L1 (Fig. 6b). The only noteworthy changes between groups in the CCL3-L1 gene were at p + 480, where four of 13 IP infant samples were homozygous for C, whereas for EU infants, three of 13 were homozygous for G (ancestral), therefore earmarking this site as a potential candidate for altered CCL3 production.

**Fig. 3.** Spontaneous and PHA-stimulated release of CCL3, CCL4 and CCL5 from in vitro PBMC cultures and peripheral levels of CCL3, CCL4 and CCL5 for HIV-1-uninfected mothers (neg control) and for HIV-1-infected mothers whose infants remained HIV-1-uninfected (EU, exposed–uninfected) or were infected intrapartum (IP) or in utero (IU). Data are presented as medians (horizontal bar), 25th and 75th percentiles (boxes), 10th and 90th percentiles (bars) and outliers (×). Sample numbers and significant differences between groups are indicated.
A large proportion of infants born to HIV-1-infected mothers escape HIV-1 infection, even in the absence of any antiretroviral intervention, providing testament to the presence of protective immune processes. This study set out to test prospectively whether fetal CC chemokine production primed by HIV-1 in utero can protect against HIV-1 transmission with subsequent exposure at delivery. The main findings were as follows: (i) HIV-1 exposure in utero (i.e. being to born to an HIV-positive mother) resulted in increased infant production of CCL3 and CCL4, but decreased CCL5 production; (ii) HIV-exposed infants who failed to respond with elevated CCL3 production (suggesting a deficiency in their capacity to produce CCL3) were more vulnerable to acquiring HIV-1 infection at delivery; (iii) mothers transmitting intrapartum also presented with a deficient CCL3-production phenotype evident in plasma, suggesting that the underlying nature of deficient infant CCL3 production was genetic and not due to a difference in in utero exposure between EU and IP infants (supported by similar levels of soluble immune-activation markers in cord-blood plasma of EU and IP infants 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); (iv) 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. However, copy number did not account entirely for the deficiencies in CCL3 production associated with risk of transmission, suggesting the presence of inactive copies of CCL3-L1 in IP infants; (v) mutations in CCL3 gene regions or lack of appropriate transcription factors or differential mRNA stability may be contributing factors to reduced production. Our results highlight the fact that copy number of CCL3-L1 per se does not always dictate levels of CCL3 production and that copies of CCL3-L1 or CCL3 in different individuals represent different abilities to be induced to produce protein. Whatever the contributing factors, it is clear that the association with susceptibility to HIV-1 lies at the level of induction of gene expression of CCL3/CCL3-L1, culminating in quantitative differences in protein production.

We propose the following model for the role of CCL3 in protective immunity to HIV-1. Given that most vertical transmission occurs with R5 isolates (LaRussa et al., 2002; Ometto et al., 1995), it is not unexpected that the CC chemokines, being natural ligands for the HIV-1 coreceptor CCR5, may influence this mode of transmission through non-cytolytic inhibition of infection. The fact that copy number of CCL3-L1 in particular associates with risk of transmission, as does induced production of CCL3,
suggests a ‘more expression, more protein’ quantitative effect, identifying CCL3-L1 as playing a more prominent role in protection than CCL3, which did not vary from two copies p.d.g. in all individuals tested. In this regard, proteins produced from the CCL3 and CCL3-L1 genes have been shown to have distinct biological functions, with CCL3-L1 being 30-fold more potent at inhibiting HIV-1 infection (Aquaro et al., 2001; Menten et al., 1999; Nibbs et al., 1999, 2001). Post-translational modification by amino-terminal truncation of CCL3-L1 by CD26/DPP (dipeptidyl peptidase), present in plasma and highly expressed on a number of cell types (Van Damme et al., 1999), gives rise to a

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**Fig. 6.** CCL3 and CCL3-L1 gene structures and SNPs identified by DNA sequencing. Schematic representation of the gene structures of CCL3 (a) and CCL3-L1 (b), showing SNPs identified from a total of 86 mother and child sequences of approximately 1240 bp (CCL3) and 1550 bp (CCL3-L1) each. Positions are numbered based on +1 being the mRNA start site. Open boxes indicate exons 1–3; solid lines between exons indicate introns 1 and 2; arrows indicate approximate binding positions of primers used for PCR amplification of CCL3 and CCL3-L1. The first three SNPs in the CCL3 gene (*) were always associated with each other (TTT haplotype); possession of the TT haplotype at positions +199 and +545 has previously been identified to be associated with low risk of HIV-1 infection (Gonzalez et al., 2001). Possession of a T at +89 has not been reported previously in humans, but does occur in rhesus and langur monkeys (Goila et al., 2001). The frequency of individuals within each group (C-C, EU-C, IP-C, IU-C: uninfected control, exposed–uninfected, intrapartum-infected, in utero-infected infants, respectively; C-M, EU-M, IP-M, IU-M: uninfected control, non-transmitting, intrapartum-transmitting, in utero-transmitting mothers, respectively) are tabulated below the respective genes, with sample numbers in each group shown.
variant form that is even more potent an agonist and HIV-1 inhibitor (Proost et al., 2000; Struyf et al., 2001). It stands to reason that increased copies of CCL3-L1 that encode protein with greater anti-HIV activity would present a distinct advantage at first encounter with HIV-1, purely on the basis of the ability of CCL3 to block HIV-1 entry into target cells through steric hindrance or downregulation of the CCR5 receptor. However, our data would also suggest that this mechanism is unlikely to be the only one at play and, in particular, that CCL4 and CCL5 cannot compensate for lack of CCL3, further supporting the hypothesis that immune functions unique to CCL3 are involved in protection from HIV-1.

A successful antiviral response, facilitated by CCL3, could be envisaged to occur in two phases, the first involving acute inflammatory effects of CCL3, which establish the recruitment of specific cell types in response to HIV-1 challenge, a concentration-dependent process. A deficit in CCL3 production at this point may alter all subsequent events substantially. The second phase would involve the effects of CCL3 on adaptive immunity. Studies in mice immunized with protein have shown that, if given in addition, chemokines such as lymphotactin (Lillard et al., 1999), CCL5 (Lillard et al., 2001) and, more recently, CCL3 and CCL4 (Lillard et al., 2003) could potentiate both humoral and cell-mediated adaptive mucosal and systemic immunity. The distinct differences in activities of CCL3 and CCL4 (Lillard et al., 2003) can help to explain why increased levels of CCL4 primed by exposure to HIV-1 in utero did not compensate for lack of CCL3 production in those infants who became infected. In particular, CCL3, but not CCL4, promotes both mucosal and systemic cytotoxic T-lymphocyte responses. CCL3 also promotes strong antigen-specific serum IgG and IgM responses, enhances T-helper type 1 responses and modulates costimulatory molecules on T cells and antigen-presenting cells (Lillard et al., 2003). Interestingly, we have observed previously (Kuhn et al., 2001a, b) that Env-specific, interleukin 2-dependent cellular immune responses were only detected in cord blood of exposed-uninfected infants and not in infants who subsequently become infected, consistent with the notion that deficient CCL3 production may compromise the development of primary immune responses to HIV-1. In further support of this, Wasik et al. (1999) also observed that HIV-1 Env-specific T-helper cell responses detected in exposed-uninfected infants were associated with the enhanced expression of CC chemokines. The fact that we do not observe a threshold of CCL3 production that is fully protective against transmission suggests that CCL3 alone is insufficient and may be an important part of the multifactorial immune responses necessary to protect against HIV-1 at varying extents of viral exposure.

In contrast to the mothers transmitting intrapartum, mothers transmitting intrauterine had elevated peripheral levels of CCL3. These levels correlated with higher levels of spontaneous release from PBMCs of CCL3, suggesting that increased peripheral production was probably the result of activated cells in the peripheral circulation. As sample numbers are small, it will be important to verify this further, as it suggests that consequences of CCL3 may be different for in utero infection. Whether this is merely a marker of the very much elevated immune activation that is characteristic of these mothers or that CCL3 itself impacts negatively on transmission due to high levels of CCL3 at the maternal-fetal interface remains to be established. CCL3 produced by placental cytotrophoblasts has been shown to attract monocytes, natural killer and T cells to sites adjacent to the trophoblast (Drake et al., 2001) and the extent of production may determine cell infiltration and likelihood of protection from, or infection with, HIV-1.

Our findings are consistent with the novel findings on gene copy number (Gonzalez et al., 2005) in raising the importance of CCL3 in HIV susceptibility. We also show that ‘not all CCL3-L1 gene copies are created equal’ and it will be important to identify the precise genetic determinants that define levels of CCL3 production, so that genotypic assays can be developed that identify individuals at increased risk of infection or hastened disease progression. Mutations within some of the duplicated copies may render them less or non-functional; this may explain why absolute copy number per se does not associate with protection, but the copy number in relation to the specific population median does (Gonzalez et al., 2005).

Given the challenges in developing successful vaccines to HIV-1 (Brander & Walker, 2003; Stratov et al., 2004), it would be advantageous if non-specific immune parameters, able to provide protection and/or augment HIV-specific responses, were identified. For example, a recent macaque study demonstrated proof of principle that an artificially created CCL5 could be an effective micobicide for prevention of vaginal simian–human immunodeficiency virus infection (Lederman et al., 2004). CCL3 would represent an excellent adjuvant for inclusion in an HIV vaccine construct, but the question remains as to how to compensate for the loss of function in vaccinees that have a deficient CCL3—production phenotype. Host genetics need to be taken into consideration in vaccine evaluation, and vaccines should ideally elicit immune responses that produce sufficient CCL3 at the site of subsequent antigen–HIV-1 encounter. This underscores the need to find natural or other molecules that, under certain conditions of stimulation, may compensate for lack of CCL3 function. Therefore, it is imperative to delineate the precise roles in the immune response of CCL3, aside from its non-cytolytic inhibitory effect on HIV-1, as its role in driving the development of adaptive immunity may be crucial to protection.

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