Copy number variation (CNV) is becoming increasingly important as a feature of human variation in disease susceptibility studies. However, the consequences of CNV are not so well understood. Here, we present data exploring the functional consequences of CNV of CCL3L1 in 55 independent UK samples with no known clinical phenotypes. The copy number of CCL3L1 was determined by the paralogue ratio test, and expression levels of macrophage inflammatory protein-1α (MIP-1α) and mRNA from stimulated monocytes were measured and analysed. The data show no statistically significant association of MIP-1α protein levels with copy number. However, there was a significant correlation between copy number and CCL3L1:CCL3 mRNA ratio. The data also provide evidence that expression of CCL3 predominates in both protein and mRNA, and therefore the observed variation of CCL3 is potentially more important biologically than that of CNV of CCL3L1.

Genes and Immunity (2012) 13, 374–379; doi:10.1038/gene.2012.5; published online 5 April 2012

Keywords: CCL3L1; MIP-1α; gene expression; copy number

INTRODUCTION

Copy number variation (CNV) is a frequent form of variation throughout the human genome. Over the last few years much research has been carried out to show the significant contribution of CNV to the variation observed between individuals and their influence upon disease susceptibility.

Although CNV has been shown to contribute to the heritable variation in human gene expression,1 the full functional impact of CNVs is not completely recognised. At a genome-wide level, CNV can directly alter the expression level of genes within the copy variable region.1–2 However, there is less progress in understanding the functional consequences of specific copy variable loci, particularly those CNVs that are multi-allelic.

The CCL3L1/CCL4L1 copy variable region is located on chromosome 17q12,3 and extends over 90 kb. Each repeat unit contains a single copy of CCL3L1 and CCL4L1 and is flanked by the gene TBC1D3. The repeat region is situated adjacent to two very closely related, and commonly copy invariant genes CCL3 and CCL4, and is thought to have evolved by duplication of the invariant CCL3 and CCL4 region and successive divergence.4 Consequently, members of each paralogous pair exhibit a high degree (96%) of nucleotide and protein similarity, with CCL3 and CCL3L1 being identical at 67/70 residues in the mature protein and 747/780 sites in the coding sequence, and CCL4 and CCL4L1 being identical at 68/69 residues in the mature protein and 644/667 sites in the coding sequence.

The genes CCL3 and CCL3L1 encode macrophage inflammatory protein (MIP)-1α, isoforms LD78α and LD78β, respectively. MIP-1α is a low molecular weight β-chemokine. The chemokine acts as a pro-inflammatory cytokine, inducing a wide variety of immune cells, particularly CD8+ T cells and immature dendritic cells, and being inhibited by IL-4, IL-10 and IL-13.5,6 It is secreted from most macrophages, predominantly macrophages, in response to stimulus, and functions by attracting lymphocytes and macrophages to sites of infection and inflammation, with the isoform LD78β being twofold more efficient at chemoattracting human monocytes and lymphocytes than the LD78α isoform.7 For this reason CNV of CCL3L1 has the potential to influence both immunological disorders and auto-immunity.

Furthermore MIP-1α is a natural ligand for CCR5, the co-receptor used by HIV-1 virus for cell entry.8–9 The isoform LD78β has been shown to be the most potent agonist for CCR5,7 with a superior antiviral activity10 and a 10-fold higher EC50 in inhibiting viral replication than LD78α.7 CNV of CCL3L1 has thus been suggested to limit HIV-1 entry into cells,11 and is implicated in HIV-1 progression,10,12–18 although this is disputed.19–22

The aim of the study was to explore the functional consequences of the multi-allelic copy variable gene CCL3L1. Expression of both CCL3 and CCL3L1 mRNA was measured and compared and also the variation in expression level in relation to the diploid copy number was assessed. Additionally, the variation of MIP-1α protein levels relative to copy number was investigated.

RESULTS

Variation in copy number

Copy number measurement of CCL3L1 by parologue ratio test (PRT) was performed on all 55 independent UK samples. The three independent PRT systems assigned concordant copy numbers for 51/55 samples (to within 0.5 of the integer value for 75% of samples and to within 0.75 for 93%). The four samples that showed some discordance were typed with the two microsatellite assays, which allowed confident integer copy number calling.23 The calibrated unrounded copy number distribution is shown in Figure 1 and shows that the samples all cluster around the predicted integer with discernable gaps. Variation in CCL3L1 copy number between 0–3 was observed (see Table 1), with a copy number of 2 being the most common. As the three different PRT measurement systems show a high level of agreement in all samples typed this provides no evidence for differences in the copy number neither between CCL3L1 and CCL4L1, nor of CNV in the reference genes CCL3 and CCL4.

Centre for Genetics and Genomics and School of Biology, University of Nottingham, Nottingham, UK. Correspondence: Dr D Carpenter, Centre for Genetics and Genomics and School of Biology, University of Nottingham, Medical School, Queens Medical Centre, Nottingham, NG7 2UH, UK. E-mail: danielle.carpenter@nottingham.ac.uk
Received 16 September 2011; revised 5 March 2012; accepted 5 March 2012; published online 5 April 2012
Correlation of copy number with protein production

In order to deduce a suitable time point to measure protein expression, monocytes were isolated from an initial cohort of seven individuals, and supernatants collected at 2, 4, 8, 24 and 48 h post LPS stimulation. Protein was measured by ELISA, with a lower detection limit of 0.1 ng ml\(^{-1}\), and despite a single outlier sample, the time course shows an initial increase in protein production over the first 4 h, which levelled off by 8 h and then declined gradually for all copy numbers (see Supplementary Figure 1). There was no detectable protein expression from the unstimulated cells. This is as expected as MIP-1\(\alpha\) is not constitutively expressed but is induced upon stimulation. For measurement of protein levels all cell supernatants were subsequently harvested 4 h post stimulation and MIP-1\(\alpha\) production from macrophages was measured in triplicate for all samples.

There is a high level of sequence similarity between the two isoforms of MIP-1\(\alpha\) that are encoded by CCL3 and CCL3L1, and we are not aware of any commercially available antibodies that can reliably distinguish between them. As our dataset contained one individual who had a copy number of zero (zero copy individuals make up \(\sim 2\%\) of the UK population) we were able to measure the specificity of a number of commercial antibodies (both monoclonal and polyclonal) (AbCam, Cambridge, UK; R&D systems, Abingdon, UK; Lifespan Biosciences, Seattle, WA, USA) for the LD78\(\alpha\) isoform. There were no antibodies tested that were completely specific to LD78\(\beta\) and thus all available antibodies measured both MIP-1\(\alpha\) isoforms. Therefore, the ELISA results presented in Figure 2 are for total MIP-1\(\alpha\) expression, as with other previously published reports that also only measure total MIP-1\(\alpha\) expression.

Figure 2. MIP-1\(\alpha\) protein expression levels (ng ml\(^{-1}\)) classified by integer CCL3L1 copy number.

One major advantage of investigating mRNA transcripts is that it is possible to differentiate between the transcripts of CCL3 and CCL3L1 by use of sequence-specific primers. Again a time trial was performed on an initial seven samples with total RNA being isolated from the lysed macrophages 2, 4, 8, 24 and 48 h post stimulation with LPS, and subsequent measurement of CCL3L1 by real time PCR. There was an initial high expression of CCL3L1-specific mRNA at 2 h post stimulation that declined sharply over time (Supplementary Figure 1b). Consequently, all RNA was collected 2 h post stimulation. Without stimulation there was no detectable mRNA production for either CCL3 or CCL3L1. It is also however, suggestive evidence for a weak correlation of MIP-1\(\alpha\) expression and copy number ($r = 0.2707; P = 0.0309$).

Furthermore, considering that the zero copy samples correspond to CCL3-encoded protein expression only, at least for this individual it shows the background level of expression of the copy constant CCL3 protein, LD78\(\alpha\), against which other samples can be compared. Therefore, in comparing the MIP-1\(\alpha\) expression level for all copy number samples to that of the zero copy samples, the data actually suggest that the majority of the expressed MIP-1\(\alpha\) protein is composed of the LD78\(\alpha\) isoform. Additionally, within all three classes of copy number, at least some individuals have a level of expression for total MIP-1\(\alpha\) less than that observed with the zero copy samples, suggesting that the expression of LD78\(\alpha\) is also variable.

The sandwich ELISA was used to measure MIP-1\(\alpha\) expression in sera at the time of extraction. There was no protein detected in any of the samples, with a lower detection limit of 0.1 ng ml\(^{-1}\).

To look at the variation in protein expression within each individual, 10 volunteers from the 55 agreed to be re-tested. These 10 repeat samples comprise four one-copy individuals, three two-copy individuals and three three-copy individuals. A comparison of the original MIP-1\(\alpha\) expression and the expression in the repeat samples is shown in Supplementary Figure 2a. The data show a single individual, sample F, to have a very high MIP-1\(\alpha\) expression in the original experiment. However, for this individual there was also evidence for MIP-1\(\alpha\) expression in the unstimulated cells, potentially because of clinical symptoms, although not symptomatic at the time of blood extraction. For this individual only the repeat sample data has been used in the further analysis. It should be noted that sample F was the only one that had any protein expression in the unstimulated cells. Analysis of repeat measures found that neither of the within subjects factors (time and measurement) were significant for measurement of MIP-1\(\alpha\) expression.

Correlation of copy number with mRNA transcript

One major advantage of investigating mRNA transcripts is that it is possible to differentiate between the transcripts of CCL3 and CCL3L1 by use of sequence-specific primers. Again a time trial was performed on an initial seven samples with total RNA being isolated from the lysed macrophages 2, 4, 8, 24 and 48 h post stimulation with LPS, and subsequent measurement of CCL3L1 by real time PCR. There was an initial high expression of CCL3L1-specific mRNA at 2 h post stimulation that declined sharply over time (Supplementary Figure 1b). Consequently, all RNA was collected 2 h post stimulation. Without stimulation there was no detectable mRNA production for either CCL3 or CCL3L1. It is also
is 0.18, and for the three-copy samples is 0.52, altogether give a mean ratio of 0.34. The mean ratio of the one-copy samples suggests that over time the expression of CCL3 would be expected but the data actually predicted to approximate one for the two-copy samples as equal data would suggest that there is a greater proportion of CCL3 clusters for each copy number. Additionally, the evidence for a drop in the ratio of CCL3 mRNA expression shows a statistically significant correlation (\(r = 0.052\)). For each copy number the clusters of data show a spread of expression, levels of between zero- and fivefold greater expression relative to GAPDH, suggesting that the inherent expression of CCL3 is variable. The data for the CCL3L1-specific transcripts show a statistically significant correlation of expression with copy number (\(r = 0.633; P < 0.0001\)), suggesting CCL3L1 mRNA transcript level increases with higher copy numbers. This also suggests that the potential for post-transcriptional regulation of MIP-1\(\alpha\) as the correlation with copy number is not preserved in the protein.

The ratio of CCL3L1:CCL3 mRNA transcript was measured using PRT for all samples at 2 h post stimulation (see Figure 4). The data show a statistically significant correlation (\(r = 0.877\), \(P = 0.0001\)) between copy number and CCL3L1:CCL3 mRNA ratio, with clear clusters for each copy number. Additionally, the CCL3L1:CCL3 ratio data would suggest that there is a greater proportion of CCL3 mRNA transcript than CCL3L1; as the CCL3L1:CCL3 ratio would be predicted to approximate one for the two-copy samples as equal quantities of transcripts would be expected but the data actually give a mean ratio of 0.34. The mean ratio of the one-copy samples is 0.18, and for the three-copy samples is 0.52, altogether suggesting that the there are \(\sim 2 \sim 3\) times more CCL3 transcripts than CCL3L1, for all copy numbers of CCL3L1 at 2 h post stimulation.

Furthermore, the ratio of CCL3L1:CCL3 was measured by PRT for the seven time trial samples (see Figure 5). The time trial data give evidence for a drop in the ratio of CCL3L1:CCL3 for these samples over the first 12 h, followed by a relative plateauing of the ratio. Such a drop in the ratio would suggest that the decay rate of the transcripts of CCL3 and CCL3L1 is not equal. The ratio measurements would suggest that it is the decay of CCL3L1 that is faster than that for CCL3, suggesting that over time the CCL3 transcripts can predominate. If we assume that the relative mRNA levels are preserved at the protein level, then as the CCL3 mRNA transcripts

Figure 3. mRNA expression of (a) CCL3 and (b) CCL3L1 by integer CCL3L1 copy number. The mRNA expression levels of both CCL3 and CCL3L1 were calculated using the \(\Delta \Delta Ct\) method and mRNA expression of GAPDH as the endogenous control.

Figure 4. Comparison of the ratio of CCL3L1:CCL3 mRNA expression measured by PRT between integer CCL3L1 copy numbers. The bold black bar represents the mean ratio for each copy number.

Figure 5. Time trial graph of CCL3L1:CCL3 mRNA expression, measured by PRT, from isolated macrophages post LPS stimulation for seven initial samples. These samples comprised of two one-copy samples (\(\bullet\)), two two-copy samples (\(\Delta\)) and three three-copy samples (\(\bullet\)). There was no expression detected from unstimulated cells.

The protein. Confirmation by a specific antibody is required, however.

We also observed two samples with elevated mRNA expression for both CCL3 and CCL3L1, one for a one-copy sample and the other for a three-copy sample. However, both had a CCL3:CCL3L1

Genes and Immunity (2012) 374 – 379 © 2012 Macmillan Publishers Limited
ratio that was within range for the copy number. This could suggest that a potentially shared enhancer or promoter element causes raised expression of both CCL3 and CCL3L1. Further sequence work may identify some variant at the nucleotide level that could influence expression of both mRNAs in these samples.

To look at the consistency of mRNA expression within each individual, the 10 repeat samples were evaluated. A comparison with the original CCL3L1 mRNA expression and the expression in the repeat samples is shown in Supplementary Figure 2b. The repeat measure analysis found that neither of the within subjects factors (time and measurement) were significant for the measurement of CCL3L1 mRNA expression, though there is some disparity for the three three-copy samples (i1, i2 and j). However, comparing this data with the ratio data for the repeat samples (Supplementary Figure 2c), it can be seen that the ratio data do not differ significantly within individuals, and thus the disparity observed in the CCL3L1 mRNA expression for the higher copies is mirrored in the CCL3 expression also.

DISCUSSION

In this study we sought to explore the functional consequences of CNV at CCL3L1 through analysis of variation in expression of CCL3L1 mRNA and protein. We found no significant association between copy number and MIP-1α protein expression. Although the data presented here are the characteristic of a European population, with a narrow range and mean copy number of 2, a population with a higher mean copy number, for example Africans, may be useful to explore protein expression of higher copy numbers. Furthermore, as our study suggests that the majority of MIP-1α products are of the isoform LD78α, then it is possible that any significant association between copy number and LD78β expression is being masked by the higher levels of LD78α. However, until a specific antibody can be developed, this cannot be proven.

The mature proteins, LB78α and LD78β, differ at only three amino acids situated at position 3, 39 and 47. The dispersed distribution of these amino-acid substitutions throughout the mature protein does potentially make the generation of a specific antibody to one or other of the isoforms more difficult, though these sites are not evolutionary conserved. Nevertheless, the substitution of a serine by proline at position three could alter the secondary Figure 2c), it can be seen that the ratio data do not differ significantly within individuals, and thus the disparity observed in the CCL3L1 mRNA expression for the higher copies is mirrored in the CCL3 expression also.

The predominance of LD78α is interesting as it suggests that the variation observed with CCL3 is potentially more consequential, biologically, than the CCL3L1 CNV. Indeed, functionally, although LD78β is a more potent agonist than LD78α, LD78β nevertheless has agonist activity, and therefore, even if less potent it can outnumber LD78β three to one. Although a prior study has established that LD78β is twofold more chemoattractive than LD78α, if the expression of LD78β in a two-copy individual is only 15% of the total MIP-1α expression, then the LD78α isoform will still account for most of the biological activity. It is thus possible that the variation in expression observed for CCL3 is as likely to influence susceptibility to infectious disease and HIV progression as the CNV of LD78β.

Our data do not support a previous study, which found a significant association of copy number with MIP-1α expression. However, unfortunately it is not possible to directly compare the data as MIP-1α was collected 4 h post stimulation in this study, whereas it was collected 48 h post stimulation previously. Furthermore, while their ELISA system was the same as ours, the prior study did not contain any zero copy samples with which to compare their background levels of CCL3-encoded protein. While the previous study did not specifically look at the separate mRNA transcripts, the ratio of CCL3L1:CCL3 was assessed and found to be significantly correlated with copy number, which is in agreement with our observations, though we cannot assess whether, like our data, there is a predominance of CCL3 transcript or not.

There have been a number of studies that have found associations between CNV of CCL3L1 and HIV-1 progression. The biological rationale is that there is strong competition between the MIP-1α isoform LD78β and HIV-1 for the receptor CCR5; thus, the more LD78β there is, the greater the competition. However, there are other studies that dispute this reported association, and in particular a series of highly powered studies published recently failed to replicate the association, while another study demonstrated the problems with the measurement of CCL3L1 copy number and how this may confound associations with copy number. As our data suggest that the product of CCL3L1 is appreciably less abundant than that of CCL3, this would call into question the biological premise behind the associations observed between HIV-1 progression and increased CCL3L1 copy number.

To conclude, in exploring the functional consequences of CNV of CCL3L1 we find no statistically significant association of MIP-1α protein levels with copy number. In contrast, we find evidence...
that it is CCL3 that predominates at both the protein and mRNA level and therefore variation of CCL3 expression has potentially more impact biologically than the CNV of CCL3L1.

MATERIALS AND METHODS

Study population

This study utilizes 55 independent volunteers from the University of Nottingham student and staff body, with 10 randomly selected repeat volunteers (see Supplementary Figure 2), and were taken with full consent from individuals and under local ethical approval. All samples were of UK origin with no known clinical phenotype.

Sample preparation

In all, 20 ml of whole blood was taken from each volunteer at ~0930 hours (+/−10 min), from which sera, genomic DNA and monocytes (a primary source of MIP-1α) were isolated. Two millilitres of the whole blood were removed and spun down at 13 000 × g for 2 min to generate 1 ml of serum per sample, which was stored at −80°C.

Peripheral blood mononuclear cells were isolated from the remaining 18 ml of whole blood via density gradient centrifugation over Ficoll-paque (Sigma, Gillingham, UK). Monocytes were isolated using positive selection by means of CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and re-suspended in cell culture media, supplemented with foetal bovine serum, antibiotics and glutamine. Monocytes were transferred into 96-well plates to a concentration of 0.5 × 10⁶ cells per well and left overnight to mature to macrophages. As only macrophages adhere to the base of the cell culture plate confirmation that all the cells isolated were macrophages was possible. Cells were then either left untreated or stimulated with 10 ng ml⁻¹ of LPS (Sigma); varying LPS concentrations (1 μg ml⁻¹ − 10 ng ml⁻¹) were tested and 10 ng ml⁻¹ was found to be the most appropriate (data not shown).

Genomic DNA was prepared from the remaining mononuclear cells using reagents from Qiagen (Crawley, UK).

Measurement of gene copy number by the PRT

The copy number of CCL3L1 was measured from genomic DNA using the PRT as previously described. 23,26,27 Briefly, the PRT method is essentially a PCR-based assay using a single pair of primers to simultaneously amplify two specific products in a single reaction, one from a single-copy reference locus and the other from a copy variable test locus of interest. The copy number of the test locus is then estimated from the ratio of test to reference PCR products. In this study a single tube triplex assay was performed using three independent PRT assays, which span the copy variable region (CCL3), CCL3L1 (assay termed CCL4L1) and a long repeat sequence (assay termed LTR61A) to give three measures of copy number, which are then averaged into a single unrounded copy number value (see Figure 7). Fragment analysis of the test and reference loci was carried out by electrophoresis on an ABI3100 36 cm capillary using POP-4 polymer with an injection time of 30 s. Products from the single PCR reactions were mixed with 10 μl HiDi formamide with ROX-500 marker (Applied Biosystems, Warrington, UK). GeneMapper software (Applied Biosystems) was used to extract the peak areas for the three PRT systems and calculate the ratio of test to reference. Copy number values were calculated by calibrating the ratios from each experiment with four ECACC HRC-1 samples (http://www.hpacultures.org.uk/) of known copy number (C0075 with a copy number (CN) = 1; C0150 with CN = 2; C0007 with CN = 3; and C0077 with CN = 4), which were included in every experiment in duplicate. All experimental samples were repeated in a separate PCR to confirm copy number value.

For further confirmation of gene copy number two microsatellite PCRs were performed for each sample, as described previously. 23

Measurement of MIP-1α protein

The extent of MIP-1α secreted by monocytes post LPS (S. minnesota) stimulation and the amount of MIP-1α present in the serum at time of extraction was measured using a sandwich MIP-1α ELISA system (R&D systems), according to manufacturer’s instructions. Assays were performed in duplicate on serial dilutions of recombinant MIP-1α (LD78) protein of known concentration (R&D systems) to generate a standard curve. Assays were performed in duplicate for each individual sample and measured in triplicate.

Measurement of CCL3 and CCL3L1 mRNA transcripts

Total mRNA was prepared from lysed LPS-stimulated macrophages using a mini-prep (Qiagen) kit and reverse-transcription performed. RNA preparation was performed in duplicate for each individual sample.

Real time PCR was performed using SYBR green chemistry on an ABI7500 machine (Applied Biosystems) and the well established relative quantification ∆∆Ct method. Primers (Invitrogen, Paisley, UK) were designed using Primer Express (Applied Biosystems). CCL3-specific mRNA transcripts were amplified using a final concentration of 0.1 μM CCL3QF (5'-TGG CTC TCT GCA ACC AGT TC-3') and CCL3QR (5'-CAC TGG CTG CTC GTC TCA AA-3'), CCL3L1-specific mRNA transcripts were amplified using a final concentration of 0.1 μM CCL3L1QF (5'-GCT CTC TGC AAC CAG GTC-3') and CCL3L1QR (5'-CAC TGG CTG CTC GTC TCA AA-3'), and the constitutively expressed endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified using a final concentration of 0.1 μM GAPDHF (5'-ATC ATC AGC AAC GCC TCC TG-3') and GAPDHR (5'-AGT CTT CTG GGT GCC AG-3'). Primer concentrations were adjusted to

Figure 7. A schematic diagram showing the chromosomal region (17q12) containing the CCL3L1/CCL4L1 copy variable region on a chromosome with two copies of this region. The approximate locations of the primers are identified; the CCL3 system has a set of primers that simultaneously amplify sequences in a reference locus (CCL3) and a test locus (CCL3L1); the CCL4A system has a set of primers that simultaneously amplify sequences in a reference locus (CCL4) and a test locus (CCL4L1); the LTR61A system has a set of primers that simultaneously amplify sequences in a reference locus (an LTR sequence on chromosome 10) and a test locus (an LTR sequence within the copy variable region). This figure is modified from Walker et al. 23
improve yield according to the manufacturer’s instructions and all had efficiency greater than 95%. For every individual the mRNA expression level of CCL3 and CCL3L1 relative to GAPDH was calculated, real time PCR assays were performed in triplicate for each individual sample and for all primer pairs.

The ratio of CCL3L1:CCL3 mRNA was also calculated using a novel cDNA PRT system. For each sample a single PCR was carried out using 5 ng cDNA and 0.5 U Taq DNA polymerase (NEB, Hitchin, UK) in a buffer with final concentrations of 50 mM Tris – HCl pH 8.8, 12.5 mM ammonium sulphate, 1.4 mM magnesium chloride, 7.5 mM 2-mercaptoethanol, 125 µM m 0 and 200 µM each dNTP. Products were amplified with 1 µM each of primers FAM-labelled CCL3CRNAF (5'-AAT CAT GCA GGT CTC CAC T-3') and CCL3CR (5'-AAT CAT GCA GGT CTC CAC T-3'), for 22 cycles of 95 °C for 30 s, 55 °C for 30 s, 70 °C for 1 min followed by a final hold at 70 °C for 40 min. CCL3 products were 170 bp and CCL3L1 products were 170 bp and can be readily distinguished by fragment analysis carried out by electrophoresis on an ABI3100. Products from the single PCR reactions were mixed with 10 µl HiDi formamide with ROX-500 marker (Applied Biosystems), and run on an ABI3100 36 cm capillary using POP-4 polymer with an injection time of 10 s. GeneMapper software (Applied Biosystems) was used to extract the peak areas for each sample and the ratio was calculated by comparing the transcript peak to the specific CCL3 transcript peak. All experimental samples were repeated in a separate PCR to confirm CCL3L1:CCL3 mRNA ratio.

Statistical analysis
Correlations between groups of copy number data and either protein expression or mRNA expression was assessed using a Spearman’s correlation in SPSS V16 (SPSS, Chicago, IL, USA) and figures were drawn with the software GraphPadPrism v4 for Windows (GraphPad Software, San Diego, CA, USA). Repeat measures were analysed in SPSS using a general linear model with repeated measures, with the within subject factors defined as time and measurement and the between subject factor defined as copy number.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work and DC was supported by a Wellcome Trust grant (number 083929) awarded to JALA and RJP.

REFERENCES
1. Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N et al. Relative impact of nucleotide and copy number variation on gene expression phenotypes. Science 2007; 315: 848–853.
2. Henrichsen CN, Vinckenbosch N, Zollner S, Chaignat E, Pradervand S, Schutz F et al. Segmental copy number variation shapes tissue transcriptomes. Nat Genet 2009; 41: 424–429.
3. Modi WS. CCL3L1 and CCL4L1 chemokine genes are located in a segmental duplication at chromosome 17q12. Genomics 2004; 83: 735–738.
4. Irving SG, Zipfel PF, Balke J, McBride OW, Morton CC, Burd PR et al. Two inflammatory mediator cytokine genes are closely linked and variably amplified on chromosome 17q. Nucleic Acids Res 1990; 18: 3261–3270.
5. Berkmann N, John M, Roessens G, Jose PJ, Barnes PJ, Chung KF. Inhibition of macrophage inflammatory protein–α expression by IL-10. Differential sensitivities in human blood monocytes and alveolar macrophages. J Immunol 1995; 155: 4412–4418.

6. Standiford T, Kunkel SL, Liebler JM, Burdick MD, Gilbert AR, Strieter RM. Gene expression of macrophage inflammatory protein-1alpha from human blood monocytes and alveolar macrophages is inhibited by interleukin-4. Am J Respir Cell Mol Biol 1993; 9: 192–198.
7. Menten P, Stryuf S, Schmutzey E, Wuyts A, De Clercq E, Schols D et al. The LD78b isoform of MIP-1α is the most potent CCR5 agonist and HIV-1-inhibiting chemokine. J Clin Invest 1999; 104: R1–R5.
8. Choe H, Farzan M, Sun Y, Sullivan N, Rollins B, Ponath PD et al. The [beta]-Chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 1996; 85: 1135–1148.
9. Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM et al. CCR5: A RANTES, MIP-1alpha, MIP-1beta Receptor as a Fusion Co-factor for Macrophage-Tropic HIV-1. Science 1996; 272: 1955–1958.
10. Aquaro S, Menten P, Stryuf S, Proost P, Van Damme J, De Clercq E et al. The LD78b Isoform of MIP-1α Is the Most Potent CC-Chemokine Inhibiting CCR5-dependent human immunodeficiency virus type 1 replication in human macrophages. J Virol 2001; 75: 4402–4406.
11. Xin X, Shioda T, Kato A, Liu H, Sakai Y, Nagai Y. Enhanced anti-HIV-1 activity of C-chemokine LD78(βα), a non-allelic variant of MIP-1(α/β/Δ). FEBS Letters 1999; 457: 219–222.
12. Shostakovich-Koretskaya L, Catano G, Chykarenko ZA, He W, Gornalusse G, Mummidii S et al. Combinatorial content of CCL3L1 and CCL4L1 gene copy numbers influence HIV/AIDS susceptibility in Ukrainian children. AIDS 2009; 23: 679–688.
13. Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G et al. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science 2005; 307: 1434–1440.
14. Dolan MJ, Kulkarni H, Camargo JF, He W, Smith A, Anaya J-M et al. CCL3L1 and CCR5 influence cell-mediated immunity and affect HIV-AIDS pathogenesis via viral entry-independent mechanisms. Nat Immunol 2007; 8: 1324–1336.
15. Kulkarni H, Marconi VC, Agan BK, McArthur C, Crawford G, Clark RA et al. Role of CCL3L1-CCR5 genotypes in the epidemic spread of HIV-1 and evaluation of vaccine efficacy. PLoS ONE 2008; 3: e3671.
16. Ahuja SK, Kulkarni H, Catano G, Agan BK, Camargo JF, He W et al. CCL3L1-CCR5 genotype influences durability of immune recovery during antiretroviral therapy of HIV-1-infected individuals. Nat Med 2008; 14: 413–420.
17. He W, Kulkarni H, Castiblanco J, Shimizu C, Aluyen U, Maldonado R et al. Reply to: “CCL3L1 and HIV/AIDS susceptibility” and “experimental aspects of copy number variant assays at CCL3L1”. Nat Med 2009; 15: 1117–1120.
18. Huij K, Sadam M, Karki T, Avi R, Krispin T, Paap P et al. CCL3L1 copy number is a strong genetic determinant of HIV Seropositivity in caucasian intravenous drug users. J Infect Dis 2010; 201: 730–739.
19. Urban TJ, Wentrob AC, Fellay J, Colombo S, Shianna KV, Gumbos C et al. CCL3L1 and HIV/AIDS susceptibility. Nat Med 2009; 15: 1110–1112.
20. Fellay J, Ge D, Shianna KV, Colombo S, Ledergerber B, Cirilli ET et al. Common genetic variation and the control of HIV-1 in humans. PLoS Genet 2009; 5: e1000791.
21. Shao W, Tang J, Song W, Wang C, Li Y, Wilson CM et al. CCL3L1 and CCL4L1: variable gene copy number in adolescents with and without human immunodeficiency virus type 1 (HIV-1) infection. Genes Immun 2007; 8: 224–231.
22. Bhattacharya T, Stanton J, Kim Y-E, Kunstman KJ, Phair JP, Jacobson LP et al. CCL3L1 and HIV/AIDS susceptibility. Nat Med 2009; 15: 1112–1115.
23. Walker S, Jayakantakul S, Armour JAL. Multiplex paralogue ratio tests for accurate measurement of multiallelic CNVs. Genomics 2009; 93: 98–103.
24. Townsend JR, Barcellos LF, Nibbs RJ. Gene copy number regulates the production of the human chemokine CCL3-L1. Eur J Immunol 2002; 32: 3016–3026.
25. Field SF, Howson JM, Maier LM, Walker S, Walker NM, Smyth DJ et al. Experimental aspects of copy number variant assays at CCL3L1. Nat Med 2009; 15: 1115–1117.
26. Armour JAL, Palla R, Zeeuwen PLJM, den Heijer M, Schalkwijk J, Hollox EJ. Accurate, high-throughput typing of copy number variation using paralogue ratios from dispersed repeats. Nucleic Acids Res 2007; 35: e19.
27. Carpenter D, Walker S, Prescott N, Schalkwijk J, Armour JAL. Accuracy and differential bias in copy number measurement of CCL3L1. BMC Genomics 2011; 12: 418.

Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)