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Copy number, linkage disequilibrium and disease association in the *FCGR* locus

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The response of a leukocyte to immune complexes (ICs) is modulated by receptors for the Fc region of IgG (FcγRs), and alterations in their affinity or function have been associated with risk of autoimmune diseases, including systemic lupus erythematosus (SLE). The low-affinity FcγR genomic locus is complex, containing regions of copy number variation (CNV) which can alter receptor expression and leukocyte responses to IgG. Combined paralogue ratio tests (PRTs) were used to distinguish three intervals within the *FCGR* locus which undergo CNV, and to determine *FCGR* gene copy number (CN). There were significant differences in *FCGR3B* and *FCGR3A* CNV profiles between Caucasian, East Asian and Kenyan populations. A previously noted association of low *FCGR3B* CN with SLE in Caucasians was supported [OR = 1.57 (1.08–2.27), $P = 0.018$], and replicated in Chinese [OR = 1.65 (1.25–2.18), $P = 4 \times 10^{-4}$]. There was no association of *FCGR3B* CNV with vasculitis, nor with malarial or bacterial infection. Linkage disequilibrium (LD) between multi-allelic *FCGR3B* CNV and SLE-associated SNPs in the *FCGR* locus was defined for the first time. Despite LD between *FCGR3B* CNV and a variant in FcγRIIB (I232T) which abolishes inhibitory function, both reduced CN of *FCGR3B* and homozygosity of the FcγRIIB-232T allele were individually strongly associated with SLE risk. Thus CN of *FCGR3B*, which controls IC responses and uptake by neutrophils, and variations in *FCGR2B*, which controls factors such as antibody production and macrophage activation, are important in SLE pathogenesis. Further interpretations of contributions to pathogenesis by FcγRs must be made in the context of LD involving CNV regions.

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INTRODUCTION

The low-affinity receptors for the Fc region of IgG, FcγRII and FcγRIII, play a key role in regulation of the immune response to IgG. Following cross linking of the receptors by immune-complexed antigen, they are important in phagocytosis and presentation of complexed antigen, as well as in responses such as cytokine production. The FcγRs are predominantly activatory, with the exception of the FcγRIIB receptor which has an inhibitory effect on signalling through the activatory FcγRs as well as the B cell receptor (1).

The human 1q23.3 locus contains five *FCGR* genes (*FCGR2A, FCGR2B, FCGR2C, FCGR3A* and *FCGR3B*) encoding the FcγRII and FcγRIII receptor families. There is high sequence similarity between the genes, in part due to an ancestral segmental duplication, which produced *FCGR3B* and formed the pseudogene *FCGR2C* from copies of the 5′ end of *FCGR2B* and the 3′ end of *FCGR2A* (2). The duplicated regions are 98% identical (3), and arose following the division of the great apes from the new world monkeys (4,5). The locus is complicated by the presence of more recent, >99% identical, copy number variable (CNV) regions, the exact limits and number of which have not been conclusively defined (6–8). The homology in the region of the ancestral duplication is likely to facilitate CNV formation (9).

Polymorphisms in the low-affinity FcγRs have been associated with a variety of autoimmune diseases, foremost among them systemic lupus erythematosus (SLE). SLE is clinically diverse, but is unified by autoantibody production, deposition of immune complexes (ICs) in organs including the joints, skin and kidneys, and a subsequent inflammatory response to the deposited ICs. The inflammation in these organs results in tissue damage leading to a diverse range of clinical features, from skin rashes and cytopenias through to organ-threatening glomerulonephritis (10). Failure of FcγR-mediated clearance of ICs and control of inflammatory responses are thought to be a predisposing factor to SLE.

Alterations in the function of FcγRs have been associated with SLE. A substitution in FcγRIIA where histidine replaces arginine at position 131 (R131H, rs1801274) increases the affinity of binding to IgG2 (11), and increases phagocytosis of IgG2 opsonin. In FcγRIIIA, a valine for phenylalanine substitution (V176F, rs396991) lowers affinity for IgG1 and IgG3, and prevents binding to IgG4 (12). Two isoforms of FcγRIIIB are encoded by haplotypes of four SNPs: the FcγRIIIB-HNA1a isoform has reduced N-linked glycosylation compared with the FcγRIIIB-HNA1b isoform, and has increased affinity for IgG3, mediating increased phagocytosis of IgG1 and IgG3 ICs (13). The low-affinity alleles of all three receptors have been linked to risk of SLE in linkage and association studies (14). The presence of CNV regions can lead to apparent violation of Hardy–Weinberg equilibrium, and can lead to exclusion of probes binding within these regions from genome-wide association studies (GWAS) (8). This may be the reason that only FcγRIIA-131R, and not SNPs in FcγRIIIB and FcγRIIIA, was identified as associated with SLE by GWAS (15).

The disruption of the inhibitory function of FcγRIIB due to the conversion of an isoleucine to a threonine in the transmembrane domain of the inhibitory receptor (I232T, rs1050501) (16) is also associated with SLE in Asians (17) and Caucasians (18). This appears to be due to a failure of the threonine form of the receptor (FcγRIIB-232T) to partition to lipid rafts, perhaps due to the polar nature of the threonine, or disruption of a protein sequence necessary for raft entry (16,19). Exclusion from lipid rafts abolished the inhibition of activatory signalling initiated by crosslinking of FcγRIIIB and the activatory FcγR or B cell receptor by IC (16).

Copy number (CN) variable areas may cover ∼5% of the genome, and contribute to the genetic variation between individuals (7). CN of the low-affinity *FCGR* genes and the association of CN with disease have recently been investigated. Studies using multiplex ligand probe amplification (MLPA) have investigated gene CN across the locus in Caucasians (6,20). qPCR methods have also been used to determine CN of *FCGR3B* alone in Caucasians (21–24) or in Chinese (23,25). In these studies, low CN of *FCGR3B* was associated with SLE (22,23) or SLE nephritis (21) in Caucasians, although both low and high CN was also recently associated with SLE (24) and no association with SLE was reported in Chinese (23,25). These studies are limited, however, by the requirement to categorize the continuous distribution of data generated by qPCR assays into discrete CN 'bins' (21,22,24) or alternatively to compare the means (23). The limitations of the qPCR method may also be responsible for the wide discrepancy between the percentage of Caucasian individuals with low *FCGR3B* CN determined by qPCR [approximately 10–25% (22), and 40% (21)] and MLPA methods (5–7%) (6,20). A more accurate method of CN designation is therefore needed to confirm the association with SLE.

It is important to clarify the effect of *FCGR3B* CN in disease, and determine if it is independent of the effect of other polymorphisms in the locus. In this study, we used an assay combining three paralogue ratio tests (PRTs) to define three CNV regions within the *FCGR* locus, and determine the prevalence of variation of these regions in Caucasian, East Asian and Kenyan populations. Further, the previous association of low *FCGR3B* CNV in SLE in Caucasians was supported, and an association with SLE was identified in a second ethnic group, Hong Kong Chinese. We describe differences in linkage disequilibrium (LD) in the *FCGR* region between ethnic groups for the SLE-associated SNPs and with *FCGR3B* CNV, while taking into account *FCGR3A* CNV. We show that although there is LD between *FCGR3B* CN and *FCGR2B-I232T*, both show an independent effect on SLE risk.

RESULTS

Copy number in the *FCGR* locus

The qPCR-based CNV assays previously used to interrogate the *FCGR* locus (21–25) have been shown to be limited by the requirement to make discrete CN calls from continuous data distributions (23,26). Therefore, we developed an assay involving a combination of three PRTs to better analyse CNV in the *FCGR* locus. Both PRT and qPCR compare the signal generated at a variable locus to that at a reference locus. The ratio between amplified products, and thus relative gene CN, can then be determined. The key advantage to the PRT assay is that a single pair of primers amplifies two genomic loci, producing products which differ in size by a

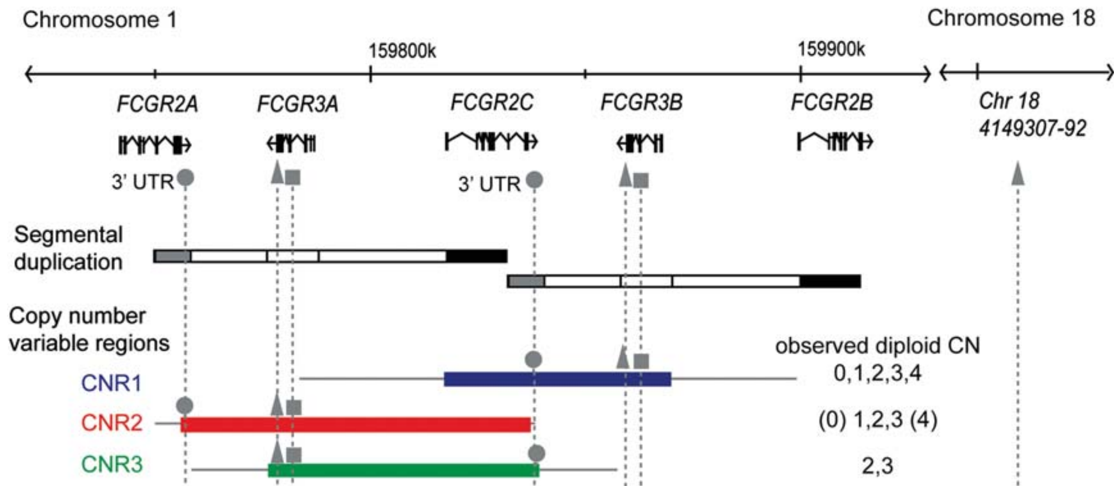


Figure 1. CNV and paralogue ratio test (PRT) in the *FCGR l* ocus. A segmental duplication resulted in the production of *FCGR3B*, as well as *FCGR2C* which combines the 5′ end of *FCGR2B* and the 3′ end for *FCGR2A*. Repeated black, grey and white regions reflect areas of homology within the genes. The PRT primer pairs used in this study to determine copy number variation (CNV) are PRT-2C$^{3'}$/2A$^{3'}$ (circle) and PRT-3B/3A (square), which each bind at and amplify two locations in the genome on Chromosome 1; and PRT-3(A+B)/Chr18 (triangle) which binds at three locations—two on chromosome 1 and one on chromosome 18. Relative gene locations are derived from the Database of Genomic Variants, human genome build 36 (hg18). Three CNV regions are identified by variation at PRT primer binding sites (marked): CNR1 (blue) includes CNV at the PRT primer sites in *FCGR2C* and *FCGR3B*; CNR2 (red) includes CNV at the PRT primer sites in *FCGR2A* 3′-UTR and *FCGR3A*, but not in *FCGR2C 3′-UTR*; and CNR3 (green) includes CNV at the PRT primer sites in *FCGR3A* and *FCGR2C 3′-UTR*. Solid colour bars indicate areas suggested to be included in the CNV interval and thin grey lines indicate maximum plausible extent. Diploid copy number variations of each region observed in this study are noted, with other probable variations in brackets. For further details of the relationship between relative PRT product, underlying gene CN and CNV regions, see Supplementary data online.

few nucleotides, in the same tube. In contrast, in qPCR, the separate reference and CNV reactions introduce variability, with significant plate-to-plate variation (23). Two PRT assays developed in this study compare the CN of a region in *FCGR3B* with one in *FCGR3A*, and CN of a region in the *FCGR2C* 3′-untranslated region (3′-UTR) with one in the *FCGR2A* 3′-UTR (Fig. 1, Supplementary Material, Fig. S1A and B). These were used in conjunction with a third previously described PRT assay which compared total CN in both *FCGR3B* and *FCGR3A* with a region on Chromosome 18 (27). Comparison of these three PRT assays allowed CN of *FCGR3A, FCGR3B* and the 3′-UTR of *FCGR2A* and *FCGR2C* to be determined, without the requirement of a restriction enzyme digest as used previously (27) (full description of PRT in Supplementary Material; Figs S1 and S2). *FCGR2B* was previously shown not to undergo CNV (6,20). The CN variation over these loci suggested at least three CNV regions: CNR1, CNR2 and CNR3 (Fig. 1, Supplementary Material, Fig. S1C and D). The more common CNR1 and CNR2 show similarity to, and extend, previously identified CNV regions (6,20), and a novel CNR3 is seen primarily in East Asians. Both duplication and deletion were observed at CNR1 and CNR2 (and thus the CNV at these sites is multi-allelic), whereas only a duplication of CNR3 was able to be identified (Supplementary Material, Fig. S2E and Table S1).

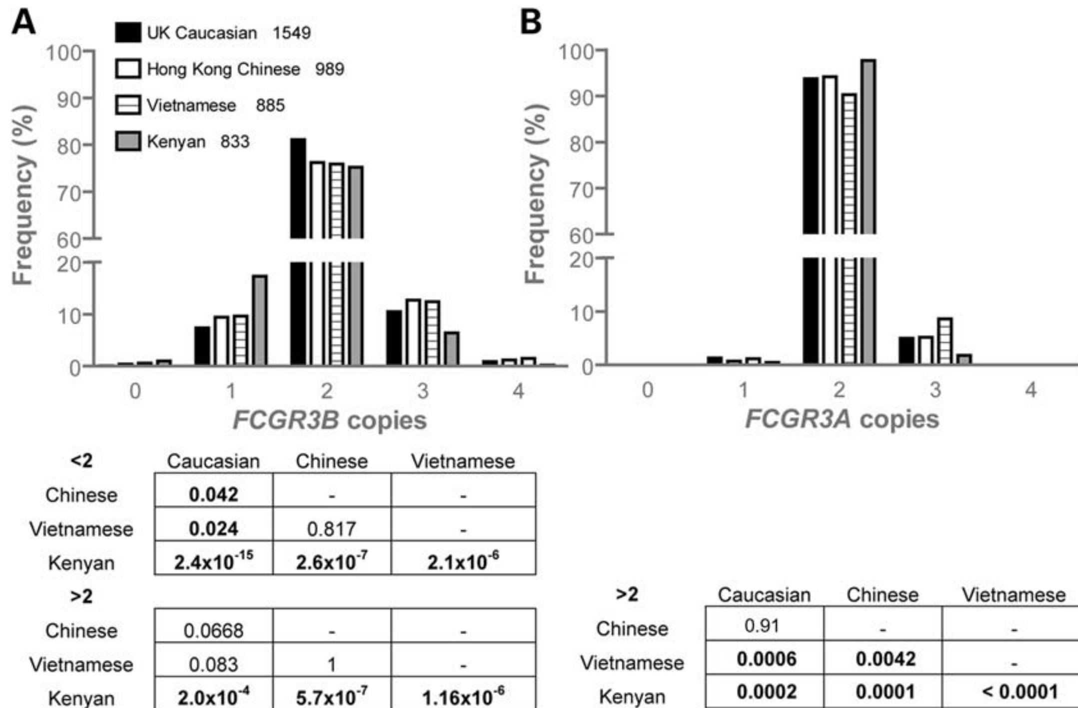
Variation in *FCGR* CNV between ethnic groups

Previous work, based on a small number of individuals, raised the possibility of ethnic variation in *FCGR* CN profiles (27). To investigate this, the triple PRT assay was used to determine CN of *FCGR3B* and *FCGR3A* in large cohorts of Caucasian, Hong Kong Chinese, Vietnamese Kinh and Kenyan individuals (Fig. 2). There was significant ethnic variation in the CN profiles for both *FCGR3B* and *FCGR3A*. Low *FCGR3B* CN (0 or 1 copies) was least common in Caucasians, intermediate in Chinese and Vietnamese populations and highest in the Kenyan population. *FCGR3B* high CN (3 or more copies) was most common in the Chinese and Vietnamese populations. The percentage of Caucasian individuals with *FCGR3B* low CN (7.4%) is similar to the 5–7% previously found using MPLA and an assay combining PRT, restriction enzyme digest and short terminal repeat amplification to determine CN (6,27) and considerably lower than the 10–40% found using qPCR (21,22). Overall, there was less variation in *FCGR3A* CN, with high CN more common than low. *FCGR3A* 3CN was most common in the Vietnamese population, and least common in Kenyans.

Low CN of *FCGR3B* is associated with SLE, but not with vasculitis

Previous studies, using qPCR methodology, had shown association of low CN of *FCGR3B* with SLE nephritis (21,22) and SLE (22,23) in Caucasians but not Asians (23,25). These findings are complicated by a recent qPCR study which showed that individuals with low and high CN were both increased in an SLE cohort (24).

Therefore, we analysed *FCGR* CNV in cohorts of SLE patients and controls using the triple PRT assay (Fig. 3A). The association with low CN, but not high CN, was supported by Caucasian SLE cohorts from the UK and Sweden. These cohorts individually showed a trend for increased low CN with SLE (Fig. 3A) and combined as a single cohort by meta-analysis (Fig. 3B) showed significant association of



<2	Caucasian	Chinese	Vietnamese
Chinese	**0.042**	-	-
Vietnamese	**0.024**	0.817	-
Kenyan	**2.4x10^{-15}**	**2.6x10^{-7}**	**2.1x10^{-6}**

>2	Caucasian	Chinese	Vietnamese
Chinese	0.0668	-	-
Vietnamese	0.083	1	-
Kenyan	**2.0x10^{-4}**	**5.7x10^{-7}**	**1.16x10^{-6}**

>2	Caucasian	Chinese	Vietnamese
Chinese	0.91	-	-
Vietnamese	**0.0006**	**0.0042**	-
Kenyan	**0.0002**	**0.0001**	**< 0.0001**

Figure 2. Ethnic differences in *FCGR3B* and *FCGR3A* CN profiles. (**A**) Distribution of copy number of *FCGR3B* in four control cohorts from different ethnic backgrounds. The Caucasian controls are from the UK cohort. Statistical differences between the populations are tested for *FCGR3B* diploid copy number <2 or >2 versus the remainder, and the *P*-value for a Fisher's exact test is shown. (**B**) Distribution of copy number of *FCGR3A* in the four control cohorts from different ethnic backgrounds. Statistical differences between the populations are tested for 1 versus 2,3 diploid copy number, and the significance for a Chi-squared test of independence (df = 2) is shown. Bold values show the significant *P*-value.

low CN with SLE [odds ratio (OR) 1.57, 95% confidence interval (CI) 1.08–2.27; $P = 0.018$]. We also tested the association of low CN of *FCGR3B* with SLE in a Hong Kong Chinese cohort, and found a strong association of low CN with SLE (OR 1.65, 95% CI 1.25–2.18; $P = 4 \times 10^{-4}$) with a similar effect size to that in Caucasians (Fig. 3A), despite previous qPCR studies which found no association in different Chinese cohorts (23,25). This association was the strongest in the sub-group of Chinese patients with lupus nephritis, and demonstrates for the first time the association of *FCGR3B* low CN with SLE in a non-Caucasian population. The combined OR of this effect, by meta-analysis, was 1.62 (95% CI 1.30–2.03) (Fig. 3B). Thus *FCGR3B* low CN is one of the strongest common variants reported to increase risk of SLE (28). The amount of *FCGR3A* CNV did not vary significantly between SLE cases and controls (Supplementary Material, Table S2).

We also investigated the association of *FCGR3B* CN with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). AAV is a systemic autoimmune disease distinct from SLE, with anti-neutrophil cytoplasmic antibodies, but not with IC deposition. An initial association with AAV and low *FCGR3B* CN by qPCR (22) was not confirmed (23). We thus used the triple PRT assay to analyse the CNV of a combined UK Caucasian vasculitis cohort, and showed no difference in the CN profile compared with controls (Fig. 3C), suggesting that the initial finding may have been a false positive resulting from inconsistencies in the qPCR assay.

No association of *FCGR3B* CN with malaria or bacterial sepsis

FCGR3B is primarily expressed on neutrophils and *FCGR3A* on monocytes and macrophages. Both are likely to play a role in the defence against infection. We investigated whether CN of either *FCGR3B* or *FCGR3A* influences risk of developing severe malaria in Vietnamese and Kenyan populations from areas with endemic malaria. The higher incidence of SLE-associated low *FCGR3B* CN in the Kenyan population may be due to positive selective pressure by conferring protection from infectious disease. In Africa, endemic malaria is a major contributor to mortality in children (29), and malaria is known to exert a strong evolutionary selective effect (30). The *FCGR* CN profiles of two Kenyan cohorts comprised of children treated for severe malaria at Kilifi District Hospital were compared with cord blood controls. In neither of the Kenyan cohorts, nor in a cohort of Vietnamese adults treated for severe malaria, was there any association of CN of *FCGR3B* (Fig. 4) or *FCGR3A* (Supplementary Material, Table S2).

Severe bacterial sepsis is also a major factor in child mortality in Africa (29). We investigated *FCGR3B* and *FCGR3A* CN in a cohort of children admitted to Kilifi District Hospital with blood-culture positive bacterial infection. The cohort was heterogeneous with respect to infecting bacteria, but those detected most frequently were *Streptococcus pneumoniae, Salmonella typhi, Haemophilus influenzae* and *Escherichia coli*. There was no difference in the CN profiles of *FCGR3B*

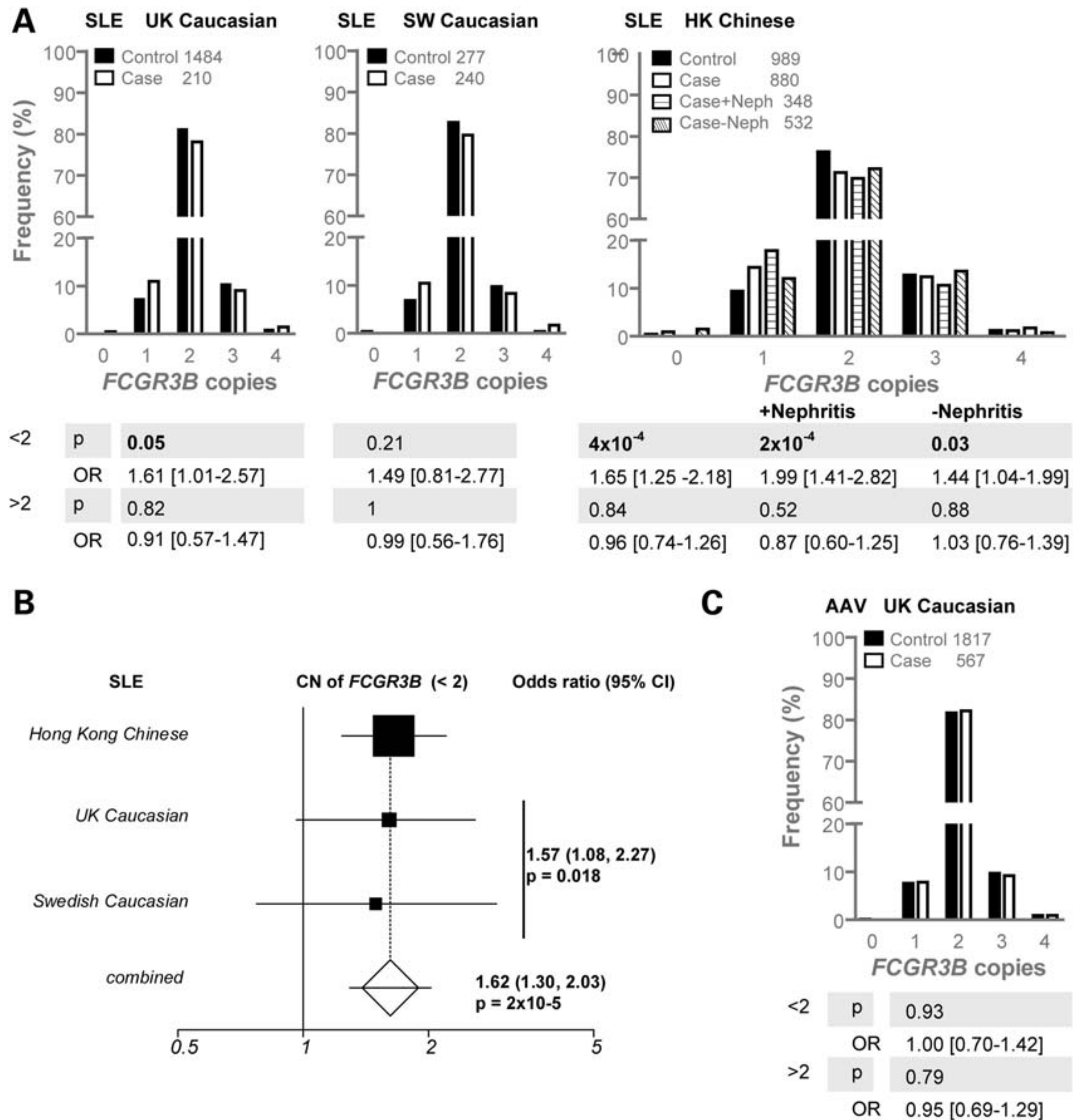


Figure 3. Association of *FCGR3B* CN with SLE and vasculitis. (**A**) Distribution of copy number of *FCGR3B* in three systemic lupus erythematosus (SLE) case cohorts compared with their controls: UK Caucasian, Swedish (SW) Caucasian and Hong Kong (HK) Chinese. The HK Chinese cases were subsequently split into those with (+neph) and without (−neph) nephritis. Numbers of individuals in each cohort are noted. Statistical significance for a difference between cases and controls was tested for *FCGR3B* diploid copy number of <2 or >2 versus the remainder by a Fisher's exact test. OR, odds ratio (95% confidence interval); *P*, significance value. Bold values show the significant *P*-value. (**B**) Meta-analysis (random effects model) of <2 versus 2,3 and 4 diploid copies of *FCGR3B* between SLE cases and controls in all three SLE cohorts (main graph) or Caucasian cohorts alone (bar on right). Box size is proportional to sample size. (**C**) Distribution of copy number of *FCGR3B* in an ANCA-associated vasculitis (AAV) Caucasian cohort and controls. Controls included those used as controls for the UK SLE cohort.

in this cohort compared with controls (Fig. 4). There was, however, a weak association between high *FCGR3A* CN and disease (OR 1.92, 95% CI 1.03–3.56; $P = 0.039$) (Supplementary Material, Table S2).

Confirmation of association of FcγRIIB-232T homozygosity with SLE in Caucasians

Homozygosity of the allele for the FcγRIIB-232T isoform has previously been associated with SLE, initially in East Asians (17) and more recently in Caucasians (18). We have added to this data by analysing two new Caucasian SLE cohorts from UK and Swedish populations, further demonstrating a significant association of FcγRIIB-232T allele homozygosity with SLE risk (Supplementary Material, Table S3). When all studies involving Caucasian cohorts were analysed in a meta-analysis, there was a strong association with SLE (OR 2.26, 95% CI 1.49–3.41) (Supplementary Material, Fig. S3). The Hong Kong Chinese cohort had previously been typed for the FcγRIIB-I232T polymorphism, and showed a non-significant trend towards an increased percentage of FcγRIIB-232T allele homozygotes in SLE, which strengthened the overall association by meta-analysis in East Asian cohorts (18).

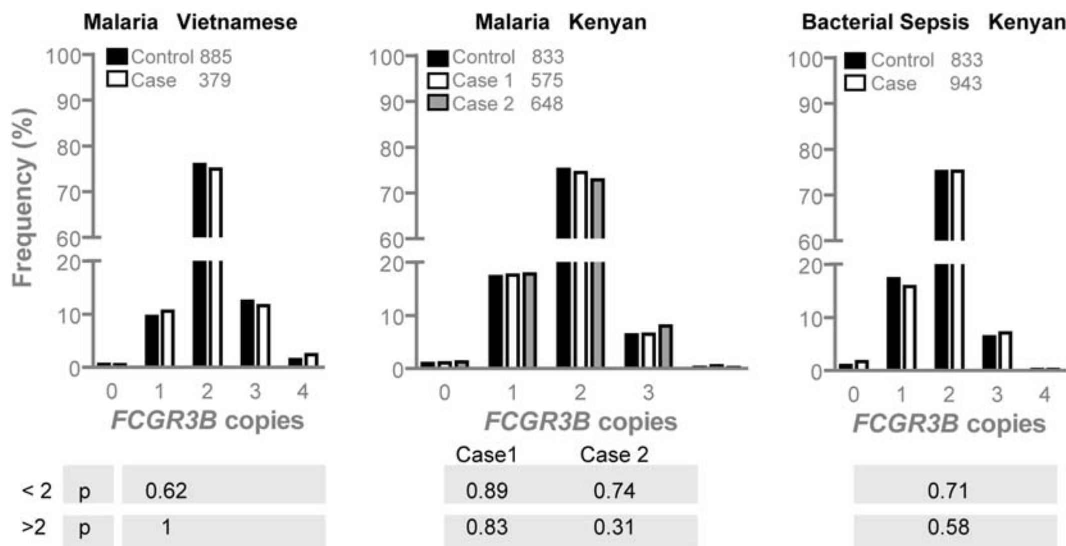


Figure 4. No association of *FCGR3B* CN with malaria or bacterial sepsis. Distribution of copy number of *FCGR3B* in four case cohorts compared with cord blood controls. A single Kenyan control group is used for all three Kenyan case cohorts. Numbers of individuals in each cohort are noted. Statistical significance for a difference between cases and controls are tested for *FCGR3B* diploid copy number of <2 or >2. *P*, significance value for a Fisher's exact test.

LD between the *FCGR2B-I232T* polymorphism and *FCGR3B* CN

It was thought important to determine the degree of LD between the multi-allelic *FCGR3B* CNV and the FcγRIIB-I232T polymorphism, as these neighbouring variants are both associated with SLE risk. Indeed, our initial observations showed that low *FCGR3B* CNV was more common in individuals with the FcγRIIB-232T isoform, suggesting that these SLE-associated alleles might be inherited together to some degree (Supplementary Material, Fig. S4). This raised the possibility that the observed disease association attributed to one variant may be primarily due to the other variant. LD between the FcγRIIB-I232T SNP and *FCGR3B* CNV was determined in control cohorts both at the haplotype level, using estimated phased haplotypes, as well as at the genotype level. There was weak-to-moderate LD in the Caucasian and Chinese control cohorts, as measured by D′, which varied depending whether LD calculation compared the deletion haplotype or the duplication haplotype to the remainder (Fig. 5, Supplementary Material, Table S4).

LD between other *FCGR* SNPs and *FCGR3B* CN

Multiple SNPs within the low-affinity *FCGR* locus are associated with SLE. This further raises the question of whether all the disease-associated variants act independently, or whether LD across the locus influences these reported associations. Therefore, we also typed the FcγRIIA-R131H and FcγRIIIA-V176F polymorphisms in a subset of the SLE cohorts (Supplementary Material, Table S5), and in the Kenyan and Vietnamese control cohorts (Supplementary Material, Table S6). The proportion of FcγRIIA-131R homozygotes was increased in the SLE cohorts, although the association was not significant, perhaps due to relatively small numbers (Supplementary Material, Table S5). In both the Chinese and Caucasian SLE cohorts, the OR was 1.3, which correlated with that noted in two SLE GWAS (15,31). FcγRIIIA-V176F did not show a significant association with SLE.

There is some evidence that there is LD between the SLE-associated SNPs FcγRIIB-I232T and FcγRIIA-R131H or FcγRIIIA-V176F in East Asian populations (32–34), and between FcγRIIA-R131H and FcγRIIIA-V176F in European Caucasian populations and an African American population (35–37). In these studies, however, CN of *FCGR3A* has not been taken into account, and, to our knowledge, no formal assessment of LD between SLE-associated SNPs and CNV in the *FCGR* locus has been performed. We investigated the degree of LD between SLE-associated SNPs FcγRIIB-I232T, FcγRIIA-R131H, FcγRIIIA-V176F and *FCGR3B* CN, controlling for *FCGR3A* CN. LD based on genotype and phase haplotype data was generally consistent (Supplementary Material, Table S4). The pattern of LD between SNPs observed in our Caucasian and East Asian control cohorts (Fig. 5) generally corresponded to that seen in previous studies. In addition, LD between FcγRIIB-I232T and FcγRIIA-R131H or FcγRIIIA-V176F was noted in the Kenyan control cohort. There was also some LD between *FCGR3B* CNV and FcγRIIA-R131H in the Chinese control cohort; however, it was the combination of SLE-associated FcγRIIA-131R homozygosity and protective *FCGR3B* high CN that was enriched, suggesting the risk due to a low *FCGR3B* CNV was not due to a linked FcγRIIA-131R allele (Supplementary Material, Fig. S4 and Table S7).

Low *FCGR3B* CNV and FcγRIIB-232T both contribute to SLE risk despite LD

Given the presence of LD between the FcγRIIB-I232T and *FCGR3B* CNV loci, we investigated whether low *FCGR3B* CNV contributed to the risk of SLE independent of the effect of FcγRIIB-232T. The SLE and control cohorts were stratified by FcγRIIB-I232T genotype and the association of

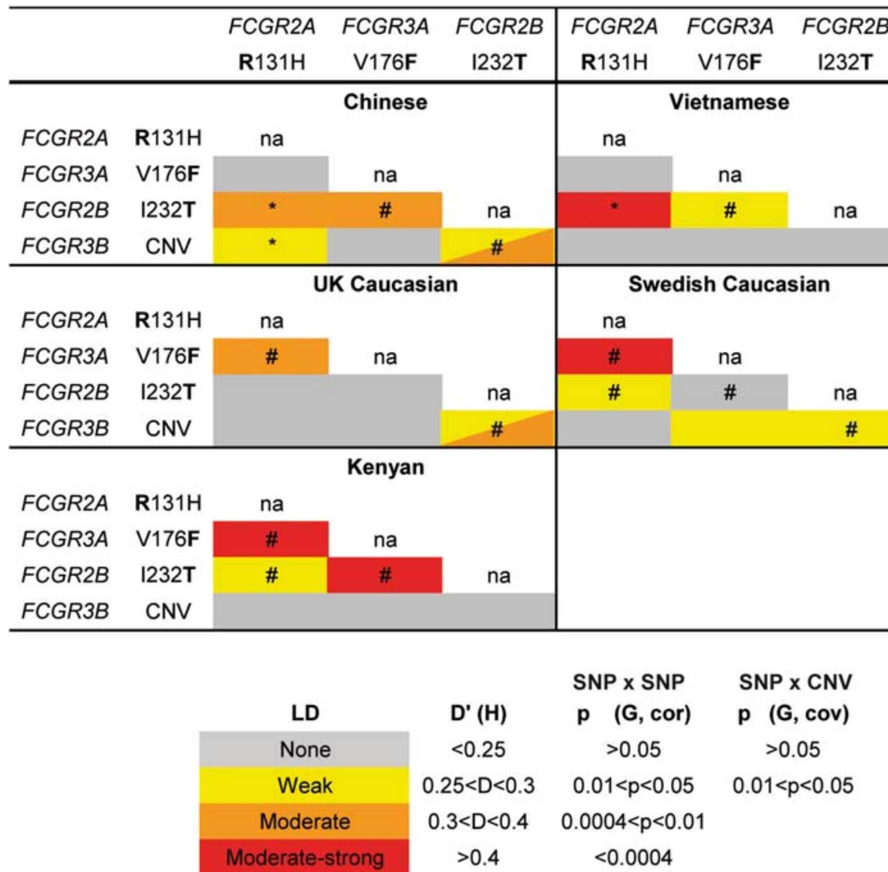


Figure 5. Linkage disequilibrium (LD) in the *FCGR* locus. LD in normal individuals between *FCGR3B* copy number variation (CNV) and single nucleotide polymorphisms (SNPs) previously associated with systemic lupus erythematosus (SLE) was determined by haplotype-based methods (D') and genotype-based methods [correlation (Gcor) and covariance (Gcov)]. Colours indicate increasing level of LD. Split colour bars indicate variation in LD when either high or low *FCGR3B* CN was compared. #Combined genotype involving two SLE risk diplotypes (*FCGR2A*-131R/R, *FCGR3A*-176F/F, *FCGR2B*-232T/T, or *FCGR3B*-low CN) is enriched. *Combined genotype involving one SLE risk and one non-risk diplotype is enriched. All individuals are from the control cohorts used in association studies.

low *FCGR3B* CNV after accounting for FcγRIIB-I232T background was determined in the Chinese and the combined Caucasian cohorts. In both populations, the significantly increased risk associated with low *FCGR3B* CNV was only observed in the presence of the FcγRIIB-232T allele, although a non-significant trend was seen in the FcγRIIB-232I homozygotes (Table 1). Numbers were too small in the FcγRIIB-232T homozygote group for meaningful analysis alone. The variation according to FcγRIIB-232T allele presence could indicate a modifying effect of FcγRIIB-I232T on the risk due to low *FCGR3B* CNV. Because there was LD between the loci, it was not possible to differentiate between whether the relative risk of the CNV was altered by the FcγRIIB-232T allele in a *cis* (within the same haplotype) or a *trans* (inherited independently on different chromosomes) manner. A test for heterogeneity of ORs across the FcγRIIB-I232T strata, however, was not significant in either cohort.

To further test whether each locus contributed to risk of SLE once the effect of the other was taken into account, we analysed the effect of *FCGR3B* CNV and the FcγRIIB-I232T SNP by logistic regression to a general linear model in the Chinese and Caucasian cohorts. After adjusting for effects due to differences between two Caucasian cohorts, the effect of *FCGR3B* CNV was significant after accounting for the effect due to FcγRIIB-I232T (Table 2). Equally, the effect of FcγRIIB-I232T was significant after adjusting for the effect due to *FCGR3B* CNV. In the Hong Kong Chinese cohort, the effect of *FCGR3B* CNV was also significant after accounting for the effect due to FcγRIIB-I232T, although the trend for the FcγRIIB-I232T SNP did not reach significance. There was no significant deviation from a multiplicative model of risk for the SNP:CNV interaction in the Chinese cohort, and it was only just significant in the Caucasian cohort. Overall, this indicated that, despite the presence of LD between the loci, *FCGR2B*-I232T and *FCGR3B* CNV have independent effects on the risk of developing SLE. The risk associated with low *FCGR3B* CN is equivalent between two ethnic groups. We therefore demonstrate a consistent, strong effect of low *FCGR3B* CN on the risk of developing SLE.

DISCUSSION

Using a triple PRT assay, we have confirmed association of low CN of *FCGR3B* with SLE. Although the association in Caucasians was previously reported, this triple PRT assay

Table 1. *FCGR3B* CN stratified by *FCGR2B-I293T* type

FCGR3B CN	UK and Swedish Caucasian[a] SLE, *n*	Cont, *n*	OR (95% CI)	*P*-value	Hong Kong Chinese SLE, *n*	Cont, *n*	OR (95% CI)	*P*-value
	418	595	*FCGR2B-232 all*		745	914	*FCGR2B-232 all*	
0,1	49	45	1.55 (1.02–2.37)	**0.049**	117	89	1.73 (1.29–2.32)	**3×10^{-4}**
3,4	43	66	0.93 (0.62–1.39)	0.76	100	134	0.90 (0.68–1.19)	0.48
	297	472	*FCGR2B-232 I/I*[b]		432	495	*FCGR2B-232 I/I*	
0,1	28	33	1.35 (0.79–2.28)	0.28	48	44	1.29 (0.84–1.99)	0.27
3,4	32	59	0.86 (0.55–1.36)	0.57	73	87	0.95 (0.68–1.34)	0.79
	89	106	*FCGR2B-232 I/T*		260	366	*FCGR2B-232 I/T*	
0,1	15	6	2.98 (1.11–8.00)	**0.037**	55	37	2.39 (1.52–3.75)	**1×10^{-4}**
3,4	7	6	1.39 (0.45–4.29)	0.58	26	43	0.83 (0.50–1.40)	0.52
	32	17	*FCGR2B-232 T/T*		53	53	*FCGR2B-232 T/T*	
0,1	6	6	0.53 (0.15–1.90)	0.34	14	8	2.02 (0.77–5.32)	0.2
3,4	4	1	2.13 (0.22–20.6)	0.65	1	4	0.24 (0.03–2.18)	0.36
Breslow–Day test of heterogeneity				0.056				0.14

CN, copy number (diploid); SLE, systemic lupus erythematosus; cont, control; OR, odds ratio; CI, confidence interval.
[a]Combined Caucasian group: the sum of UK Caucasian and Swedish Caucasian cohorts.
[b]*FCGR2B-232 I/I*, *FCGR2B-232 I/T*, or *FCGR2B-232 T/T* indicates the stratification of the data before the association of <2 or >2 diploid copies of *FCGR3B* with SLE is tested by Fisher's exact test. All individuals are *FCGR3A* CN = 2.
Bold values show the statistical significance at the 95% level.

allows clearer designation of integer values for CN. The association found in Hong Kong Chinese is novel, and is important as it validates the effect of *FCGR3B* CN in a different genetic background. The highest ORs for risk of SLE of the common polymorphisms strongly associated by GWAS are the major histocompatibility complex region (OR = 2.0), interferon regulatory factor 5 (*IRF5*, OR = 1.8) and complement receptor 3 subunit integrin alpha M (*ITGAM* OR = 1.6) (15). This places the OR for *FCGR3B* low CN, at 1.6, among the strongest genetic risk factors yet identified for SLE.

FcγRIIIB is expressed primarily on neutrophils, where it is the most common FcγR (38,39). The strong association of low CN of *FCGR3B* with SLE suggests a hitherto un-emphasized role of neutrophils in protection from SLE. Defects in IC clearance have been implicated in SLE, and macrophages from SLE patients show reduced soluble IC uptake (40,41). Neutrophils lacking FcγRIIIB had profoundly reduced soluble IC uptake, and increasing *FCGR3B* CN correlated with FcγRIIIB surface expression and increasing IC uptake (23). FcγRIIIB is also important in tethering neutrophils under flow conditions, which is required to allow extravasation into the tissues (42,43); such adhesion correlated with *FCGR3B* CN (23). FcγRIIIB appears less important in initiating superoxide release, since increased *FCGR3B* CN in individuals within a family did not cause significantly increased release upon stimulation (23). A contribution to IC clearance may be made by neutrophils trafficking to sites of IC deposition and clearing complexes once there. Reduced FcγRIIIB expression could thus lead to increased IC deposition and resulting inflammatory damage and disease.

FcγRIIIB is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor, and does not contain intrinsic cytoplasmic signalling domains or associate with signalling adaptor proteins used by other FcγRs. Instead, it is thought to colocalize with and signal through other receptors, such as complement receptor 3 (CR3) and FcγRIIA, to facilitate neutrophil functions such as phagocytosis and the oxidative burst (44–48). Therefore, it is interesting to note that polymorphisms in *ITGAM* (part of the CR3 receptor) and *FCGR2A* are strongly associated with SLE in GWA studies (15,31). Although in this study no interaction was found by logistic regression between the *FCGR2A* SLE-associated SNP and *FCGR3B* CN, an interaction with *ITGAM* was not tested. Since CR3 is important in FcγRIIIB-mediated phagocytosis in neutrophils (46), it is possible that polymorphisms which inhibit CR3 binding to complement or interaction with FcγRIIIB may exacerbate the effect of low *FCGR3B* CN.

CN of *FCGR2C* alters in parallel with both *FCGR3A* and *FCGR3B* in the CNV regions. *FCGR2C* is a pseudogene in the majority of individuals. In a Caucasian population, however, 18% of individuals had at least one *FCGR2C* allele with an open reading frame, due to a variant which changes the common stop codon in exon 3 to a glutamine (6). The expression level of *FCGR2C* on NK cells correlates to presence of this open reading frame allele, rather than total gene CN (6). Although there was an association of *FCGR2C*-ORF frequency with risk of idiopathic thrombocytopenic purpura, there was no association with total CN of *FCGR2C* (6). The increase in *FCGR2C* concomitant with *FCGR3B* in the SLE population in our study is therefore unlikely to correlate to increased *FCGR2C* expression.

This study also highlights the complexity of the low-affinity *FCGR* locus. In large cohorts from multiple ethnic groups, we were able to identify multiple individuals with variation in two different CNV regions. A third CNV region (CNR3) primarily found in East Asian populations was also identified which, although it included *FCGR3A* and *FCGR2C*, had altered breakpoints compared to the more common CNR2 region. It is likely that there are multiple breakpoints of CNV regions within the areas of homology in this locus.

A previous study has examined LD of *FCGR3B* CNV or FcγRIIIB-HNA1 polymorphisms with proximal SNPs in HapMap individuals, and found no SNPs with sufficiently high LD (assessed by r^2) to be predictive, although the study was limited by the SNP data available and did not include any SNPs within the ancestral duplication of *FCGR*

Table 2. Logistic regression to test of independence of *FCGR3B* CN and *FCGR2B-I232T* risk in SLE

FCGR2B-I232T	HK Chinese	Caucasian[a]	*FCGR3B-CN*	HK Chinese	Caucasian
TT versus IT/II			0,1 versus 2,3,4		
1 $P\ \chi^2$	0.142	**0.0003**	1 $P\ \chi^2$	**0.0003**	**0.017**
2 $P\ \chi^2$	0.211	**0.001**	2 $P\ \chi^2$	**0.0004**	**0.048**
OR (95%CI)	1.28 (0.87–1.87)	2.72 (1.48–4.99)	OR (95%CI)	1.67 (1.26–2.22)	1.55 (1.01–2.38)
Interaction					
3 $P\ \chi^2$	0.948	0.047			

HK, Hong Kong; $P\ \chi^2$, *P*-value of the Chi-square test statistic; OR, odds ratio; CI, confidence interval; 1,2,3 denote the order in which effects are adjusted for.
[a]Combined Caucasian group, comprised of UK Caucasian and Swedish Caucasian cohorts, in which the factor 'group' is used to take into account differences between the cohorts prior to determining risk due to *FCGR2B* or *FCGR3B*.
Bold values show the statistical significance at the 95% level.

genes (27). Others have assessed LD between SNPs and di-allelic CNVs (7). We examined linkage between the multi-allelic *FCGR3B* CNV and SLE-associated SNPs in East Asian, Caucasian and Kenyan control cohorts, and demonstrated weak-to-moderate LD in the *FCGR* locus. The differing degree of LD between *FCGR2B* and *FCGR3B* found when comparing the *FCGR3B* deletion haplotype or the duplication haplotype may indicate that these events formed independently, and may have subtly different breakpoints.

The *FCGR2B-I232T* polymorphism was found to be in LD with *FCGR3B* CNV in Chinese and Caucasians. The association of homozygosity of the FcγRIIB-232T allele with SLE has been well established in East Asian populations (17,18,49), and in this study we confirm our previously reported association in Caucasians (18) with an effect size (OR = 2.6) among the highest for SLE. Critically, *FCGR3B* CNV was demonstrated to independently contribute to SLE risk despite the LD with *FCGR2B-I232T*.

There were two intriguing negative findings in this study. The first was that there was no association with AAV. The evidence for association of *FCGR3B* CN with vasculitis has been contradictory—low CN was initially associated with disease (22), but this finding was not replicated in a second study using a similar qPCR assay (23). In the current study, we found no association or distinct trend in either direction. It is plausible that *FCGR3B* low CN does not have a significant effect on AAV, since ICs are not thought to play a major role in AAV pathogenesis (50). The previous association with low AAV depended upon integer CN designation from a qPCR assay, and the number of low CN individuals in Caucasian controls was high (10–25%) compared to what has been identified here with the triple PRT (7%) and previously with MLPA (5–7%) (6,20). Thus findings based solely on qPCR assays must be treated with caution, and confirmed using other methods.

There was no association of *FCGR3B* CN with severe bacterial infection or malaria in Kenyan and Vietnamese cohorts. Neutrophils are known to play a critical role in the clearance of bacteria. However, it is possible that a dosage effect of FcγRIIIB is not equally important in all infections. In the response to bacteria which elicit a largely IgG2 response, such as *Streptococcus pneumoniae*, FcγRIIA-H131 is most effective in binding the IgG isotype (14) and will be most important in binding the opsonized pathogen, whereas FcγRIIIB binding may play a lesser role. Thus to examine the effect of *FCGR3B* CN on such diseases, the analysis of larger and microbiologically more precisely defined cohorts will be required.

This study describes a triple PRT method to determine integer CN of genes in the *FCGR* locus, in particular *FCGR3B*. The heterogeneity of CNV breakpoints is highlighted, as is LD between SNPs and CNV in the region, and future studies in the *FCGR* locus will need to take this complexity into account. In this study, CN of *FCGR3B* is demonstrated to be an important risk factor in SLE across two different ethnic groups, independent of neighbouring *FCGR* SNPs. Further work needs to be carried out to accurately determine the profile of *FCGR3B* CN in other autoimmune diseases and bacterial infections, for which the triple PRT assay is highly suitable.

MATERIALS AND METHODS

Case and control cohorts

Chinese SLE cases and controls. A Hong Kong Chinese SLE patient cohort was recruited from three Hong Kong hospitals, and control samples were obtained from the Hong Kong Red Cross. In brief, patients and controls were all of self-reported Chinese ethnicity living in Hong Kong, and patients met the criteria of the American College of Rheumatology for SLE diagnosis (51), with those with a definite diagnosis of lupus nephritis designated by more than 0.5 g per day proteinuria or cellular casts seen on urine microscopy. The study was approved by the Institutional Review Board of the University of Hong Kong and Hospital Authority, Hong Kong West Cluster, New Territory West Cluster and Hong Kong East Cluster. All patients and controls gave informed consent.

Caucasian SLE cases and controls. The UK SLE cohort was obtained from the MRC/Kidney Research UK National DNA Bank for glomerulonephritis. All individuals were between the ages of 18 and 50 with a definite diagnosis of lupus nephritis based on biopsy and on clinical and serological features defined by the American College of Rheumatology. Control individuals were obtained from the British 1958 Birth Cohort, collected as part of an ongoing study following all births in England, Scotland and Wales in 1 week in 1958 (www.b58cgene.sgul.ac.uk). This cohort is an expansion of a data set previously shown to be appropriate for use as UK-wide controls (52). This study was approved by the Cambridge Local Research Ethics Committee and the Oversight Committee of the KRUK DNA Bank.

The Swedish Caucasian SLE cohort comprised patients attending the Karolinska University Hospital, Solna, who fulfilled at least 4 of the 1982 American College of Rheumatology (ACR) classification criteria for SLE. Participants were examined in person by a rheumatologist and their medical history and medical records were reviewed. Blood samples were stored in −70°C after over-night fasting. The regional ethical boards and the ethics review board at the Karolinska Institutet approved the study. All patients and controls gave informed consent.

Caucasian vasculitis cases. The ANCA-associated vasculitis cohort comprises subjects from three sources, all meeting the Chapel Hill diagnostic criteria (53):

(1) The MRC/Kidney Research UK National DNA Bank for Glomerulonephritis. Individuals were between the ages of 18 and 70 years, were ANCA seropositive and had biopsy-proven necrotizing glomerulonephritis.
(2) The UK vasculitis cohort 2 was recruited from nine centres in the UK and comprised patients seropositive for ANCA and/or with histological evidence of small vessel vasculitis.
(3) Patients recruited from the University of Birmingham. All individuals were ANCA seropositive with firm clinical and/or histological evidence of vasculitis.

Vietnamese malaria cases and controls. Severe malaria patients were recruited at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City, Vietnam. Three hundred and forty-six patients greater than 14 years of age were enrolled as part of a randomized controlled clinical trial of Artemether versus Quinine between 1991 and 1995 (54) and a further 71 patients were enrolled following the same entry criteria from 2006 to 2008. Subjects were defined as severe malaria patients if they had asexual forms of *P. falciparum* on a peripheral-blood smear and had one or more of the following as previously described (54); a score on the Glasgow Coma Scale of <11, anaemia or jaundice with a parasite count of >100 000 per mm^3 on a peripheral blood smear, renal impairment, hypoglycaemia, hyper-parasitemia and/or systolic blood pressure <80 mmHg with cool extremities. The control group comprised of 983 DNA samples extracted from the umbilical cord blood of newborn babies born at Hung Vuong Obstetric Hospital, HCMC, in 2003–2007. All samples came from unrelated individuals who were ethnic Vietnamese Kinh, as assessed by questionnaire. This study was approved by the ethical and scientific committees of the Hospital for Tropical Diseases Ho Chi Minh City.

Kenyan malaria cases and controls. The first group of severe malaria cases comprised children admitted to the high dependency unit at Kilifi District Hospital with *P. falciparum* malaria, complicated by one or more clinical features of severity (coma, prostration, multiple seizures, severe malarial anaemia or hyperparasitaemia), between 1992 and 1997 as described previously (55). A second group of severe malaria cases comprised children meeting the same criteria as children in case group 1 who were admitted to the same hospital during the period 2000–2008. The Kenyan control group was derived from cord blood samples that were collected at Kilifi District Hospital during the period 1992–2002.

Kenyan bacteraemia cases and controls. The cohort of bacteraemia cases were children (<13 years) admitted to Kilifi District Hospital with blood-culture positive bacterial infection (Gram positive and negative) between 2003 and 2008. The most frequent organisms were *Streptococcus pneumoniae, Salmonella typhi, Haemophilus influenzae* and *Escherichia coli*. The controls used for these cases were the same as those used for the group of malarial cases. Ethical approval for the collection of the Kenyan samples was given by the Kenya Medical Research Institute (KEMRI) National Scientific Steering and Research Committees.

Paralogue ratio test conditions

Three PRT primer pairs, which each co-amplified products of different lengths at two genomic locations, were used and combined to determine *FCGR3B* CN. The primer pairs PRT-2C$^{3'}$/2A$^{3'}$ co-amplified products in the 3′-untranslated region of *FCGR2C* and *FCGR2A*, PRT-3B/3A co-amplified products in *FCGR3B* and *FCGR3A* and PRT-3(A+B)/c18 [based on a published assay (27)] amplified a region of the same length in both *FCGR3A* and *FCGR3B* and a third region in Chromosome 18 (4149307–4149392, hg18). Each forward primer was labelled with HEX at the 5′ end. The PCR reaction amplified 5–10 ng of genomic DNA using Phusion Hot Start High Fidelity polymerase (Finnzymes) with the HF buffer as described by the manufacturer with the cycling conditions: 98°C for 60 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at the specified temperature for 20s and elongation at 72°C for a specified time (below). A final hold at 72°C for 7 min followed. Control samples were run to allow normalization between plates and wherever possible, plates comprised half of cases, half of controls. The co-amplified products were added to Hi-Di™ Formamide with the fluorescent GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems) and analysed on an ABI 3730 × l DNA Analyzer/Sequencer (Applied Biosystems). The HEX fluorescent peak areas corresponding to the two products were recorded by GeneMapper 4.0 software (Applied Biosystems).

PRT pair	Primer	Annealing (°C)	Elongation (s)	Product size (bp)
PRT-2C3′/2A3′	FOR 5′-CTTCATGAATTGCGCCTCAG	67	20	FCGR2C 274
	REV 5′-GCTAGAGGCCAGAAGTTCGAG			FCGR2A 279
PRT-3A/3B	FOR 5′-GATGTCCACGAATCCACTGG	65	30	FCGR3B 304
	REV 5′-GACAGGAACTCTTTACCTTCCTCG			FCGR3A 296
PRT-3(A+B)/c18	FOR 5′-TGCCCTTCATGATCTGGCC	60	5	FCGR3B/3A 75
	REV 5′-TGAGTTCAAGAAAGCAGTTTG			Chr18 86

CN designation by data clustering

The relative amounts of the co-amplified products produced in each PRT reaction were determined for each sample, and relative underlying diploid gene CN was inferred. In order to improve the discrimination between groups of individuals with different relative gene CNs, the *FCGR3B/FCGR3A* product result was plotted against *FCGR2C*$^{3'}$/ *FCGR2A*$^{3'}$, since *FCGR2C* and *FCGR3B* were expected to have the same CN. Clusters comprising a relative product amount of approximately 0.5, 1 and 1.5 for both PRT assays were designated using the 'mclust' package from Bioconductor (56,57). A Gaussian mixture model using an expectation-maximization (EM) algorithm was generated, and model-based cluster analysis was applied to group the data. The algorithm was constrained by filtering out all the cases where one or more of the intensities were zero or missing, and then the expected number of clusters was set to 3. The clusters were specified to be ellipsoidal with variable volume but constant shape. Multiple plates were normalized with controls and combined. The relative product amounts of 0.5, 1 and 1.5 corresponded to *FCGR3B:FCGR3A* diploid gene CN of 1:2, 2:2 and 3:2, respectively. CN combinations which were rare, or which formed clusters too diffuse to be called by the clustering algorithm, were called by two researchers blinded to cohort following further discrimination by plotting the *FCGR3B/FCGR3A* result against the *FCGR3(A+B)*/Chr18 result. These clusters represented the *FCGR3B:FCGR3A* diploid gene CN of 0:2, 4:2, 2:3, 2:1, 3:1 and 1:3, and assumed only one CN variable event per chromosome. *FCGR3B*:*FCGR3A* gene CN of 1:1 and 3:3 could not be discriminated from *FCGR3B*:*FCGR3A* of 2:2 due to the variability in the PRT-3(A+B)/Chr18 assay.

When adjacent regions amplified by PRT varied in CN together, the locations were assumed to be part of a single CN region. The identified regions (CNR1, CNR2, CNR3) were compared with CN regions described by a previous study using MLPA with multiple probes in each gene in the low-affinity *FCGR* locus (6,20), and the estimated ranges of CNR1 and CNR2 were extended to include surrounding gene areas based on similarities to the previous regions.

For a more detailed description of CNV assignment, see Supplementary Material.

SNP typing

The primers for the *FCGR2B*-specific long-range PCR were those used previously (58). Following cleanup of PCR products by ExoSAP-IT (GE), SNP typing was performed using a custom TaqMan Human SNP Genotyping Assay (Applied Biosystems). For *FCGR2A* and *FCGR3A* SNP typing, a pre-designed TaqMan Human SNP Genotyping Assay (Applied Biosystems) was used in accordance with the manufacturer's protocol. Fluorescence detection for allelic discrimination was performed on an Applied Biosystems 7500. Sequence detection system software (Applied Biosystems) was used to plot results and automatically call genotypes dependent on fluorescence intensities of the VIC and FAM reporter dyes.

Statistics

Association. The association of CN or SNP genotype with disease was tested by a Fisher's exact test or Chi-square contingency test where appropriate using GraphPad Prism. Meta-analysis was performed using Stats Direct software, with a random effects model to allow for variation between studies.

Linkage disequilibrium. The LD between SNPs or between SNPs and CNV was determined both at the genotype and haplotype levels. All samples used were from normal individuals from the control cohorts (UK Caucasian, Swedish Caucasian, Kenyan, Vietnamese and Hong Kong Chinese) used in the association studies. At the genotype level, LD between SNPs was determined by correlation, with the *P*-value to reject the null hypothesis that Pearson's $r = 0$ determined using GraphPad Prism. LD between a SNP and CNV was determined at the genotype level by a covariance test between SNP and CNV genotype in R.

To determine LD at the haplotype level, phase was inferred. For two SNPs, haplotype phase in both SNPs was estimated using maximum likelihood in the R genetics function LD(data), which derived the LD measures D, D' and r^2, using standard equations, as well as the chi-square statistic for linkage equilibrium. A D' over 0.25 was taken to show an appreciable level of LD between two loci. For a SNP and CNV, phased haplotypes were estimated using an maximum likelihood, in which the 3×5 table of genotypes ([0,1,2,3,4] CN × [aa aA AA] SNP genotypes) was iteratively mapped onto a 3×2 table of haplotypes ([0,1,2] CN × [a, A] SNP alleles). Hardy–Weinberg equilibrium was assumed. D' and r^2 were then determined using the same method as for two di-allelic loci, with LD for 0,1 CN versus 2 CN, 1,2 versus 0 and 0,2 versus 1 haplotype combinations calculated individually.

To determine which genotype combination was elevated due to LD, observed numbers of each diplotype combination were compared with the estimated numbers derived from the frequency of each individual diplotype.

Independence of factors. The independence of the SNP and CNV was tested by logistic regression with approximation to a general linear model (binomial family) in R, using ANOVA to test the effect of the SNP, followed by the effect of the CNV, then their interaction. The order of testing of SNP and CNV was then reversed. To combine the two Caucasian groups, a third factor was added which removed the effect of the differences between the groups prior to testing for the effect of the SNP of CNV. The OR for the risk due to each factor was expressed with 95% CIs.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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