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CCL3L1 and HIV/AIDS susceptibility

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To the Editor:

We read with interest the recent article in Nature Medicine describing the influence of variation in *CCL3L1* copy number and *CCR5* genotype on immune recovery during highly active antiretroviral therapy (HAART) in HIV-1 infected individuals[1]. The chemotactic cytokine *CCL3L1* (encoding the MIP-1αP protein) is a potent ligand for the HIV-1 coreceptor CCR5, which is essential for viral entry into human host cells[2]. The recent study is part of a series that began in 2005 with a paper reporting effects of *CCL3L1* copy number variation on HIV-1 acquisiton, viral load, and disease progression[3], followed by several publications investigating clinically correlated phenotypes in a largely overlapping set of HIV^+ individuals[1,4,5].

While these studies appear to generate considerable independent support for a role of *CCL3L1* in viral control, many of the traits considered are at least partially correlated and the studies include largely overlapping samples and presumably *CCL3L1* assay data. For these reasons we sought to re-evaluate a core set of associations related to the effect of *CCL3L1* on viral control in a large group of HIV-infected patients with known date of seroconversion enrolled in one of the nine cohorts of the Euro-CHAVI Consortium[6]

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On behalf of the Center for HIV/AIDS Vaccine Immunology (CHAVI) and the Euro-CHAVI consortia (A.T., D.B.G.), the Multicenter AIDS Cohort Study (MACS) (R.D., J.J.M.), and the Tri-Service AIDS Clinical Consortium (TACC) of the Infectious Disease Clinical Research Program (A.W., N.L.M.).

(http://www.chavi.org, *n* = 1,042), in an African-American cohort from the Tri-Service AIDS Clinical Consortium (TACC) (http://www.idcrp.org/tacc2.html, *n* = 277) or in the Multicenter AIDS Cohort Study (MACS) (http://www.statepi.jhsph.edu/macs/macs.html) (*n* = 451 HIV^+, *n* = 195 high-risk seronegative). We assayed for *CCL3L1* copy number using the method described by Gonzalez *et al.* [3] (Supplementary Methods online). A total of 1,855 subjects were successfully genotyped. Distributions of *CCL3L1* copy numbers in patients of European or African ancestry were similar to those reported elsewhere, with a median copy number of 2 or 4 in individuals of primarily European (range 0-9) or African (range 1-11) descent, respectively (Fig. 1a,b)[1,3,4,7].

We then tested for association of *CCL3L1* copy number with HIV viral load at set point by linear regression after stratifying according to ethnicity and correcting for known covariates (gender, age at seroconversion, and ancestry as determined by a principal components method described previously8), and found no evidence of association (European:, $P = 0.14$; African: $P = 0.27$) (Fig. 1c,d). Dividing the sample into the previously described "high risk" (*CCL3L1*^{low}) and "low risk" (*CCL3L1*^{high}) genotype groups (where high risk *vs.* low risk is defined as having copy number below *vs.* equal to or above the population median, respectively)[3], we again found no evidence of association, either within each population (European: $P = 0.10$; African: $P = 0.41$) or in the combined sample ($P = 0.35$) (Table 1). Furthermore, a model including known functional polymorphisms in the *CCR5* receptor (*CCR5*Δ32, *CCR5**HHE) in a subset of n=820 individuals of European descent for which *CCR5* effects had been tested previously (Fellay et al., unpublished data), showed that while the *CCR5* polymorphisms were strongly associated with viral load (*CCR5*Δ32: $\beta = -0.29 +/- 0.08$ log RNA copies, $P = 0.001$; *CCR5**HHE: $\beta = 0.14 +/- 0.05$ log RNA copies, $P = 0.005$), there remained no appreciable effect of *CCL3L1* copy number (copy number: $P = 0.24$; genotype risk group: $P = 0.12$).

We next tested whether *CCL3L1* variation influences disease progression. We used both a quantitative measure of progression introduced by Fellay *et al.*[6] (consisting of measured or estimated time to $CD4^+$ cell count <350/mm3 or initiation of antiretroviral therapy; Supplemental Methods online) and a simple case/control comparison of progressors vs. non-progressors (defined as progression to $CD4^+$ cell count <350/mm3 or antiretroviral therapy within 10 years since seroconversion *vs.* no progression within 10 years). Finally, we tested for an effect of *CCL3L1*^{low} vs. *CCL3L1*^{high} group on these measures as well as progression to AIDS 1987, AIDS 1993, or AIDS-related death using a Cox proportional hazards model. Neither *CCL3L1* copy number nor *CCL3L1*^{low} vs. *CCL3L1*^{high} genotype group assignment was associated with disease progression under any of these models ($P > 0.1$ for all tests) (Table 1).

We then tested whether *CCL3L1* copy number was associated with risk of HIV infection by comparing the copy number distributions in HIV-infected patients (HIV^+) compared with individuals who were judged to be unusually exposed to HIV but remain uninfected (called high-risk seronegative, or HSRN). Using samples from the MACS cohort we compared 451 HIV^+ to 195 HRSN individuals. This comparison was well powered to detect effects of *CCL3L1* copy number on risk of infection through mucosal exposure (the principal model of transmission in this cohort). No association was found between infection status and either copy number ($P = 0.53$) or genotype risk group ($P = 0.18$) (Table 1). In the same sample, *CCR5*Δ32 homozygosity was strongly associated with reduced risk of infection (*CCR5*Δ32/*CCR5*Δ32 genotype frequency: 4.9% in exposed uninfected vs. 0% in infected individuals, $P = 3.5 \times 10^{-6}$). Of note is the enrichment of *CCR5*Δ32 homozygotes in the HRSN sample (4.9% vs. an estimated 1% in unselected individuals of European descent)[9], demonstrating that the effective exposure in the HRSN cohort was very high and therefore that this cohort should provide sufficient power to detect additional genetic risk factors of reasonable effect

size. Notably, we also found no effect of *CCL3L1* copy number on infection risk after stratifying according to *CCR5*Δ32 genotype.

We investigated whether *CCL3L1* copy number influences *CCL3L1* mRNA expression in $CD4^+$ T lymphocytes from 122 HIV^+ patients who had not yet initiated antiretroviral therapy, using the Illumina WG-6 v3 expression array (Supplementary Methods online), and found a strong and linear increase of *CCL3L1* mRNA levels with copy number ($r^2 = 0.23$, $P = 3.0 \times 10^{-8}$, Fig. 1e). In the same samples, however, *CCL3L1* mRNA expression itself shows no correlation with HIV set point ($r^2 = 0.003$, $P = 0.51$, Fig. 1f).

These observations raise the question of why earlier studies reported positive associations which cannot be replicated here. As a possible explanation we note that measurement of *CCL3L1* copy number variation appears highly susceptible to systematic biases related to the preparation and quality of DNA samples. We observed that batch differences in input DNA amounts between cases and controls can lead to biased copy number estimates by the real-time PCR method used here, and in fact found an apparently significant association in the direction opposite to that previously reported (with higher copy number among HIV^+ cases compared with controls) before diluting DNA samples into an appropriate range (Supplementary Methods online). Additionally, we compared the results of different assays (the real-time PCR based assay used here and in the previous reports, and a recently published method based on the paralogue ratio test (PRT)[10,11]) and found that although the results were generally very highly correlated, for one comparison the association statistics from the two assays diverged markedly. Specifically, in a comparison of a small number of HIV^+ and HIV^- samples from Malawi, copy number estimated by the PRT method showed a strong association with infection status, whereas the real-time PCR-based estimates showed no association; this discrepancy appears to be explained by systematic differences in DNA degradation between case and control samples, in which degradation or shearing of DNA leads to systematic overestimation of copy number by the PRT method specifically (Supplementary Methods online). Among HIV^+ individuals, we did not observe either assay method recording a signal of association for any HIV related quantitative trait; these tests are both more statistically powerful than the case/control comparisons and far less sensitive to any “batch effects” on the copy number estimation. Although both of the assays described here are liable to different types of systematic biases, we emphasize that differences between cohorts in the distribution of DNA concentrations are presumed to be far more likely, and perhaps expected, compared with differences in DNA storage or degradation, and thus the real-time PCR method will often be expected to produce a false positive association unless input DNA amounts are carefully considered. We therefore suggest that some of the previously reported associations may reflect differences in DNA quality or concentration which systematically increase or decrease the inferred number of copies of *CCL3L1* in cases *vs.* control samples.

In summary, we find the absence of any significant effect of *CCL3L1* copy number variation on HIV-1 infection, viral load, or disease progression. We do, however, show a highly significant association of copy number variation with *CCL3L1* mRNA levels, demonstrating that the assays are sufficiently accurate to detect the intermediate biological effects of copy number variation. While there is some evidence that reduced expression of the CCR5 receptor may aid in viral control and delay progression to AIDS, there is less reason to believe that CCR5 inhibition is protective from infection without complete CCR5 blockade. Others have demonstrated that *CCL3L1*/MIP-1αP is expressed at relatively low levels compared with other CCR5 ligands, with measured serum concentrations well below its estimated EC_{50} based on *ex vivo* assays[7,12]. Indeed, concentration increases to orders of magnitude higher than those reported in both healthy and HIV-infected individuals would appear to be required to approach half-maximal occupancy by *CCL3L1*, whereas a reduction

in receptor concentration would effectively multiply the affinity of all CCR5 ligands, including the much more abundant CCL5 (RANTES) and others in addition to MIP-1αP[7]. We should note that these arguments do not apply to the postulated effects of *CCL3L1* that operate independently of direct CCR5 blockade, for example through effects on the expression of innate defense pathways. Such an explanation, however, might also be expected to drive correlations between *CCL3L1* expression and viral control in infected individuals which were not observed (Fig. 1d). We point out that a gold standard for copy number determination in this region is yet lacking, and that the current techniques are likely to be influenced by other sources of error beyond the systematic ones described here. Despite progress in cataloging sequence and structural variation in the *CCL3L1* region[13], accurate assessment of the contribution of genetic variation in such a complex region will require the development of more accurate assay methods which provide information not only about gene copy number but also gene content. Finally, we emphasize that these results do not cast any doubt on efforts to develop CCR5 antagonists (*i.e.* MIP-1αP analogs) as therapeutics for HIV prevention and treatment, but merely argue that natural variation in *CCL3L1* gene dose does not appear to have any important effects on the control of HIV-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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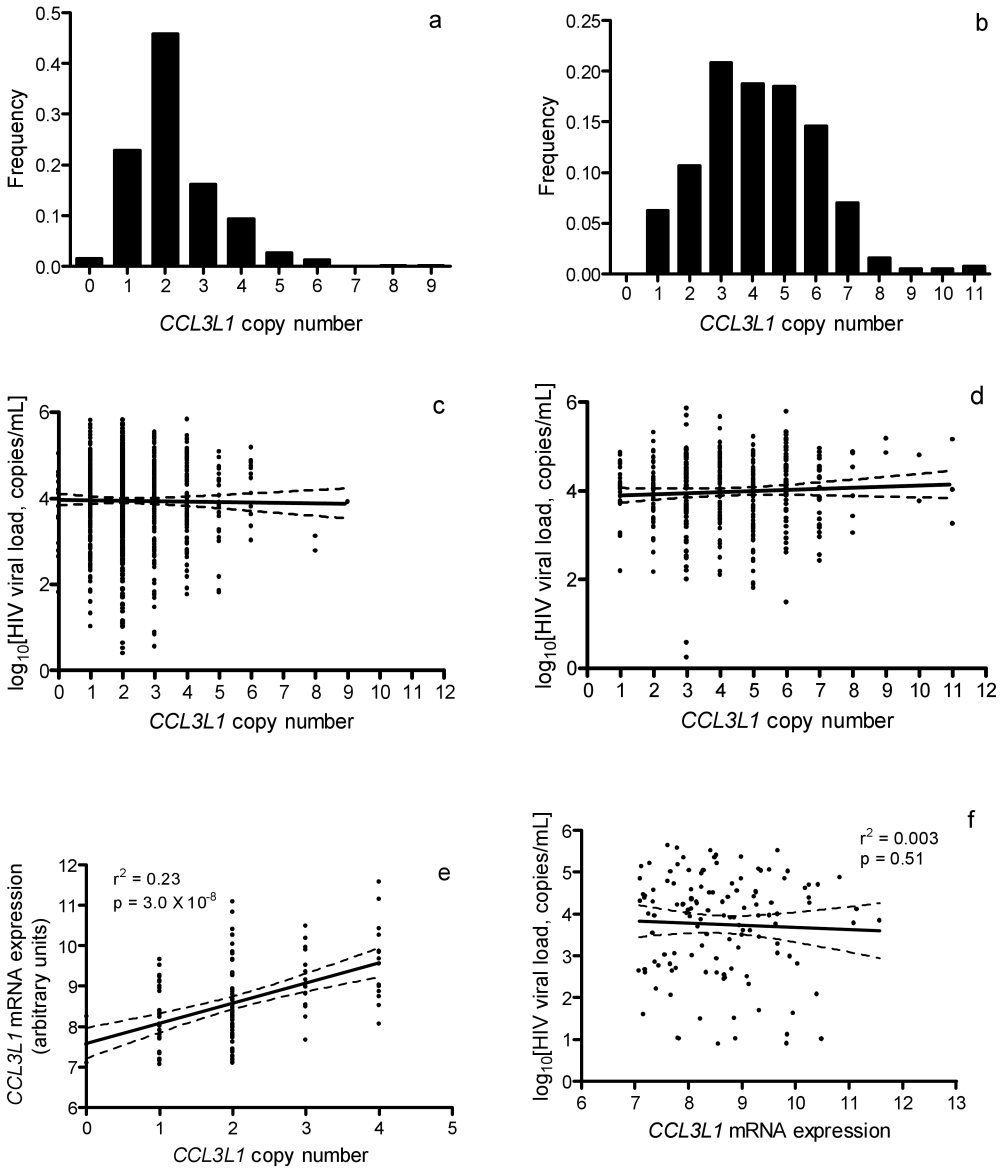

Figure 1.

(**a** and **b**) Distribution of *CCL3L1* copy number in HIV-infected individuals of recent (**a**) European or (**b**) African ancestry. Median *CCL3L1* copy number was 2 in patients of recent European descent and 4 in patients of recent African descent. (**c** and **d**) Relationship between HIV viral load at setpoint and *CCL3L1* copy number among patients of recent European (**c**) or African (**d**) ancestry. Linear regression of HIV viral load at setpoint on *CCL3L1* copy number showed no significant effect of *CCL3L1* dose (European: $r^2 = 0.0006$, $P = 0.14$; African: $r^2 = 0.0022$, $P = 0.27$). (**e** and **f**) Relationship among *CCL3L1* copy number, *CCL3L1* mRNA expression, and viral load at set point. Specific expression of *CCL3L1* mRNA in $CD4^+$ T cells was determined using the Illumina WG-6 v3 expression array. *CCL3L1* expression in $CD4^+$ T lymphocytes showed a strong correlation with copy number, but was not associated with viremia.

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Table 1

Results of statistical tests for association of *CCL3L1* copy number or genotype risk group (GRG) status with HIV infection risk or HIV-related outcomes.

Continuous Traits	**n**	**beta**	**r^2**	**p-value**
Set point vs. copy number (EUR)	1138	-.0342	0.0006	0.14
Set point vs. copy number (AFR)	366	.0244	0.0022	0.27
Set point vs. *CCL3L1*^{high}/*CCL3L1*^{low} (EUR)	1138	-.1022	0.0019	0.10
Set point vs. *CCL3L1*^{high}/*CCL3L1*^{low} (AFR)	366	.0741	0.0021	0.41
Set point vs. *CCL3L1*^{high}/*CCL3L1*^{low} (combined)	1504	-.0395	0.0006	0.42
Time to progression vs. copy number (EUR)	682	6.64	0.0002	0.90
Time to progression vs. *CCL3L1*^{high}/*CCL3L1*^{low} (EUR)	682	62.5	0.0001	0.65
Binary Traits	n (progressor)	n (nonprogressor)	Odds Ratio	p-value
Progressor/Nonprogressor vs. copy number (EUR)	611	71	1.14	0.33
Progressor/Nonprogressor vs. CCL3L1high/CCL3L1low (EUR)	611	71	1.25	0.46
Survival Analysis	n	Hazard ratio	95% CI	p-value
Time to progression vs. *CCL3L1*^{high}/*CCL3L1*^{low} (EUR)	744	0.95 +/- 0.09	0.78 – 1.15	0.59
Tests for Association with Infection Status	n (HIV^{+})	n (HRSN)	Odds Ratio (95% CI)	p-value
HIV infection status vs. copy number	451	195	0.86 – 1.08	0.53
HIV infection status vs. *CCL3L1*^{high}/*CCL3L1*^{low}	451	195	0.52 – 1.13	0.18

EUR, individuals of recent European ancestry; AFR, individuals of recent African ancestry (African Europeans and African Americans). Beta, odds ratios, hazard ratios and p-values are reported for the *CCL3L1* copy number or GRG term after adjusting for gender, age at seroconversion, and population structure. R^2 values represent the fraction of variation explained by the *CCL3L1* copy number or GRG term before correction for other covariates.