INTRODUCTION

Chemokines are a large superfamily of low-molecular-weight (~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 homoeostatic 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 Colobran et al.). Three CC chemokines, namely CCL3 (formerly macrophage inflammatory protein-1α), CCL4 (formerly macrophage inflammatory protein-1β) and CCL5 (formerly RANTES), were shown to demonstrate HIV-1 suppressor activity early on, which was later attributed to their binding to the CCR5 receptor, the co-receptor RS 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, as well as by downregulation of CCR5 surface expression, however whether these chemokines have 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 Colobran et al.). Of interest in this study is CCL3, which is encoded by two functional genes, namely CCL3L and CCL3La and in addition to these two genes, a third ‘pseudogene’ (CCL3Lb) has been described, which has been recently reported to have novel 3′ exons giving rise to alternatively transcribed mRNA species. CCL3 is present as two copies per diploid genome, whereas CCL3La and CCL3Lb (collectively termed CCL3L) have been shown to exist in variable copies per diploid genome. 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 RS HIV-1 infection compared with the CCL3 isoform. CCL3 copy number variation (CNV) and its role in HIV-1 infection have been the focus of a number of studies, however, not all studies agree to a role for CCL3 CNV. In a meta-analysis of nine published studies, recently conducted by Liu et al., lower CCL3 CN was associated with higher risk of HIV-1 infection when one takes into account the specific population as different populations differ with respect to CCL3 CN means. Although in vitro studies show CCL3 to be less potent than CCL3La at RS 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. 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 (SAAs) and Caucasians in an earlier study. There are certain advantages to studying the role of haplotypes rather than a one-SNP-at-a-time approach in candidate genes studies, first, 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. Variation in a population tends to be ‘structured into haplotypes that are likely to be transmitted as a unit’. Also, ethnically divergent population groups differ considerably regarding their haplotypic structures and select SNPs that can ‘tag’ more than one haplotype.

In a recent study conducted on serodiscordant Zambian couples, two CCL3 SNPs, namely rs50299410 (3′-untranslated region (UTR)) and rs34171309 (exon 3), were found to be significantly associated with lower viral load and increased risk of HIV-1 acquisition, respectively.20 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 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 CN was evaluated.

RESULTS

Cohort

A total of 314 HIV-1-infected SAA mothers and their infants (and 4 additional unmatched infants, that is, 318 infants), recruited as part of 4 mother-to-infant HIV-1 transmission cohorts in Johannes-
burg, South Africa, were used in this study. A detailed description of the four 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 (NT) 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 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 four cohorts. The median MVLs (copies per ml) and CD4 counts (cells per μl) for the broad groups are also shown in Table 1.

Antiretroviral drugs

As this study aimed to investigate the role of select genetic variations on mother-to-infant HIV-1 transmission, the role had by the various antiretroviral drugs administered in the four cohorts needs to be addressed. Study participants were classified according to whether the mothers were administered no NVP (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 mNVP (with MVLs and CD4 counts). Single-dose NVP administration to the mother during labour serves to only reduce IP HIV-1 infant infection as mNVP does not exert its protective effect by reducing the MVL (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 before 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.

Table 1. Distribution of study participants according to cohorts previously described and MVL and CD4 counts

|                    | Cohortsa | Total N | VL (copies per ml) log_{10} | CD4 (cells per μl) Median (range) |
|--------------------|----------|---------|-----------------------------|----------------------------------|
|                    | 1 2 3 4  | N       |                            |                                  |
| 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 | –                        | –                                |
| mNVP-stratified group characteristics |          |         |                            |                                  |
| mNVP               |          |         |                            |                                  |
| 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 | –                        | –                                |

Abbreviations: EU, exposed uninfected; INF, total infected infant; mNVP, maternal Nevirapine; MVL, maternal viral load; NT, non-transmitting; TR, transmitting. –, Not applicable (EU infants) and not determined (INF infants). aDescribed in Kuhn et al.22
Homozygosity for Hap-A3 was rarely detected. the least prevalent of the haplotypes with an allelic frequency of 5%. An average allelic frequency of Hap-A1 is also a prevalent haplotype in this population group with Hap-2SNP was found in an average allelic frequency of Hap-A1 and Hap-A3, that is, 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’.

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, that is, 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’.

Table 2. Frequencies (%) of CCL3 haplotypes in the SAA mother and infant groups

| Allelic frequency | Control mothers N = 112–114 | HIV-1 + ve mothers N = 299–308 | Total infants N = 313–315 |
|-------------------|-------------------------------|-------------------------------|--------------------------|
| 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 | Control mothers N = 112–114 | HIV-1 + ve mothers N = 299–308 | Total infants N = 313–315 |
|---------------------|-------------------------------|-------------------------------|--------------------------|
| 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                     |

Abbreviations: SAA, South African African; SNP, single nucleotide polymorphism; WT, wild type.

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 among SAA mother and infant pairs. Figure 1 shows the SNPs as well as the nucleotide (minor allele) composition of these two haplotypes 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 (that is, an individual having both haplotypes) as 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 (LD) between all minor alleles in the two haplotypes (Hap-A1 and Hap-A3) and 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 noticeable 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 ~18.9% (averaged across the three groups) and heterozygosity for Hap-2SNP was found in ~31% of the SAA population group. Hap-A1 is also a prevalent haplotype in this population group with an average allelic frequency of ~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
VL 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 with a cross-sectional study where the rate of MTCT would be at least twofold less. Thus, to determine the role of CCL3 haplotypes on markers of disease progression, mothers were also analysed in the absence of TR mothers (that is, NT mothers). Strong trends in the total mother group were seen with Hap-2SNP heterozygosity as well as Hap-A3 homozygosity combined with Hap-2SNP homozygosity (that is, at least one copy of Hap-2SNP) and lower CD4 count (P = 0.059 and 0.056, respectively), which both proved to be significant upon analysis of the NT mothers alone (P = 0.034 and 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
Percentage representation of the CCL3 haplotype genotypes (homozygotes and heterozygotes) in both the infant and mother groups is shown as bar graphs in supplementary Figure 1.
In EU infants, Hap-A1 heterozygosity was significantly lower compared with INF-infants (Table 4). Hap-A1 heterozygosity was also significantly lower in IU than IP-infants, with the magnitude of the reduction greater in EU infants than INF infants (Figures 2a and 3a). This result strongly suggests that Hap-A1 is having a role in protection from HIV-1 infection.

When Hap-A1 was analyzed as a whole group, the protective effect of WT/Hap-A1 occurred at the level of the TR group, that is, in the discordant combination where the protective effect of WT/Hap-A1 was abrogated. 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, Hap-A1 was not present in almost all discordant mother–infant pairs (Figures 2a and 3a).

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

Hap-A1 comparisons between all groups of TR 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 with INF infants (P = 0.06; odds ratio (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 with EUs (P’s = 0.09–0.15 across comparisons), the ORs were low and ranged from 0.29 to 0.32 (Table 4). Given that the IU group is fairly small (n = 20), and given that the infected infant group of unknown (that is, 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 with 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 having 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 with their M+I+ and M−I− counterparts (Figure 2a).

### Table 3. VL and CD4 count comparisons (Mann–Whitney U-test) between HIV-1 positive total and NT mothers harbouring (positive) and not harbouring (negative) select CCL3 haplotypes

| Haplotype status | Total mothers | NT mothers | P | N | Median | Range |
|------------------|---------------|------------|---|---|--------|-------|
| VL (copies per ml) log₁₀ | | | | 0.192 | 68 | 4.40 | 2.60–5.88 |
| Hap-A1 | | | | | 0.192 | 206 | 4.25 | 1.70–5.88 |
| Positive | | | | | | 28 | 4.15 | 2.60–5.73 |
| Negative | | | | | | 248 | 4.24 | 1.70–5.88 |
| Hap-A3 | | | | | | 82 | 4.29 | 2.60–5.88 |
| Positive | | | | | | 179 | 4.26 | 1.70–5.88 |
| Negative | | | | | | 89 | 4.27 | 2.60–5.88 |
| Hap-2SNP (Het) | | | | | | 25 | 4.14 | 16–1146 |
| Positive | | | | | | 259 | 4.49 | 16–1555 |
| Negative | | | | | | 25 | 4.14 | 16–1146 |
| CD4 count (cells per μl) | | | | | | | |
| Hap-A1 | | | | | | | |
| Positive | | | | | | | |
| Negative | | | | | | | |
| Hap-A3 | | | | | | | |
| Positive | | | | | | | |
| Negative | | | | | | | |
| Hap-2SNP (Het) | | | | | | | |
| Positive | | | | | | | |
| Negative | | | | | | | |
| Hap-2SNP (Het + Hom) | | | | | | | |
| Positive | | | | | | | |
| Negative | | | | | | | |

Abbreviations: NT, non-transmitting; SNP, single nucleotide polymorphism; VL, viral load. Grey shading: highlights strong trends (0.05 < P < 0.1) and significant (P < 0.05) associations. a For Hap-A1 and Hap-A3, the presence of at least one copy was scored as positive, that is, both heterozygous and homozygous individuals. b Het: only individuals heterozygous for Hap-2SNP were scored as positive. c Het + Hom: both heterozygous and homozygous Hap-2SNP were scored as positive.
A role for Hap-A3 in maternal HIV-1 transmission

Comparison of infected infant groups to EU infants with respect to Hap-A3 heterozygosity

Table 4. Comparison of HIV-1-EU infants and -infected infant groups with respect to Hap-A1 heterozygosity

| Infant groups | Unadjusted | MVL adjusted | mNVP adjusted | MVL and mNVP adjusted |
|---------------|------------|--------------|---------------|------------------------|
|               | OR CI P    | OR CI P      | OR CI P       | OR CI P                |
| 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 |

Abbreviations: CI, confidence interval; EU, exposed uninfected; INF, total infected infant; INF-2, INF minus the IP-infected infants, that is, in utero infected infants; IP, intrapartum; IU, infected in utero; mNVP, maternal Nevirapine; MVL, maternal viral load; OR, odds ratio; WT, wild type. Grey shading: highlights strong trends (0.05 < P < 0.1) and significant (P < 0.05) associations.

Role of CCL3 haplotypes and CCL3L gene CN

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

As we have previously seen within this cohort, EU infants have statistically higher CCL3L CN compared with INF infants (P = 0.004). When we compared the possession of at least one copy of Hap-A1 (that is, 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 with 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...
Table 5. Comparison of HIV-1-infected NT mothers and TR mothers groups with respect to heterozygosity for Hap-A3 and homozygosity for Hap-2SNP

| Mother groups | Unadjusted | MVL adjusted | mNVP adjusted | MVL and mNVP adjusted |
|---------------|------------|--------------|---------------|-----------------------|
|               | OR | CI | P | OR | CI | P | OR | CI | P | OR | CI | P |
| WT/Hap-A3     |    |    |   |    |    |   |    |    |   |    |    |   |
| 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 IJ-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-A2 |       |       |       |       |       |       |       |       |       |       |       |       |
| 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 IJ-TR   | 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 |

Abbreviations: CI, confidence interval; IP-TR, intrapartum-transmitting; IJ-TR, in utero-TR; mNVP, maternal Nevirapine; MVL, maternal viral load; NT, non-transmitting; OR, odds ratio; SNP, single nucleotide polymorphism; TR-2, total TR minus the IP-TR mothers, that is, in utero-TR + mothers of unknown mode of transmission; WT, wild type. Grey shading, highlights strong trends (0.05 < P < 0.1) and significant (P < 0.05) associations.

Table 6. Mann–Whitney U-test comparisons of CCL3 CN as well as CCL3L CN and CCL3 haplotypes between infant groups

| Comparison infant group | CCL3 haplotype | N | CCL3L CN median | CCL3L CN range | P |
|-------------------------|---------------|---|-----------------|----------------|---|
| EU                      | NA            | 233 | 5 | 1–10 | 0.004 |
| INF                     | NA            | 80  | 4 | 1–10 | 0.136 |
| CCL3 Hap-A1 Total       | +             | 81  | 5 | 2–8 | 0.001 |
|                         | –             | 230 | 4 | 1–10 | 0.005 |
| EU                      | +             | 65  | 5 | 3–8 | 0.006 |
|                         | –             | 166 | 4 | 1–10 | 0.154 |
| INF                     | +             | 16  | 4.5 | 2–8 | 0.141 |
|                         | –             | 64  | 4 | 1–10 | 0.101 |
| CCL3 Hap-A3 Total       | +             | 33  | 5 | 3–10 | 0.665 |
|                         | –             | 276 | 5 | 1–10 | 0.679 |
| EU                      | +             | 24  | 4.5 | 3–7 | 0.679 |
|                         | –             | 206 | 5 | 1–10 | 0.154 |
| INF                     | +             | 9   | 5 | 3–10 | 0.154 |
|                         | –             | 70  | 4 | 1–8 | 0.141 |
| CCL3 Hap-A25NP Total    | +             | 111 | 5 | 2–10 | 0.001 |
|                         | –             | 198 | 4 | 1–10 | 0.011 |
| EU                      | +             | 86  | 5 | 3–8 | 0.011 |
|                         | –             | 144 | 4 | 1–10 | 0.029 |
| INF                     | +             | 25  | 5 | 2–10 | 0.029 |
|                         | –             | 54  | 4 | 1–7 | 0.029 |

Abbreviations: CN, copy number; EU, exposed uninfected; INF, total infected infants; NA, not applicable. NA for comparison on CCL3 CN between two infant groups. Grey shading: highlights significant (P < 0.05) associations. *The presence (+) or absence (−) of at least one copy of the haplotype (that is, heterozygotes and homozygotes scored equally).

To determine whether 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 (http://www.hapmap.org) and CCL3L CN 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).

As high CCL3 CN is significantly associated with infant protection from HIV-1 infection, and as 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 CCL3 CN warrants further investigation. To determine whether there is an additive effect of CCL3 Hap-A1 and high CCL3 CN, we compared EU infants and INF-2 with both these genetic factors to those with neither. Table 7 shows that both these genotypes (P = 0.033) is not more statistically significant than each genotype alone, that is, suggesting no additive effect. However, when we compared the same groups with respect to having either one of these protective genotypes, that is, at least one copy of CCL3 Hap-A1 or high CCL3 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 vivo to be inhibitory to HIV-1 entry. An in vivo role for CCL3 in HIV-1 disease has largely been shown through animal model
Role of CCL3 haplotypes and CCL3L copy number in HIV-1 infection

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Table 7. Comparison (Fisher’s exact test) of EU and INF-2 infant groups with respect to CCL3 Hap-A1 and high CCL3L CN

| CCL3 Hap-A1 | OR | CI  | P    |
|-------------|----|-----|------|
| EU vs INF-2 | 0.36 | 0.15–0.90 | 0.028 |
| High CCL3L CN | 0.37 | 0.19–0.72 | 0.002 |
| CCL3 Hap-A1 and High CCL3L CN | 0.27 | 0.08–0.93 | 0.033 |
| CCL3 Hap-A1 or high CCL3L CN | 0.34 | 0.18–0.65 | 0.0008 |

Abbreviations: CI, confidence interval; CN, copy number; EU, exposed uninfected; INF-2, total infected infants after exclusion of intrapartum-INFs (that is, in utero-enriched infected group); OR, odds ratio. *The presence of at least one copy of CCL3 Hap-A1 (that is, homozygotes and heterozygotes); **High CCL3L CN ≥ 5. *Individuals with CCL3 Hap A1 (at least one copy) and a high CCL3L CN. **Individuals with either CCL3 Hap A1 (at least one copy) or a high CCL3L CN.

studies involving both mice and non-human primates. A role for CCL3 in HIV-1 infection in humans has been suggested primarily through genetic association studies, where low CCL3L gene CN has been shown in numerous studies to be associated with increased adult and infant/child HIV-1 susceptibility. A role for CCL3 in cell-mediated immune responses to HIV-1 has also been suggested in studies by Shalekoff et al and Dolan et al. As CCL3 is encoded by two functional genes (CCL3 and CCL3L), the contribution of CCL3, in combination with CCL3L CN, on HIV-1 disease in a SAA population group was investigated. Although the CCL3 isoform is far less potent than the CCL3L 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 CN. However, if CCL3L is far more potent than CCL3, then much less protein may be required in vivo, and small changes in the abundance of CCL3L 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 understood.

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, 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 CN data for the same cohort 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. 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, had 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 with 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 with predominantly IU-infected infant–mother pairs (that is, 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. were the first to find an association with two SNPs (C/T p = 0.045 and C/T p = 0.045; Figure 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, but no effect was seen on disease progression within the same group. Two other studies, however, both investigating SNPs associated with HIV-1 risk of infection in the same intravenous-drug-using cohort (ALIVE), found opposite associations for different SNPs that are present in Hap-A1. Shrestha et al. report an increased susceptibility among AA intravenous-drug-users with homozygosity for the minor allele of C/T p = 0.045 (which they state to be in LD with C/T p = 0.045 and A/G p = 0.045), whereas Modi et al. report three SNPs, two correlating to C/T p = 0.045 and G/T p = 0.045 (which they report to be in LD with C/T p = 0.045 and A/G p = 0.045) 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 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 with the NT mothers. Hap-A3 heterozygosity was significantly higher in IP-TR mothers compared with 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 has 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 target-rich regions raising the question as to...
whether the three 3′-UTR Hap-A3 SNPs form part of any microRNA target sequences that function to modulate CCL3 RNA transcription. Although Hap-2SNP homozygosity was also higher in IP-TR mothers compared with NT mothers, the small sample numbers in this comparison make 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 introns but in introns 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 first the numbers in this cohort are not large and the absence of correction for multiple tests makes 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 CN 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 CN in the total and as in the 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 CN and CCL3 haplotypes 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 CN imply? A number of possibilities come to mind, first, as Hap-A1 was higher in the EU infants compared with the INF infants (particularly in the IU-enriched-infected group: INF-2), and EU infants have significantly higher CCL3L CN compared with 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 as the association was not more significant than either genotypic feature alone. However, having Hap-A1 or high CCL3L CN was very significantly (P < 0.001) associated with high CCL3L CN in the EU infants compared with the INF-2 infants, and more significant than the individual associations. Logistic regression analysis of CCL3 Hap-A1 and CCL3L CN 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 CN, however, if so, one would expect to see a similar relationship in the corresponding mothers that 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 CN distributions. A number of studies have investigated LD between SNPs and regions of CNV in an attempt to try and find reliable ‘tag’ SNPs for CN polymorphisms and the general consensus seems to be that there is a high degree of LD between simple biallelic CN polymorphisms and the surrounding SNPs, however, multiallelic CN polymorphisms (like CCL3L) tend to show less LD with surrounding SNPs. Unexpectedly, this data also showed a significant association between a Hap-A1-associated SNP and CCL3L CN, although it was not as strong as the association seen. Similar comparisons (Mann–Whitney U-test) made this result tentative as indicated by the small sample numbers in this study. Although, we cannot be certain that Hap-A1 is in fact present in this population group, it seems likely that it is as it appears to be present in AA population groups. Thus, given these two contrasting results for two African population groups makes it unlikely that there is LD between CCL3 Hap-A1 and CCL3L CN, 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 differential.
dis-equilibrium. The statistical significance of the LD between each of the allele pairs was evaluated by the approximate $\chi^2$ described by Liu et al. 39

Development of a real-time PCR assay for haplotype genotyping As both haplotypes were comprised of alleles in complete LD, a SNP unique to each haplotype (that is, 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 WT and mutant (that is, Hap-A1 or Hap-A3)) and one common primer (that is, reverse 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 modified 3’-end base (5’-TAGCATAGACAGACATCATAY-3’) and a standard reverse primer (5’-TGGAGGACTGTGACTTGT-3’). For Hap-A3, the best results were obtained by using a combination of a lock nucleic acid modified WT (that is, ‘C’ base) detection reverse primer, a standard mutant (that is, ‘T’ base) detection reverse primer (5’-GAGGACTGGAAGCCGAR-3’) and a common forward primer (5’-GAAGACTCAAAGGAAGAGG-3’). Each 10 μl reaction contained 1× Applied Biosystems SYBR Green PCR Master Mix (Applied Biosystems, Foster City, 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 min. To analyse the PCR data, the cycle threshold, (Ct, the minimal fluorescence above which a sample is determined positive) was determined and used to calculate DCt values (difference in Ct) by subtracting the Ct of the WT reaction from the mutant reaction. Heterozygous individuals had DCt values between 0 and 1.5, whereas homozygous WT and mutant individuals had DCt values $\geq 7$ and $\geq 7$, respectively. Plasmid DNA harbouring cloned CCL3 genes with the two respective haplotypes as well as genomic DNA from individuals of known genotype were used to optimize the assays.

Hardy–Weinberg equilibrium All genotypic data were tested for deviation from Hardy–Weinberg equilibrium using the conventional Monte Carlo exact test of Guo and Thompson30 implemented through the freely available online computer program Tools For Population Genetic Analyses (TFPPGA: version 1.3; http://www.marksgeneticssoftware.net/_vti_bin/softhtml.exe/tfppga.html).

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 which are as follows (i) Stratification of mother and infants according to single dose mNVP, and no maternal NVP (noNVP) revealed that the mNVP subgroup had unexpectedly significantly more infected infants in total compared with the noNVP subgroup (31.5% vs 18.3%; $P=0.013$, but as expected, significantly proportionally less IP infants in the mNVP subgroup compared with the noNVP subgroup (11/46, 24% vs 18/25, 72%; $P=0.001$) as 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 with 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 IP. Thus, in trying to determine whether 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 haplotypes 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 of ORs of genotype frequency differences (Simple Interactive Statistical Analysis).12 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 MVL and mNVP dose, as well as and to test for the interaction between CCL3 Hap-A1 and CCL3L CNV. Mann–Whitney U-tests were carried out using the SPSS software version 15.0 for Windows (SPSS Inc., Chicago, IL, USA), and the level of statistical significance for these analyses was also set at $P<0.05$. No adjustment was made for multiple comparisons in this study.

CONFLICT OF INTEREST The authors declare no conflict of interest.

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