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<th><strong>Title</strong></th>
<th>Copy number, linkage disequilibrium and disease association in the FCGR locus</th>
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<td><strong>Author(s)</strong></td>
<td>Niederer, HA; Willcocks, LC; Rayner, TF; Yang, W; Lau, YL; Williams, TN; Scott, JAG; Urban, BC; Peshu, N; Dunstan, SJ; Hien, TT; Phu, NH; Padyukov, L; Gunnarsson, I; Svenungsson, E; Savage, CO; Watts, RA; Lyons, PA; Clayton, DG; Smith, KG</td>
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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 × 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 FcγRIIB 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, 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 FcγRIIA, a valine for phenylalanine substitution (V176F, rs396991) lowers affinity for IgG1 and IgG3, and prevents binding to IgG4 (12). Two isoforms of FcγRIIB are encoded by haplotypes of four SNPs: the FcγRIIB-HNA1a isoform has reduced N-linked glycosylation compared with the FcγRIIB-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 FcγRIIB and FcγRIIA, 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 FcγRIIB 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 parologue 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 CN 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 CN, while taking into account FCGR3A CN. 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 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
To investigate this, the triple PRT assay was used to determine the possibility of ethnic variation in the FCGR2A CN of both previously described PRT assay which compared total sites in the genome on Chromosome 1; and PRT-3A(+)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.

Variation in FCGR CN 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 CN 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
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 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 \( FCGR3B \)
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 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.
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 CN 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 CN 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 CN was not due to a linked FcγRIIA-131R allele (Supplementary Material, Fig. S4 and Table S7).

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

Given the presence of LD between the FcγRIIB-I232T and FCGR3B CN loci, we investigated whether low FCGR3B CN 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...
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-232T 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-232T 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-232T, although the trend for the FcγRIIB-232T 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 CNV is equivalent between two ethnic groups. We therefore demonstrate a consistent, strong effect of low FCGR3B CNV 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...
Neutrophils lacking Fcγ from SLE patients show reduced soluble IC uptake (40,41). Clearance have been implicated in SLE, and macrophages a role of neutrophils in protection from SLE. Defects in IC FcγR3B CN of FcγR3B is the most common FcγR element receptor 3 subunit integrin alpha M (23). FcγRIIIB is expressed primarily on neutrophils, where it is the most common FcyR (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 FcyRIIIB had profoundly reduced soluble IC uptake, and increasing FCGR3B CN correlated with FcyRIIIB surface expression and increasing IC uptake (23). FcyRIIIB 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). FcyRIIIB 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 FcyRIIIB expression could thus lead to increased IC deposition and resulting inflammatory damage and disease.

FcyRIIIB 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 FcyRs. Instead, it is thought to colocalize with and signal through other receptors, such as complement receptor 3 (CR3) and FcyRIIA, 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 FcyRIIIB-mediated phagocytosis in neutrophils (46), it is possible that polymorphisms which inhibit CR3 binding to complement or interaction with FcyRIIIB 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 CN or FcyRIIIB-HNA1 polymorphisms with proximal SNPs in HapMap individuals, and found no SNPs with sufficiently high LD (assessed by r²) 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 1. FCGR3B CN stratified by FCGR2B-1293T type

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<th>UK and Swedish Caucasian(^a)</th>
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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 I/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.
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. 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.

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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 CNV 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 FcγRIIB 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 FcγRIIB 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.
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 and/or histological evidence of small vessel vasculitis.

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-2C3/2A3′ 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 × 1 DNA Analyzer/Sequencer (Applied Biosystems). The HEX fluorescent peak areas corresponding to the two products were recorded by GeneMapper 4.0 software (Applied Biosystems).

<table>
<thead>
<tr>
<th>PRT pair</th>
<th>Primer</th>
<th>Annaling (°C)</th>
<th>Elongation (s)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRT-2C3/2A3′</td>
<td>FOR 5′-CTTCTATGAATTGCGCCTCAG</td>
<td>67</td>
<td>20</td>
<td>FCGR2C 274</td>
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<td></td>
<td>REV 5′-GCTAGGGCCAGAAGTTTCGAG</td>
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<td></td>
<td>FCGR2A 279</td>
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<tr>
<td></td>
<td>FOR 5′-GATGGCCAGAATCCACTGG</td>
<td>65</td>
<td>30</td>
<td>FCGR3B 304</td>
</tr>
<tr>
<td></td>
<td>REV 5′-GACAGGAACTTTTACCTTCCTG</td>
<td></td>
<td></td>
<td>FCGR3A 296</td>
</tr>
<tr>
<td></td>
<td>FOR 5′-TGCCCTTCATGATCTGCCC</td>
<td>60</td>
<td>5</td>
<td>FCGR3B/3A 75</td>
</tr>
<tr>
<td>PRT-3(A+B)/c18</td>
<td>REV 5′-TGAGTTCAGAAGAAGCTTG</td>
<td></td>
<td></td>
<td>Chr18 86</td>
</tr>
</tbody>
</table>

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.

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.

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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 \textit{FCGR3B}/\textit{FCGR3A} product result was plotted against \textit{FCGR2C}^+/\textit{FCGR2A}^3, since \textit{FCGR2C} and \textit{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 \textit{FCGR3B}/\textit{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 \textit{FCGR3B}/\textit{FCGR3A} result against the \textit{FCGR3}(A+B)/Chr18 result. These clusters represented the \textit{FCGR3B}/\textit{FCGR3A} diploid gene CN of 0:2, 2:2, 2:3, 2:1, 3:1 and 1:3, and assumed only one CN variable per chromosome. \textit{FCGR3B}/\textit{FCGR3A} gene CN of 1:1 and 3:3 could not be discriminated from \textit{FCGR3B}/\textit{FCGR3A} of 2:2 due to the variability in the PRT-\textit{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 \textit{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 \textit{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 \textit{FCGR2A} and \textit{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

\textit{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.

\textit{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 \(D\), \(D^r\) and \(r^2\), using standard equations, as well as the chi-square statistic for linkage equilibrium. \(A^r\) 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 \(3 \times 5\) table of genotypes \([0,1,2,3,4]\) \(CN \times [a, aA, AA]\) SNP genotypes was iteratively mapped onto a \(3 \times 2\) table of haplotypes \([0,1,2]\) \(CN \times [a, A]\) SNP alleles. Hardy–Weinberg equilibrium was assumed. \(D^r\) 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.

\textit{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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REFERENCES


