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Influence of intragenic *CCL3* haplotypes and *CCL3L* copy number in HIV-1 infection in a sub-Saharan African population

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Abstract

Two *CCL3* haplotypes (HapA1 and Hap-A3) and two polymorphic positions shared by the haplotypes (Hap-2SNP) were investigated together with *CCL3L* copy number (CN), for their role in HIV-1 disease. Hap-A1 was associated with protection from *in utero* HIV-1 infection: exposed-uninfected infants had higher representation of WT/Hap-A1 than infected infants (excluding intrapartum-infected infants), which maintained significance post maternal Nevirapine (mNVP) and viral load (MVL) correction (P=0.04; OR=0.33). Mother-infant pair analyses showed the protective effect of Hap-A1 is dependent on its presence in the infant. Hap-A3 was associated with increased intrapartum transmission: WT/Hap-A3 was increased in intrapartum vs. non-transmitting mothers, and remained significant post mNVP and MVL correction (P=0.02; OR=3.50). This deleterious effect of Hap-A3 seemed dependent on its presence in the mother. Hap-2SNP was associated with lower CD4 count in the non-transmitting mothers (P=0.03). *CCL3* Hap-A1 was associated with high *CCL3L* CN in total (P=0.001) and exposed-uninfected infants (P=0.006); the effect was not additive, however having either Hap-A1 or high *CCL3L* CN was more significantly (P=0.0008) associated with protection from *in utero* infection than Hap-A1 (P=0.028) or high *CCL3L* CN (P=0.002) alone. Linkage disequilibrium between Hap-A1 and high *CCL3L* CN appears unlikely given that a Nigerian population showed an opposite relationship.

Keywords

CCL3; *CCL3L*; HIV-1; Haplotypes; SNPs; Mother-to-child-transmission

Conflict of interest The authors declare no conflict of interest.

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Introduction

Chemokines are a large superfamily of low-molecular-weight (approximately 8-15 kDa), structurally related cytokines that mediate their biological functions through recruitment of cells bearing seven-transmembrane G-protein-coupled receptors, as part of homeostatic immune cell-trafficking or during inflammatory responses. Most chemokines can be divided into two major subgroups on the basis of the arrangement of the two N-terminal cysteine residues, depending on whether the first two residues are adjacent (CC) or have an amino acid between them (CXC) (see review [1]). Three CC chemokines, namely CCL3 (formerly MIP-1α), CCL4 (formerly MIP-1β) and CCL5 (formerly RANTES), were shown to demonstrate HIV-1 suppressor activity early on [2], which was later attributed to their binding to the CCR5 receptor [3], the co-receptor R5 strains of HIV-1 use to gain entry into target cells. These chemokines have been shown *in vitro* to inhibit HIV-1 entry by competing with viral Env protein for binding [4-5] as well as by down-regulation of CCR5 surface expression [6]; however whether these chemokines play a similar role *in vivo* is largely unknown. A number of genetic association studies suggest a role for these and other chemokines in HIV-1 infection (see review [7]). Of interest in this study is CCL3, which is encoded by two functional genes, namely *CCL3* and *CCL3La* and in addition to these two genes, a third 'pseudogene' (*CCL3Lb*) has been described [8-9] which has been recently reported to have novel 5′ exons giving rise to alternatively transcribed mRNA species [10]. *CCL3* is present as two copies per diploid genome (pdg), whereas *CCL3La* and *CCL3Lb* (collectively termed *CCL3L*) have been shown to exist in variable copies pdg, [8-9, 11-12]. These three genes have all been mapped to a narrow region on human chromosome 17.

The two isoforms of CCL3, namely CCL3 and CCL3La, differ by only three amino acids, yet CCL3La has been shown to be 30-fold more potent at inhibiting R5 HIV-1 infection compared to the CCL3 isoform [13-14]. *CCL3L* copy number variation (CNV) and its role in HIV-1 infection has been the focus of a number of studies, however not all studies agree to a role for *CCL3L* CNV. In a meta-analysis of nine published studies, recently conducted by Liu *et al.* [15], lower *CCL3L* copy number was associated with higher risk of HIV-1 infection when one takes into account the specific population since different populations differ with respect to *CCL3L* copy number means. Although *in vitro* studies show CCL3 to be less potent than CCL3La at R5 HIV-1 inhibition, there have been reports of single nucleotide polymorphisms (SNPs) and haplotypes within *CCL3* impacting on both HIV-1 susceptibility as well as disease progression [16-18]. Interestingly, all these studies involve polymorphisms within various haplotypes that we believe are all part of larger haplotypes that we have described in South African Africans and Caucasians in an earlier study [19]. There are certain advantages to studying the role of haplotypes rather than a one-SNP-at-a-time approach in candidate genes studies, firstly, the proteins produced by an individual's genes occur in two polypeptide chains that correspond to maternal and paternal haplotypes and their folding as well as other properties are likely to be dependent on the arrangement of particular amino acid combinations [20]. Variation in a population tends to be "structured into haplotypes that are likely to be transmitted as a unit" [20]. Also, ethnically divergent population groups differ considerably regarding their haplotypic structures and select SNPs can 'tag' more than one haplotype.

In a recent study conducted on serodiscordant Zambian couples, two *CCL3* SNPs, namely rs5029410 (3′ UTR) and rs34171309 (exon 3), were found to be significantly associated with lower viral load and increased risk of HIV-1 acquisition, respectively [21]. The role of *CCL3* genetic variation in mother-to-child-transmission (MTCT) of HIV-1 has not been investigated, thus in this study we investigated the role of three *CCL3* haplotypes (one encompassing the exon 3 rs34171309 SNP), previously identified in our South African African (SAA) individuals [19], for their role in MTCT HIV-1 transmission and in HIV-1

disease progression. In addition, the combined effect of *CCL3* haplotypes and *CCL3L* copy number was evaluated.

Results

Cohort

A total of 314 HIV-1 infected South African African (SAA) mothers and their infants (and 4 additional unmatched infants i.e. 318 infants), recruited as part of 4 mother-to-infant HIV-1 transmission cohorts in Johannesburg, South Africa, were used in this study. A detailed description of the 4 cohorts is given by Kuhn *et al.* [22]. An additional 115 control, SAA HIV-1-uninfected mothers were also recruited. All available transmitting pairs (infant infected) and a random sample of approximately three non-transmitting pairs (mother infected but infant uninfected) per case were randomly selected from the cohorts. Of the 314 matching mother-infant pairs, 235 were mother-infant pairs where the infants were HIV-1 exposed but remained uninfected (EU) and their mothers are thus referred to as non-transmitting (NT) mothers. In addition there were three unmatched EU infants, thereby totalling 238 EU infants. There were 79 matched mother-infant pairs where the infant was determined HIV-1 infected and one additional unmatched infected infant thereby totalling 80 infected (INF) infants and 79 transmitting (TR) mothers. Of the 80 INF infants, 20 were infected in utero (IU; PCR positive at birth), 32 were infected intrapartum (IP; PCR negative at birth, positive at 6 weeks postpartum) and the remaining 28 were found to be infected at 6 weeks but had no birth sample available for determining the timing of transmission. The cohort individuals described by Kuhn *et al.* [22] were not all used in this study, thus Table 1 shows the distribution of this study's participants across the 4 cohorts. The median maternal viral loads (copies/ml) and CD4 counts (cells/:l) for the broad groups are also shown in Table 1.

Antiretroviral Drugs

Since this study aimed to investigate the role of select genetic variations on mother-to-infant HIV-1 transmission, the role played by the various antiretroviral drugs administered in the 4 cohorts needs to be addressed. Study participants were classified according to whether the mothers were administered no nevirapine (noNVP) or a single-dose maternal NVP (mNVP) during labour. All infants received a single dose of NVP. Table 1 also shows the distribution of mothers and infants after stratification according to maternal NVP (with maternal viral loads and CD4 counts). Single-dose NVP administration to the mother during labour serves to only reduce IP HIV-1 infant infection since mNVP does not exert its protective effect by reducing the mother's viral load (insufficient time; mNVP vs. noNVP MVL: P=0.6; Mann-Whitney U-test), but by the increased NVP in the infant acquired through placental transfer. Thus, mNVP administration (and subsequent infant NVP administration) has no effect on IU infection which has occurred prior to NVP exposure. To correct for the effect of antiretroviral drugs, in addition to analysing the data without taking drugs into account, logistic regression was used to correct for the effect of mNVP and mothers and infants that were administered other antiretroviral drugs were excluded from this part of the analysis.

Haplotype description

Two intragenic haplotypes, namely Hap-A1 and Hap-A3, each comprised of minor alleles at seven SNP positions, were previously identified in the *CCL3* gene amongst South African African (SAA) mother and infant pairs [19]. Figure 1 shows the SNPs as well as the nucleotide (minor allele) composition of these two haplotyes relative to a schematic representation of the *CCL3* gene. These two haplotypes overlap at two SNP positions as indicated on Figure 1. Thus, in addition to investigating the role of each haplotype individually in both HIV-1 mother-to-child transmission and HIV-1 disease (in the mothers), we were also able to

independently investigate the effect of the minor alleles of the A/G p+1245 (rs1719130) and C/G p+1728 (rs1063340) SNPs (termed Hap-2SNP). This is not only a count of individuals heterozygous for Hap-A1 and Hap-A3 (i.e. an individual having both haplotypes) since homozygosity for either Hap-A1 or Hap-A3 would result in homozygosity for Hap-2SNP.

Linkage disequilibrium and Hardy-Weinberg

Mother (both infected and controls) and infant groups were grouped separately and analysed for both pairwise linkage disequilibrium between all minor alleles in the two haplotypes (Hap-A1 and Hap-A3) and well as for deviation from Hardy-Weinberg for the two tag SNPs and the Hap-2SNP genotypic data. All minor alleles in both haplotypes were in perfect LD with D′=1 for all pairwise combinations and all were significant with *P*'s<0.01. In addition no significant deviations from Hardy-Weinberg equilibrium were noted for the three haplotypes.

Frequency of CCL3 haplotypes in the SAA population

The minor allele frequencies of the three haplotypes as well as the genotypic frequencies are listed in Table 2. The haplotype frequencies of the control mothers, infected mothers as well as the total infant group did not show any noticable over- or underrepresentation in any one group over the other, and comparisons between the control and infected mothers did not reveal any trends or significant differences (data not shown). Given that Hap-2SNP involves SNPs shared by Hap-A1 and Hap-A3, it is not surprising that it is the most prevalent of the haplotypes in the SAA population group with an allelic frequency of approximately 18.9% (averaged across the three groups) and heterozygosity for Hap-2SNP was found in approximately 31% of the SAA population group. Hap-A1 is also a prevalent haplotype in this population group with an average allelic frequency of approximately 13.5% whereas Hap-A3 was the least prevalent of the haplotypes with an allelic frequency of 5%. Homozygosity for Hap-A3 was rarely detected.

CCL3 haplotypes and maternal HIV-1 disease progression

Viral load and CD4 count comparisons between HIV-1 infected mothers (total and NT mothers) harbouring Hap-A1, Hap-A3 or Hap-2SNP to mothers lacking the haplotypes are shown in Table 3. The cohorts used in this study were designed to study HIV-1 MTCT, and thus a 3:1 ratio of NT:TR mothers is skewed in terms of TR mother overrepresentation compared to a cross-sectional study where the rate of MTCT would be at least two fold less. Thus to determine the role of *CCL3* haplotypes on markers of disease progression, mothers were also analysed in the absence of TR mothers (i.e. NT mothers). Strong trends in the total mother group were seen with Hap-2SNP heterozygosity as well as Hap-2SNP heterozygosity combined with Hap-2SNP homozygosity (i.e. at least one copy of Hap-2SNP) and lower CD4 count (*P*=0.059 and *P*=0.056, respectively) which both proved to be significant upon analysis of the NT mothers alone (*P*=0.034 and *P*=0.032, respectively). Hap-A1 also showed a trend of association with low CD4 count in the NT mothers (*P*=0.067).

CCL3 haplotypes and mother-to-child HIV-1 transmission

Percent representation of the *CCL3* haplotype genotypes (heterozygotes and homozygotes) in both the infant and mother groups are shown as bar graphs in supplementary Figure 1.

A role for Hap-A1 in infant protection from HIV-1 infection

Hap-A1 comparisons between all groups of transmitting mothers and NT mothers revealed no significant associations of this haplotype with any particular group (data not shown).

Table 4 shows comparisons between EU infants and infected infant groups/subgroups with respect to Hap-A1 heterozygosity (WT/Hap-A1).

EU infants had higher representation of Hap-A1 heterozygosity compared to INF infants (*P*=0.06; OR=0.53), and a trend was maintained post MVL correction(*P*=0.07; OR=0.53) and mNVP correction (*P*=0.05; OR=0.50), but was lost upon combined correction for MVL and mNVP, without much change to the OR (*P*=0.10; OR=0.55). Comparison of EU infants to IP and IU infants revealed that it is the IU infants that are underrepresented with respect to WT/Hap-A1 and although not significant when compared to EUs (*P*'s=0.09-0.15 across comparisons), the ORs we low and ranged from 0.29-0.32 (Table 4). Given that the IU group is fairly small (n=20), and given that the infected infant group of unknown (i.e. IP or IU) infection route (n=28) are likely to have a higher proportion of IU infections than IP infections (see rationale in 'Materials and Methods' section under 'Comparisons and Analyses'), we compared EU infants to infected infants (termed INF-2) after exclusion of known IP-infected infants. Table 4 shows that EU infants had significantly higher representation of WT/Hap-A1 compared to INF-2 infants (*P*=0.02; OR=0.16) and furthermore this significance was maintained post MVL (*P*=0.03; OR=0.34), mNVP (*P*=0.02; OR=0.30) and combined MVL and mNVP correction (*P*=0.04; OR=0.33). This result strongly suggests that Hap-A1 is playing a role in protection from HIV-1 *in utero* infection.

To further investigate the role of Hap-A1, we looked at mother-infant pairs with respect to concordance and discordance in possession of the WT/Hap-A1 genotype. Figure 2A shows that the protective effect of WT/Hap-A1 occurs at the level of the infant. Although concordance for the genotype (M+I+) still reveals NT/EU mother-infant pairs having higher representation than both IU-TR/IU and the TR-2/INF-2 mother-infant pairs, this is not statistically significant. In the discordant combination where the genotype is absent in the mother and present in the infant (M−I+), TR-2/INF-2 mother-infant pairs have significantly less WT/Hap-A1 than NT/EU mother-infant pairs (*P*=0.023) and although IU-TR/IU mother-infant pairs had zero representation of WT/Hap-A1, this association did not reach significance. Interestingly, the absence of the genotype in the infant (M+I−) abrogates the protective effect imparted by WT/Hap-A1; even though IU-TR/IU mother-infant pairs have no WT/Hap-A1 representation, TR-2/INF-2 mother-infant pairs have relatively high WT/Hap-A1 representation and EU/NT mother-infant pairs have fairly low WT/Hap-A1 representation compared to their M+I+ and M-I+ counterparts (Figure 2A).

A role for Hap-A3 in maternal HIV-1 transmission

Comparison of infected infant groups to EU infants with respect to Hap-A3 and Hap-2SNP failed to show a role for these two haplotypes with respect to HIV-1 infant protection (data not shown). Table 5 shows comparisons between NT mothers and TR mother groups/subgroups with respect to Hap-A3 heterozygosity (WT/Hap-A3) and Hap-2SNP homozygosity.

NT mothers had significantly lower representation of WT/Hap-A3 compared to IP-TR mothers (*P*=0.04; OR=2.94) which maintained significance post-MVL correction (*P*=0.02; OR=3.42), post-mNVP correction (*P*=0.03; OR=3.17) and post combined correction for MVL and mNVP (*P*=0.02; OR=3.50).

Minor allele homozygosity at Hap-2SNP showed a trend of underrepresentation in NT mothers compared to TR mothers and IP-TR mothers in the total group comparisons (*P*=0.08; OR=3.95 and *P*=0.06; OR=5.92, respectively), which were maintained post-MVL correction (*P*=0.06; OR=4.51 and *P*=0.08; OR=5.60, respectively). A trend was maintained only in the NT vs. IP-TR comparison post-mNVP and post the combined mNVP and MVL

correction (*P*=0.06; OR=6.06 and *P*=0.07; OR=6.05, respectively). Since IU-TR mothers had no representation of Hap-2SNP homozygosity, we compared NT mothers to TR mothers after removal of the IP-TR mother subgroup (i.e. TR-2 equivalent to the INF-2 infant group and predominantly IU-TR mothers). No significant associations were seen with respect to NT vs. TR-2 and Hap-2SNP homozygosity (Table 5).

These results point to a role for Hap-A3 in increasing the likelihood of IP mother-to-child HIV-1 transmission. Hap-2SNP also shows a trend towards increased IP transmission, however since the frequency of Hap-2SNP homozygosity is very low (2.34% in total HIV-1-infected mothers, Table 2), the confidence intervals in these comparisons are very large hence larger sample numbers would be needed to verify this result.

Concordant and discordant mother-infant comparisons with respect to WT/Hap-A3 did not reveal any significant associations, however Figure 2B clearly shows that the relationship of high WT/Hap-A3 being associated with a greater likelihood of IP transmission seemed dependent on the genotype being present in the mother since in both M+I+ and M+I-mother-infant groups, IP-TR/IP mother-infant pairs have at least double the representation of WT/Hap-A3 compared to the NT/EU mother-infant pairs. Conversely, in the M-I+ mother-infant group, IP-TR/IP mother-infant pairs have no representation of WT/Hap-A3 compared to 5.2% in the NT/EU mother-infant pairs (Figure 2B).

CCL3 haplotypes and CCL3L gene copy number

Results of Mann-Whitney *U* test comparisons in the infant groups with regard to *CCL3L* copy number (CN) alone as well as *CCL3L* CN and *CCL3* haplotypes are listed in Table 6.

As we have previously seen within this cohort [22], EU infants have statistically higher *CCL3L* CN compared to INF infants (*P*=0.004). When we compared the possession of at least one copy of Hap-A1 (i.e. homozygotes and heterozygotes) and their *CCL3L* CN, we found that within the total infant group, infants with high *CCL3L* CN were very significantly associated with possession of Hap-A1 (*P*=0.001). This was due to the EU infants as EU infants with high *CCL3L* CN also tend to possess at least one copy of *CCL3* Hap-A1 (*P*=0.006) compared to INF infants (*P*=0.141). On the other hand, possession of high *CCL3L* CN and at least one copy of Hap-A3 failed to show any significance, however when Hap-2SNP was analysed in the same manner, within the total infant group, high *CCL3L* CN and possession of at least one copy of Hap-2SNP was also significant (*P*<0.001) and seen within both the EU (*P*=0.011) and INF infants (*P*=0.029). No significant associations or trends between *CCL3L* CN and the possession of any of the three haplotypes were seen in the mothers (data not shown).

To determine if another African population showed a similar strong association with *CCL3L* CN and Hap-A1, we made use of SNP data available for the Yoruban (YRI) population group (Nigeria) from the HapMap project (www.hapmap.org) and *CCL3L* copy number data available for the same group using supplementary data from Campbell et al. [23]. Although SNP data for only two SNP positions (rs1130371 and rs1719134) found in Hap-A1 were available in HapMap, we made the assumption that Hap-A1 is present within this population group and used the rs1130371 SNP data as a putative ‘tag’ for Hap-A1 to test for the association of *CCL3L* CN and the presence or absence of the minor allele of the rs1130371 SNP (Mann-Whitney U-test). Results show that Hap-A1 was significantly associated with *CCL3L* CN however, surprisingly, and in contrast to our study population, Hap-A1 in the YRI population is associated with low *CCL3L* CN (*P*=0.005). What we did notice is that the two populations differed with respect to *CCL3L* CN distribution with the YRI population having a median *CCL3L* CN of 4 (range 2-8) whereas our study population has a median of

5 (range 1-10), which is similar to the distribution seen in healthy uninfected SAA adults (median=5, range=0-9, N=240; unpublished data).

Since high *CCL3L* CN is significantly associated with infant protection from HIV-1 infection, and since Hap-A1 is significantly associated with protection from IU HIV-1 infection (overrepresentation in EU infants vs. INF-2 infant group), the significant association of *CCL3* Hap-A1 and high *CCL3L* CN warrants further investigation. In order to determine if there is an additive effect of *CCL3* Hap-A1 and high *CCL3L* CN, we compared EU infants and INF-2 with both these genetic features to those with neither. Table 7 shows that having both these genotypes (P=0.033) is not more statistically significant than each genotype alone, i.e. suggesting no additive effect. However when we compared the same groups with respect to having either one of these protective genotypes i.e. at least one copy of *CCL3* Hap-A1 or high *CCL3L* CN, we found that this combination is more significant (P=0.0008) than the effect of each genotype alone (Table 7). Furthermore when we performed a logistic regression analysis using stratified *CCL3L* CN and *CCL3* WT/Hap-A1 on the EU vs. INF-2 data set, the effect of *CCL3L* CN remained significant (P=0.01; OR=0.38) and although *CCL3* Hap-A1 lost significance upon adjustment for the effect of *CCL3L* CN, it maintained a strong trend (P=0.055; OR=0.42). These results suggest that both these genetic factors are protective in the context of infant IU infection, however *CCL3L* CN is the stronger of the two and likely to be responsible for why an additive effect is not evident.

Discussion

CCL3, through its CCR5-mediated signalling, has been shown *in vitro* to be inhibitory to HIV-1 entry. An *in vivo* role for CCL3 in HIV-1 disease has largely been shown through animal model studies involving both mice and non-human primates [24-27]. A role for CCL3 in HIV-1 infection in humans has been suggested primarily through genetic association studies, where low *CCL3L* gene copy number has been shown in numerous studies to be associated with increased adult and infant/child HIV-1 susceptibility [10-11, 22, 28-30]. A role for CCL3 in cell-mediated immune responses to HIV-1 has also been suggested in studies by Shalekoff *et al.* [31] and Dolan *et al.* [32].

Since CCL3 is encoded by two functional genes (*CCL3* and *CCL3L*), the contribution of *CCL3*, in combination with *CCL3L* copy number, on HIV-1 disease in a South African African population group was investigated. Although the CCL3 isoform is far less potent than the CCL3La isoform at R5 HIV-1 inhibition in *in vitro* studies, this is not the only effect/function that may be of significance in an *in vivo* scenario.Furthermore, the individual contribution of these two genes to overall CCL3 production is still largely unknown mainly due to the absence of a monoclonal antibody that can differentiate the two protein isoforms. A recent study that has tried to address this question by comparing mRNA transcripts of the two genes as well as looking at CCL3 production in a Caucasian cohort, found that *CCL3* expression predominates in both mRNA and protein, and consequently one of their main conclusions is that, in terms of biological significance, the variation in *CCL3* may be potentially more relevant than *CCL3L* copy number variation [33]. However, if CCL3La is far more potent than CCL3, then much less protein may be required *in vivo*, and small changes in the abundance of CCL3La may thus have larger consequence and hence may need to be regulated more finely. Nonetheless, the contribution of the CCL3 isoform to HIV-1 disease is largely understudied.

In a previous study in which we sequenced the *CCL3* genes of 43 African mother-infant pairs, two haplotypes comprised of the minor alleles of seven SNPs (designated Hap-A1 and Hap-A3) were identified at relatively high frequencies (>5%) within this population group.

The two haplotypes overlapped in two out of the seven positions and it is highly probable that these two polymorphic positions represent an ancestral haplotype (termed Hap-2SNP in this study) which accumulated additional polymorphisms in two separate pathways resulting in Hap-A1 and Hap-A3. These two polymorphic positions are also found on a prevalent five-SNP haplotype described in a South African Caucasian population [19], further supporting Hap-2SNP representing an ancestral core haplotype. Genotypic assays designed to detect Hap-A1 and Hap-A3 in this study thus also allowed the opportunity to study the effect of these two polymorphic positions independently. In addition, the availability of *CCL3L* copy number data for the same cohort [22] allowed us to investigate the combined effect of the two genes than encode the CCL3 chemokine.

Hap-A1, found in high frequency within the SAA population group (13 % allelic frequency), is comprised of the minor alleles of a core promoter SNP, three intronic SNPs, two exonic SNPs (exons 2 and 3) and one 3′UTR SNP. This haplotype is of particular interest as it harbours SNPs that have been reported in previous genetic association studies to impact on HIV-1 susceptibility as well as disease progression [16-18, 21]. An investigation of its role in HIV-1 disease using VL and CD4 count as markers of disease progression in the mothers, revealed a strong trend of association with lower CD4 count in the NT mothers. Hap-A1 however played no role in maternal transmission of HIV-1 as evidenced by similar representation of this haplotype among TR and NT mothers (<5% difference). Hap-A1 heterozygosity on the other hand was more highly represented among EU infants when compared to INF infants. Comparison of EU infants to infected infants in the absence of the IP-infected infant group revealed a significantly higher representation of Hap-A1 in the EU infants which was maintained through all corrections performed and in addition, was significantly higher in NT/EU mother-infant pairs compared to predominantly IU-infected infant-mother pairs (i.e. after exclusion of the IP-TR/IP pairs). These results strongly suggest a protective role for Hap-A1 in acquisition of HIV-1 infection by the infant through the *in utero* mode of transmission.

A role for Hap-A1 in HIV-1 protection is consistent with select studies that have looked at one or more SNPs that are part of this haplotype. Gonzalez *et al.* [16] were the first to find an association with two SNPs (C/T p+199 and C/T p+545; Fig. 1) in African Americans (AAs) with homozygosity for the minor allele at both positions (TT) being associated significantly with lower risk of HIV-1 acquisition, however no effect was seen on disease progression within the same group. Two other studies [17-18], both investigating SNPs associated with HIV-1 risk of infection in the same intravenous-drug-using (IDU) cohort (ALIVE), found opposite associations for different SNPs that are present in Hap-A1. Shrestha *et al.* [18] report an increased susceptibility amongst AA IDUs with homozygosity for the minor allele of C/T p+954 (which they state to be in LD with C/T p+545 and A/G p+1245), whereas Modi *et al.* [17], report three SNPs, two correlating to C/T p+199 and G/T p+1428 (which they report to be in LD with C/T p+545, C/T p+954 and A/G p+1245) to be significantly elevated among highly exposed, persistently HIV-1 uninfected AAs compared with HIV-1-infected AA seroconverters. Thus both studies report opposite associations for SNPs which appear to be in LD with each other and representative of the Hap-A1 haplotype described in our African cohort. In a recent study, which looked at Zambian HIV-1 serodiscordant couples, out of 63 SNPs studied in *CCL2*, *CCL3*, *CCL4* and *CCL5*, only two SNPs, both in *CCL3*, were highlighted as playing a role; a 3′-UTR SNP was associated with lower VL in seroconverters and again a Hap-A1 SNP, G/T p+1428 (rs34171309), was found to be associated with increased risk of HIV-1 acquisition in the serodiscordant couples [21]. This is the only study to our knowledge that has reported an association for a Hap-A1-associated *CCL3* SNP in another sub-Saharan African population group, again highlighting a role for Hap-A1 in HIV-1 susceptibility, albeit an opposite effect from what we saw in our MTCT cohort. Heterosexual, intravenous and *in utero* mother-infant HIV-1 infection, are three very

different modes of transmission, and it is very likely that the role of this chemokine may differ depending on the mode of transmission.

Hap-A3 and Hap-2SNP did not show any association with regard to infant HIV-1 protection. They were however more highly represented in mothers that transmitted HIV-1 to their infants through the IP route compared to the non-transmitting mothers. Hap-A3 heterozygosity was significantly higher in IP-TR mothers compared to NT mothers and this remained significant after all corrections for MVL and mNVP. Furthermore from the concordance/discordance comparisons involving this haplotype, it appears that Hap-A3 plays a role in increased IP MTCT only when present in the mother. Hap-A3 is comprised of the minor alleles of four intronic SNPs and three 3′UTR SNPs. The 3′UTR regions of metazoan genes are microRNA (miRNA) target-rich regions raising the question as to whether the three 3′UTR Hap-A3 SNPs form part of any miRNA target sequences that function to modulate *CCL3* RNA transcription. Although Hap-2SNP homozygosity was also higher in IP-TR mothers compared to NT mothers, the small sample numbers is this comparison makes this result tentative as indicated by the large confidence intervals.

Hap-2SNP homozygosity as well as at least one copy of Hap-2SNP showed strong trends of association with lower CD4 counts in the total HIV-1-infected mothers and this proved to be significant also when NT mothers were analysed separately. The strong trend of Hap-A1 also associating with lower CD4 count in the NT mothers is probably attributable to the two SNPs in Hap-2SNP that are part of Hap-A1. The two polymorphisms that constitute Hap-2SNP are not located in the exons but in intron 2 (p+1245 A/G) and in the 3′UTR region (p+1728 C/G) and thus whether either of these two allelic variations (or both) impact on *CCL3* gene expression will be worthy of future investigation. These results do however suggest a role for CCL3 in HIV-1 disease progression.

Thus, all three *CCL3* haplotypes investigated in this study appear to have some effect within the context of HIV-1 MTCT and disease progression in a sub-Saharan African population group, however, it is important to state that firstly the numbers in this cohort are not large and the absence of correction for multiple tests make it imperative that these associations are tested in larger cohorts with more conservative statistical analyses. Studies such as these however are important to lay the groundwork regarding potential genetic features that can be looked at in larger population groups and that can be targeted in terms of determining what genetic variation impacts on CCL3 expression and function.

An unexpected finding in this study was the highly significant association that was seen with the combination of *CCL3L* copy number and two of the *CCL3* haplotypes, namely Hap-A1 and Hap-2SNP. The presence of at least one copy of Hap-A1 and Hap-2SNP was very significantly associated with high *CCL3L* copy number in the total and well as EU infant groups and also within the INF infants with regard to the Hap-2SNP alone. Even though Hap-2SNP forms part of Hap-A1 and Hap-A3, *CCL3L* copy number and *CCL3* Hap-A3 were not found to be significantly associated, suggesting that it is Hap-A1, the more prevalent of the two seven-SNP haplotypes is the major contributor in the Hap-2SNP association seen. Similar comparisons (Mann-Whitney U test) carried out in the mothers failed to show similar associations.

What does this significant association of *CCL3* Hap-A1 and *CCL3L* copy number imply? A number of possibilities come to mind, firstly since Hap-A1 was higher in the EU infants compared to the INF infants (particularly in the IU-enriched infected group: INF-2), and EU infants have significantly higher *CCL3L* copy number compared to INF infants, then a combination of the two might be expected to show an additive contribution of the two ‘protective’ traits. Comparison of EU vs. INF-2 infants with respect to having Hap-A1 and a

high *CCL3L* CN failed to show an additive effect since the association was not more significant than either genotypic feature alone. However, having Hap-A1 or high *CCL3L* CN was very significantly (P=0.0008) associated with higher representation in the EUs compared to the INF-2 infants, and more significant than the individual associations. Logistic regression analysis of *CCL3* Hap-A1 and *CCL3L* copy number in the infant groups also showed that *CCL3L* CN remains significant when accounting for *CCL3* Hap-A1, and *CCL3* Hap-A1 maintained a strong trend when accounting for *CCL3L* CN. These results suggest that these two genetic factors are both exerting a protective effect, with high *CCL3L* CN being the stronger of the two, and have probably undergone separate evolutionary selection.

Another possibility is that there may be some LD between *CCL3* Hap-A1 with high *CCL3L* copy number, however if so, one would expect to see a similar relationship in the corresponding mothers which was not the case. The infant group however is predominantly composed of EU infants and the mothers are all HIV-1 infected and so are likely to have differing *CCL3* Hap-A1 and *CCL3L* copy number distributions. A number of studies have investigated LD between SNPs and regions of copy number variation (CNV) in an attempt to try and find reliable 'tag' SNPs for copy number polymorphisms (CNPs) [23, 34-37] and the general consensus seems to be that there is a high degree of LD between simple biallelic CNPs and the surrounding SNPs however multiallelic CNPs (like *CCL3L*) tend to show less LD with surrounding SNPs [23]. To see if this result was true for another, non-infected African population group, we used data available for the Yoruban (Nigeria) population from HapMap. Unexpectedly, this data also showed a significant association between a Hap-A1-associated SNP and *CCL3L* CN, although it was the reverse association to the one seen in our study. Although we cannot be certain that Hap-A1 is in fact present in this population group, it seems likely that it is since it appears to be present in African American population groups [17-18]. Thus, given these two contrasting results for two African population groups makes it unlikely that there is LD between *CCL3* Hap-A1 and *CCL3L* copy number and the strong association more than likely reflects enrichment of two mutually exclusive protective traits in a group of infants that were exposed to HIV-1 and remained uninfected.

In conclusion, this study has examined the role of three African *CCL3* haplotypes in HIV-1 infant susceptibility, maternal HIV-1 transmissibility and HIV-1 disease progression in the mothers and suggests a role for this gene, together with *CCL3L* in HIV-1 disease. We believe this study has highlighted in particular the complexity that exists in this genome region, and the difficulty in trying to understand the contribution to HIV-1 disease of individual, closely related genes, that encode for a protein that to date cannot be easily differentiated.

Materials and Methods

Cohort HIV-1 infection status

The infant's infection status was determined by a HIV-1 DNA PCR test (Roche Amplicor version 1.5). The viral load (VL) determinations were done on maternal delivery samples using the Roche Amplicor RNA Monitor assay version 1.5 (Roche Diagnostic Systems, Inc., Branchburg, NJ) and the CD4 T-cell counts were determined using the commercially available FACSCount System from Becton Dickinson (San Jose, CA).

In the case of TR mothers, 23% in our study reported any breastfeeding vs. 16% of NT mothers (not significant). The average duration of breastfeeding for those who initiated any breastfeeding was 14 days. By extrapolation the effect of breastfeeding would be negligible in this cohort. Moreover as breastfeeding and IP transmission are thought to occur across mucosal surfaces which is different to IU transmission, combining the majority IP with a

tiny proportion of breastfeeding would not confound the genetic outcomes with transmission likelihood

This study was approved by the University of the Witwatersrand Committee for Research on Human Subjects and the Institutional Review Board of Columbia University and signed informed consent was obtained from all mothers who participated in the study.

Linkage Disequilibrium between haplotype SNPs

Although the minor alleles involved in these two haplotypes (Hap-A1 and Hap-A3) were previously confirmed to be syntenic by cloning out of respective haplotypes and in addition the SNPs were always found associated with each other in all individuals that were genotyped [19], to further confirm that all the alleles were in complete linkage, pairwise linkage disequilibrium (LD) between every two minor allele combination in the haplotype (i.e. 21 pairwise combinations for a 7-SNP haplotype) was estimated using the method described by Lewontin [38]. The linkage disequilibrium coefficient D was calculated ($D_{ij} = HF_{ij} - p_i p_j$) and subsequently normalized (D') or standardized by the maximum value it can take (D_{max}) using the formula $D'_{ij} = D_{ij}/D_{max}$. HF_{ij} is the frequency of the haplotype carrying alleles i and j, p_i and p_j are the frequencies of alleles i and j respectively and D_{max} is either $\min[p_i p_j, (1 - p_i)(1 - p_j)]$ if $D_{ij}<0$ or $\min[(1- p_i) p_j, p_i(1 - p_j)]$ if $D_{ij}>0$. D' values are defined in the range [−1 to 1] with a value of '1' representing perfect disequilibrium. The statistical significance of the linkage disequilibrium between each of the allele pairs was evaluated by the approximate chi-square described by Liau *et al.* [39].

Development of a real-time PCR assay for haplotype genotyping

Since both haplotypes were comprised of alleles in complete linkage disequilibrium, a SNP unique to each haplotype (i.e. not shared between haplotypes) was selected for development of a real-time PCR assay for the detection of the haplotype thereby using the SNP position as a 'tag' or representation of the haplotype (see Figure 1 for tag SNPs). We developed SYBR Green real-time PCR assays to detect the SNPs using allele-specific PCR with two allele-specific primers designed with their 3′-end bases complementary to one of two SNP variants present [termed wild type i.e. WT and mutant (i.e. Hap-A1 or Hap-A3)] and one common primer (i.e. reverse or forward depending on orientation of allele-specific primers). Two PCR reactions were thus conducted for each sample, one with each of the WT and mutant primers. For the detection of Hap-A1, both allele-specific primers (forward primers) were designed with a lock nucleic acid (LNA) modified 3′-end base (5′-TAGCATGACAGCATCACTA**Y**-3′) and a standard reverse primer (5′-TGGAGACCTGCATGATTCT-3′). For Hap-A3, the best results were obtained by using a combination of a LNA modified WT (i.e. 'C' base) detection reverse primer, a standard mutant (i.e. 'T' base) detection reverse primer (5′-GAGCAGTTGAGGAAGGCA**R**-3′) and a common forward primer (5′-GAAGAGTCAAGGAGAAAGAAGG-3′). Each 10 μl reaction contained 1×Applied Biosystems SYBR Green PCR Master Mix (Applied Biosystems, CA, USA), 10 pmol of each forward and reverse primer, and ~30-80 ng DNA template. The PCR reactions were run in an Applied Biosystems 7500 Real-Time PCR system. Initial 10 min denaturation at 95 °C was followed by 40 cycles of 15 s denaturation at 95 °C, annealing at 60 °C for 20 s and extension at 72 °C for 1 minute. To analyze the PCR data, the cycle threshold, (C_T, the minimal fluorescence above which a sample is determined positive) was determined and used to calculate DC_T values (difference in C_T) by subtracting the C_T of the WT reaction from the mutant reaction. Heterozygous individuals had DC_T between 0 and 1.5, whereas homozygous WT and mutant individuals had DC_T values ≥7 and ≥-7, respectively. Plasmid DNA harboring cloned *CCL3* genes with the two respective haplotypes as well as genomic DNA from individuals of known genotype [19], were used to optimize the assays.

Hardy-Weinberg Equilibrium

All genotypic data was tested for deviation from Hardy-Weinberg equilibrium using the conventional Monte Carlo exact test of Guo and Thompson [40] implemented through the computer program TFPGA (Tools for Population Genetic Analyses version 1.3; 1997: author Mark. P. Miller).

Comparisons and Analyses

In addition to comparing NT mothers and EU infants to TR and INF infant groups and subgroups (IP and IU), respectively, a number or other comparisons were made:

i. Stratification of mother and infants according to single dose maternal NVP (mNVP) and no maternal NVP (noNVP) revealed that the mNVP subgroup had unexpectedly significantly more infected infants in total compared to the noNVP subgroup (31.5% vs. 18.3%; P=0.013), but as expected, significantly proportionally less IP infants in the mNVP subgroup compared to the noNVP subgroup (11/46, 24% vs. 18/25, 72%; P<0.001) since mNVP is known to affect IP transmission/infection. Out of the 23 infants of unknown mode of infection within these NVP stratified groups, 22/23 infants fell within the mNVP group compared to 1/23 that fell within the noNVP group (P<0.001), suggesting that the higher number of infections in the mNVP group is due to these infants of unknown infection route, and further suggesting that a large proportion of these infants are likely to have been infected *in utero*. Thus in trying to determine if one of the *CCL3* haplotypes was showing an association with *in utero* HIV-1 infection, we also analysed the data by comparing EU infants to infected infants after removal of the IP-infected infants from the group. This subgroup was designated as INF-2.

ii. *CCL3* genotypes found to be associated with either infant susceptibility of maternal transmissibility were further analysed by looking at the level of concordance or discordance between mother-infant pairs with respective genotypes.

Fisher's exact tests were used to calculate statistical significances and exact 95% confidence intervals (CI) of odds ratios (OR) of genotype frequency differences (*SISA*: Simple Interactive Statistical Analysis [41]). Two-sided tests were used and the level of statistical significance for analyses was set at P<0.05. Logistic regression was used to adjust for the effect of maternal viral load (MVL) and maternal NVP (mNVP) dose, as well as and to test for the interaction between *CCL3* Hap-A1 and *CCL3L* copy number. Mann-Whitney U-tests were carried out using the SPSS software (version 15.0; SPSS Inc.) and level of statistical significance for these analyses was also set at P<0.05. No adjustment was made for multiple comparisons in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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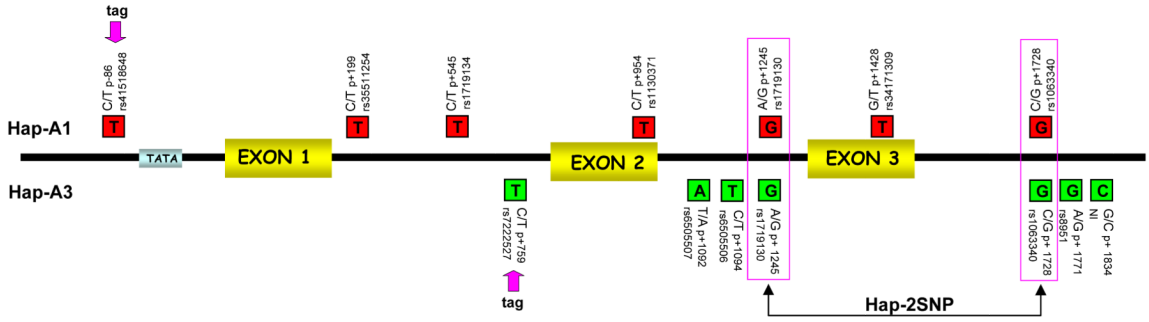

Figure 1.

Schematic representation of the *CCL3* gene and associated haplotypes. Nucleotides in red blocks indicate minor alleles making up Hap-A1 and information above each block shows the SNP, gene positions as well as dbSNP accession numbers. Similarly, green blocks indicate Hap-A3. The two SNP positions shared by Hap-A1 and Hap-A3, i.e. labelled Hap-2SNP, are bordered with pink rectangles. The two SNP positions used to design real-time SYBR green assays are indicated with arrows labelled ‘tag’.

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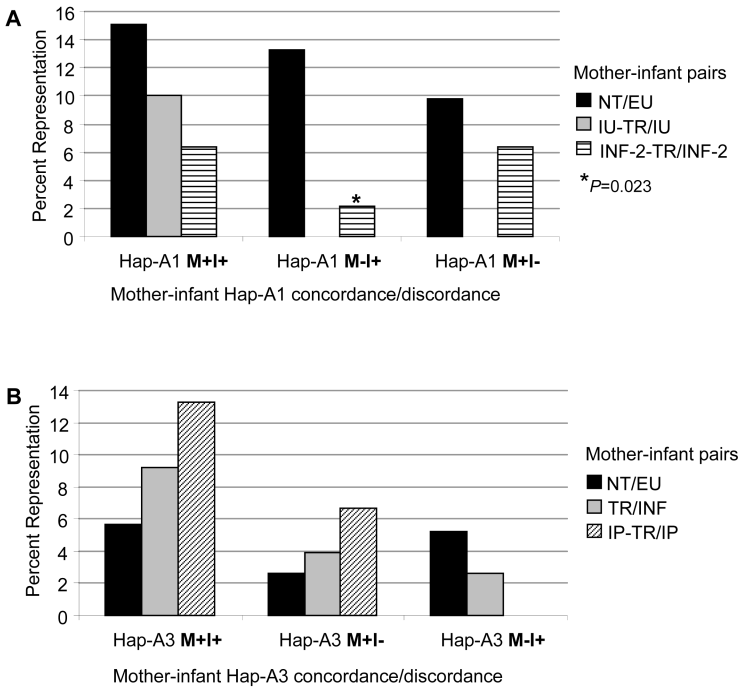

Figure 2.

Comparison of select mother-infant pair groups with respect to percent representation of concordance (mother positive and infant positive: M+I+), and discordance (mother positive and infant negative: M+I-; mother negative and infant positive: M-I+) for at least one copy (i.e. heterozygous or homozygous) of *CCL3* haplotypes Hap-A1 (A) and Hap-A3 (B). NT/EU refers to non-transmitting mothers and their corresponding exposed uninfected infants. IU-TR/IU refers to *in utero*-transmitting mothers and their corresponding IU-infected infants. INF-2-TR and INF-2 refers to transmitting mothers (after exclusion of intrapartum-transmitting mothers) and their corresponding infants. TR/INF refers to transmitting mothers and their corresponding infected infants. IP-TR/IP refers to intrapartum-transmitting mothers and their corresponding infants. * in (A) denotes significance in comparison between NT/EU mother-infant Hap-A1 pairs and INF-2-TR/INF-2 mother-infant Hap-A1 pairs (*P* value shown on figure).

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Table 1

Distribution of study participants according to cohorts previously described and maternal viral load and CD4 counts

	Cohorts[1]				Total	Viral Load (copies/ml) log_{10}	CD4 (cells/:l)
	1	2	3	4			
	N=				N=	Median (range); N=	Median (range)
Total group characteristics							
NT mothers	98	53	22	62	235	4.12 (1.70-5.88); 212	458 (16-1655); 219
TR mothers	22	26	8	23	79	4.77 (2.60-5.87); 70	366 (25-1026); 70
EU infants	99	54	22	63	238	-	-
INF infants	23	26	8	23	80	-	-
Maternal nevirapine (NVP) stratified group characteristics							
Maternal NVP							
NT mothers	9	35	0	56	100	4.08 (2.60-5.88); 100	391 (16-1146); 91
TR mothers	5	20	0	21	46	4.75 (2.60-5.87); 45	342 (25-1011); 40
EU infants	9	35	0	57	101	-	-
INF infants	5	20	0	21	46	-	-
No maternal NVP							
NT mothers	88	18	6	0	112	4.25 (1.70-5.88); 106	521 (39-1655); 107
TR mothers	17	6	1	0	24	4.81 (2.92-5.79); 23	414 (127-1026); 21
EU infants	89	19	6	0	114	-	-
INF infants	18	6	1	0	25	-	-

[1] Described in Kuhn *et al.* (2007)

-: Not applicable (EU infants) and not determined (INF infants)

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Table 2

Frequencies (%) of *CCL3* haplotypes in the SAA mother and infant groups

	Control Mothers N=112-114	HIV-1+ve Mothers N=299-308	Total Infants N=313-315
Allelic frequency			
Hap-A1	13.72	12.62	14.13
Hap-A3	4.82	4.87	5.43
Hap-2SNP	19.64	17.39	19.65
Genotypic frequency			
Hap-A1 / WT	25.66	23.99	24.44
Hap-A1 / Hap-A1	1.77	0.66	2.22
Hap-A3 / WT	9.65	9.09	10.86
Hap-A3 / Hap-A3	0.00	0.32	0.00
Hap-2SNP / WT	30.36	30.10	33.55
Hap-2SNP / Hap-2SNP	4.46	2.34	2.88

WT: wildtype

Table 3

Viral load and CD4 count comparisons (Mann-Whitney *U* test) between HIV-1 positive total and non-transmitting (NT) mothers harbouring (positive) and not harbouring (negative) select *CCL3* haplotypes

Viral Load (copies/ml) log $_{10}$								
	Total mothers				**NT mothers**			
Haplotype status	N=	Median	Range	*P*=	N=	Median	Range	*P*=
Hap-A1 [1]								
positive	68	4.40	2.60-5.88	0.192	51	4.26	2.60-5.88	0.192
negative	206	4.25	1.70-5.88		154	4.12	1.70-5.88	
Hap-A3 [1]								
positive	28	4.15	2.60-5.73	0.735	18	3.98	2.60-5.70	0.769
negative	248	4.24	1.70-5.88		190	4.13	1.70-5.88	
Hap-2SNP (Het)[2]								
positive	82	4.29	2.60-5.88	0.212	62	4.19	2.60-5.88	0.304
negative	179	4.26	1.70-5.88		136	4.12	1.70-5.88	
Hap-2SNP (Het+Hom)[3]								
positive	89	4.27	2.60-5.88	0.233	65	4.18	2.60-5.88	0.342
negative	179	4.26	1.70-5.88		136	4.12	1.70-5.88	
CD4 count (cells/μl)								
	Total mothers				**NT mothers**			
Haplotype status	N=	Median	Range	*P*=	N=	Median	Range	*P*=
Hap-A1 [1]								
positive	68	417	16-1146	0.215	52	403	16-1146	0.067
negative	213	458	39-1655		160	477	39-1655	
Hap-A3 [1]								
positive	25	378	39-891	0.090	17	443	39-891	0.306
negative	259	449	16-1555		198	458	16-1555	
Hap-2SNP (Het)[2]								
positive	83	414	16-1146	0.059	64	428	16-1146	0.034
negative	188	461	51-1555		142	485	51-1555	
Hap-2SNP (Het+Hom)[3]								
positive	88	414	16-1146	0.056	66	428	16-1146	0.032
negative	188	461	51-1555		142	485	51-1555	

[1] For Hap-A1 and Hap-A3, the presence of at least one copy was scored as positive i.e. both heterozygous and homozygous individuals.

[2] Het: only individuals heterozygous for Hap-2SNP were scored as positive.

[3] Het + Hom: both heterozygous and homozygous Hap-2SNP were scored as positive.

Grey shading: highlights strong trends ($0.05<P<0.1$) and significant ($P<0.05$) associations

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Table 4

Comparison of HIV-1 exposed uninfected infants and infected infant groups with respect to Hap-A1 heterozygosity

WT/Hap-A1	Unadjusted			MVL adjusted			mNVP adjusted			MVL and mNVP adjusted		
	OR	CI	*P*	OR	CI	*P*	OR	CI	*P*	OR	CI	*P*
Infant groups												
EU vs. INF	0.53	0.28-1.03	0.06	0.53	0.26-1.06	0.07	0.50	0.25-1.01	0.05	0.55	0.27-1.12	0.10
EU vs. IP	0.96	0.40-2.25	0.92	1.00	0.41-2.42	1.00	0.82	0.33-2.02	0.66	0.81	0.33-2.05	0.67
EU vs. IU	0.29	0.07-1.29	0.10	0.32	0.07-1.48	0.15	0.27	0.06-1.23	0.09	0.31	0.07-1.41	0.13
EU vs. INF-2	0.16	0.12-0.83	0.02	0.34	0.13-0.92	0.03	0.30	0.12-0.81	0.02	0.33	0.12-0.93	0.04

MVL: Maternal viral load; mNVP: Maternal Nevirapine; EU: Exposed uninfected infants; INF: Total infected infants; IP: Intrapartum infected infants; IU: Infants infected *in utero*; INF-2=Total infected infants (INF) minus the IP-infected infants, i.e. *in utero* infected infants + infected infants of unknown mode of infection; Grey shading: highlights strong trends ($0.05<P<0.1$) and significant ($P<0.05$) associations

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Table 5

Comparison of HIV-1-infected non-transmitting mothers and transmitting mother groups with respect to heterozygosity for Hap-A3 and homozygosity for Hap-2SNP

Mother groups	**Unadjusted**			**MVL adjusted**			**mNVP adjusted**			**MVL and mNVP adjusted**		
WT/Hap-A3	**OR**	**CI**	***P***	**OR**	**CI**	***P***	**OR**	**CI**	***P***	**OR**	**CI**	***P***
NT vs. TR	1.76	0.77-3.99	0.18	1.86	0.79-4.39	0.16	0.85	0.44-1.65	0.64	0.77	0.39-1.54	0.47
NT vs. IP-TR	2.94	1.07-8.13	0.04	3.42	1.18-9.90	0.02	3.17	1.12-8.91	0.03	3.50	1.20-10.26	0.02
NT vs. IU-TR	1.39	0.30-6.48	0.68	1.40	0.28-6.98	0.68	1.21	0.25-5.76	0.81	1.34	0.27-6.67	0.72
Hap-2SNP/Hap-2NP	OR	CI	*P*	OR	CI	*P*	OR	CI	*P*	OR	CI	*P*
NT vs. TR	3.95	0.86-18.24	0.08	4.61	0.95-22.41	0.06	3.32	0.71-15.55	0.13	3.76	0.77-18.27	0.10
NT vs. IP-TR	5.92	0.92-37.97	0.06	5.60	0.83-37.83	0.08	6.06	0.93-39.40	0.06	6.05	0.87-42.18	0.07
NT vs. TR-2	3.33	0.54-20.54	0.20	4.08	0.61-27.21	0.15	2.51	0.38-16.66	0.34	3.56	0.53-23.73	0.19

MVL: Maternal viral load; mNVP: Maternal Nevirapine; NT: Non-transmitting mothers; TR: Total transmitting mothers; IP-TR: Intrapartum transmitting mothers; IU-TR: *in utero* transmitting mothers; TR-2=Total transmitting mothers (TR) minus the IP-TR mothers, i.e. *in utero* transmitting + mothers of unknown mode of transmission; Grey shading: highlights strong trends ($0.05<P<0.1$) and significant ($P<0.05$) associations

Table 6

Mann-Whitney *U* test comparisons of *CCL3L* copy number (CN) as well as *CCL3L* CN and *CCL3* haplotypes between infant groups

Comparison Infant group	CCL3 Haplotype[1]	N=	*CCL3L* CN Median	*CCL3L* CN Range	*P*
CCL3L CN					
EU	NA	233	5	1-10	0.004
INF	NA	80	4	1-10	
CCL3 Hap-A1					
Total	+	81	5	2-8	0.001
	−	230	4	1-10	
EU	+	65	5	3-8	0.006
	−	166	4	1-10	
INF	+	16	4.5	2-8	0.141
	−	64	4	1-10	
CCL3 Hap-A3					
Total	+	33	5	3-10	0.665
	−	276	5	1-10	
EU	+	24	4.5	3-7	0.679
	−	206	5	1-10	
INF	+	9	5	3-10	0.154
	−	70	4	1-8	
CCL3 Hap-2SNP					
Total	+	111	5	2-10	<0.001
	−	198	4	1-10	
EU	+	86	5	3-8	0.011
	−	144	4	1-10	
INF	+	25	5	2-10	0.029
	−	54	4	1-7	

[1] presence (+) or absence (−) of at least one copy of the haplotype (i.e. heterozygotes and homozygotes scored equally)

NA: Not applicable for comparison on *CCL3L* CN (copy number) between two infant groups
Grey shading: highlights significant ($P<0.05$) associations

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Table 7

Comparison (Fisher's exact test) of exposed uninfected (EU) and Infected (INF-2) infant groups with respect to *CCL3* Hap-A1 and high *CCL3L* copy number

EU vs. INF -2	OR	CI	*P*
CCL3 Hap-A1[1]	0.36	0.15-0.90	0.028
High *CCL3L* CN[2]	0.37	0.19-0.72	0.002
CCL3 Hap-A1 **and** High *CCL3L* CN[3]	0.27	0.08-0.93	0.033
CCL3 Hap-A1 **or** High *CCL3L* CN[4]	0.34	0.18-0.65	0.0008

[1]Presence of at least one copy of *CCL3* Hap-A1 (i.e. homozygotes and heterozygotes)

[2]High *CCL3L* Copy number: ≥5

[3]Individuals with *CCL3* Hap A1 (at least one copy) and a high *CCL3L* CN

[4]Individuals with either *CCL3* Hap A1 (at least one copy) or a high *CCL3L* CN

EU: Exposed uninfected infants

INF-2: Total infected infants after exclusion of intrapartum-infected infants (i.e. *in utero* enriched infected group)