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Original article

Fc-gamma receptor polymorphisms differentially influence susceptibility to systemic lupus erythematosus and lupus nephritis

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Abstract

Objective. To determine relevant Fc-gamma receptor (FcγR) polymorphisms in relation to susceptibility to SLE and LN, and to determine the functional consequences of genetic associations found.

Methods. Using multiplex ligation-dependent probe amplification, copy number regions (CNRs) and relevant known functional single nucleotide polymorphisms of FcγRII and FcγRIII were determined in a LN-enriched cohort of 266 Dutch Caucasian SLE patients and 919 healthy Caucasian controls. Expression of FcγRs on leukocytes was assessed using flow cytometry.

Results. In multivariable analysis, low copy number of CNR1 (including *FCGR3B*; odds ratio (OR) 2.04; 95% CI: 1.29, 3.23), *FCGR2A*-131RR (OR 2.00; 95% CI: 1.33, 2.99), and the 2B.4 haplotype of *FCGR2B* (OR 1.59; 95% CI: 1.13, 2.24), but not *FCGR2C* open reading frame, were significantly (all $P < 0.01$) and independently associated with susceptibility to SLE. The 2B.4 haplotype was negatively associated with LN and led to surface expression of FcγRIIb on neutrophils and monocytes.

Conclusion. This study is the first to investigate the most relevant and functional single nucleotide polymorphisms and copy number variations of FcγRII and FcγRIII polymorphisms in one study population, enabling the determination of the individual contribution of each polymorphism in multivariable analysis. Three polymorphisms were shown to be independently associated with susceptibility to SLE. The novel findings of a negative association of the 2B.4 haplotype with LN, and increased expression of FcγRIIb on neutrophils and monocytes as a result of this 2B.4 haplotype warrant future research in the role of these cells and FcγRs in the pathogenesis of SLE and LN.

Key words: Fc-gamma receptor, gene polymorphisms, systemic lupus erythematosus, lupus nephritis, Caucasian, FcγRIIb, FcγRIIc

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BASIC SCIENCE

Rheumatology key messages

- Three low-affinity Fc-gamma receptor polymorphisms are significantly and independently associated with susceptibility to SLE.
- The 2B.4 haplotype results in *de novo* expression of the inhibitory FcγRIIb on neutrophils and monocytes.
- Whereas the 2B.4 haplotype is positively associated with SLE, it is negatively associated with lupus nephritis.

Introduction

SLE is an autoimmune disease that is characterized by the formation of autoantibodies, deposition of immune complexes and inflammation, and can affect virtually every organ system in the body. Aetiologically, a multifactorial model is presumed, including genetic, hormonal and environmental factors, in which defective clearance and degradation of apoptotic cells is thought to have a pivotal role in developing SLE [1, 2].

The receptors for the Fc portion of IgG [Fc-gamma receptors (FcγRs)] play an important role in the clearance of immune complexes and presentation of the complexed antigen, and provide pro- or anti-inflammatory regulation of immune cell responses [3]. There are three families of FcγRs of which FcγRI is a high-affinity receptor and FcγRII and FcγRIII are low-affinity receptors [4]. Within these families different subclasses exist. FcγRs trigger activating signalling pathways, except FcγRIIb, which triggers inhibitory signalling pathways. FcγRIIIb is a distinct receptor expressed on neutrophils that is not known to associate with signalling molecules. Although it may activate cells in some cases, it may also function as a decoy receptor, helping to clear immune complexes [5, 6]. Failure of FcγR-mediated clearance of immune complexes and control of inflammatory responses are thought to be predisposing factors for the development of SLE [7].

The relationship between SLE and FcγRs has been extensively studied [8]. The *FCGR2/3* locus on chromosome 1q23.3 that encodes the low-affinity FcγRs is subject to both single nucleotide polymorphisms (SNPs) and copy number variation (CNV) (Fig. 1) [9]. Indeed, several of these polymorphisms lead to an altered function of FcγRs [10–13]. Meta-analyses have shown an increased risk for the development of SLE for these polymorphisms, with considerable differences between ethnic groups [14–20]. However, these meta-analyses only studied the effect of a single polymorphism and did not take linkage disequilibrium into account.

Furthermore, studies on the relationship between two specific polymorphisms and susceptibility to and clinical manifestations of SLE are very limited or even absent. The first, *FCGR2C*-open reading frame (ORF), is the result of a SNP in exon 3 on *FCGR2C* that leads to an ORF, instead of the more common stop codon (*FCGR2C*-Stop) [21, 22]. If an ORF is present, then FcγRIIc is expressed on natural killer cells and is able to induce antibody-dependent cell-mediated cytotoxicity and a rise in intracellular Ca^{2+} [23, 24]. *FCGR2C*-ORF has not been previously described in relation to SLE. The other, a SNP in the promoter region of FcγRIIb, also known as 2B.4, was found to be more frequently present in SLE patients compared with healthy controls, although the functional consequences of this SNP are not completely clear [25, 26].

Studies on associations between FcγR polymorphisms and specific clinical manifestations of SLE have mainly focused on LN, defined according to ACR criteria (proteinuria and/or cell casts) or biopsy proven. Associations with LN were found for <2 copies of *FCGR3B* and the *FCGR3A*-158F allele [18, 27, 28], but not for other polymorphisms [14, 19, 20]. Only a limited number of studies investigated the relationship between FcγR polymorphisms and other clinical SLE manifestations. These studies were performed in patient groups of different ethnic backgrounds and reported various associations without a distinct direction [29–31].

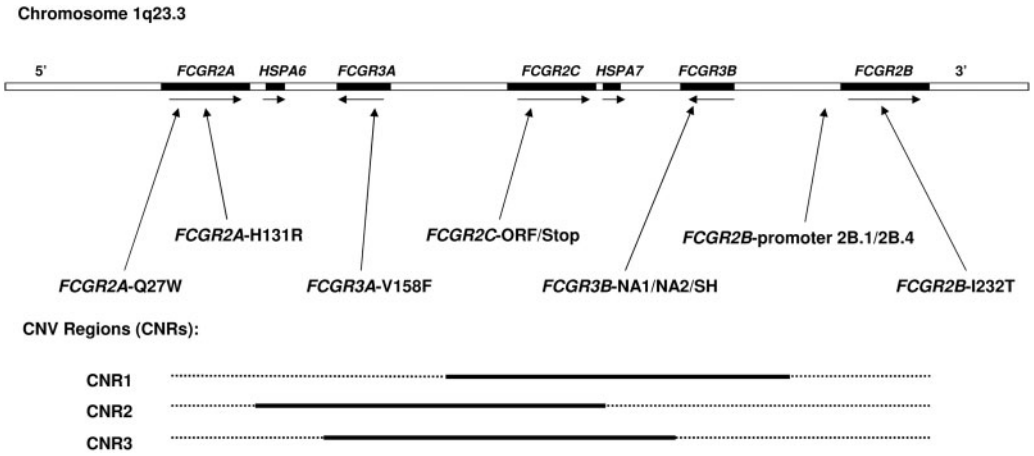
The aim of the present study is to determine associations between several known and one novel (*FCGR2C*-ORF) genetic polymorphisms and susceptibility to SLE in Caucasian patients. Secondly, to investigate the relationship between specific polymorphisms and renal disease in SLE.

Materials and patients

Subjects

DNA was available from 266 Dutch Caucasian SLE patients and 919 healthy Caucasian controls (199 from the Netherlands, 156 from Australia, 478 from Austria, 86 from the UK) who were randomly selected. SLE patients were recruited from two longitudinal SLE cohorts from VU University Medical Center (VUmc) (n = 98) and University Medical Center Groningen (UMCG) (n = 86), and from the first and second Dutch Lupus Nephritis Study (n = 82) [32]. In both Dutch Lupus Nephritis Studies, patients with a biopsy-proven proliferative LN were included. Both VUmc and UMCG provide primary through tertiary care for SLE patients. Data obtained from these patients include sex and disease duration (in years). Cumulative manifestations according to the updated revised ACR criteria for SLE were available for all SLE patients [33]. These manifestations include malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, nephrological involvement (i.e. presence of proteinuria >0.5 g/24 h and/or cell casts), neurological involvement (i.e. epilepsy and/or psychosis), haematological involvement (haemolytic anaemia, thrombopenia, leukopenia and lymphopenia), immunological involvement (presence of anti-ds DNA, anti-Sm or aCL and/or presence of lupus anticoagulant) and presence of ANA. Other recorded data were a history of biopsy-proven LN and, if present, the classification of LN according to the WHO classification system [34]. In the case of combined LN classes, the most prominent class in the biopsy was

Fig. 1 Overview of SNPs and CNV at the *FCGR2/3* locus



The approximate extent of CNRs, which show both duplication and deletion, is indicated by black lines at the bottom. SNPs and haplotypes investigated are indicated with arrows. *FCGR2A*-Q27W: SNP with unknown function; *FCGR2A*-H131R: 131H variant has increased binding affinity to IgG; *FCGR3A*-V158F: 158V variant has increased binding affinity to IgG; *FCGR2C*-ORF/Stop: *FCGR2C* contains variants that can (*FCGR2C*-ORF) or cannot (*FCGR2C*-Stop) be expressed; *FCGR3B*-NA1/NA2/SH: haplotypes determined by six SNPs, respectively encoding the HNA1a/HNA1b/HNA1c antigenic variants of HNA1; *FCGR2B*-promoter 2B.1/2B.4: haplotypes in the promoter of *FCGR2B* that influence expression; *FCGR2B*-I232T: 232T variant has decreased signalling capacity. CNR: copy number variable region; SNP: single nucleotide polymorphism; CNV: copy number variation; HNA: human neutrophil antigen.

recorded. For this study, both definitions of LN were used, namely by clinical presence of proteinuria and/or cell casts (i.e. according to ACR criteria), and/or by histology (i.e. biopsy-proven LN). It should be noted that histological evidence through biopsy is the current gold standard for LN, whereas the ACR criteria for LN are less invasive and use a clinically more feasible method, but are not as reliable as histology since proteinuria and cell casts may have other causes. In this paper, LN according to ACR criteria will be termed nephrological manifestations, whereas histological evidence of LN will be termed biopsy-proven. Written informed consent was obtained from all patients. The VUmc Medical Ethics Committee approved this study.

Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) was performed as described extensively by Breunis *et al.* [23]. In short, MLPA probes were designed specifically for the *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A* and *FCGR3B* genes on multiple sites in the genes. In this way, CNV regions for *FCGR2C*, *FCGR3A* and *FCGR3B* could be defined (Fig.1). Probes were also included to detect the following SNPs: rs1801274 (*FCGR2A* c.497A > G [p.H131R]), rs9427397 and rs9427398 (combined rs201218629), *FCGR2A* c.184C > T and c.185A > G, p[Q27W], rs1050501 (*FCGR2B* c.695T > C [p.I232T]), rs396991 (*FCGR3A* c.526G > T [p.V158F]) and *FCGR3B* haplotypes (NA1/NA2/SH). The assay also contained a probe specific for the stop codon in exon 3 of the *FCGR2C* gene, rs759550223 c.169T [p.57X] and a non-specific *FCGR2B/C* probe to detect the ORF in exon 3 [p.57Q]. Probes were also included for the splice site mutation at the border of exon 7–intron 7 in *FCGR2C* (rs76277413 c.798 + 1 A > G), to distinguish the non-expressed nonclassical *FCGR2C*-ORF variant from the classical *FCGR2C*-ORF that is typically expressed on NK cells, monocytes and neutrophils [22]. Because the nonclassical *FCGR2C*-ORF variant is not expressed [22], it was grouped with *FCGR2C*-Stop in all statistical analyses and expression studies. The assay also contained non-specific probes for the promoter regions of *FCGR2B* and *FCGR2C*; *FCGR2B/C* –386C > G (rs143796418 in *FCGR2B*, rs149754834 in *FCGR2C*) and *FCGR2B/C* –120A > T (rs780467580 in *FCGR2B*, rs34701572 in *FCGR2C*)). These promoter polymorphisms make up the promoter haplotypes 2B.1, 2B.2 and 2B.4 as described by Su *et al.* [35]. Haplotype 2B.1 consists of a G at position –386 and a T at position –120 (nucleotide positions relative to start codon of FCGR2B or FCGR2C), haplotype 2B.2 consists of –386C and –120T, haplotype 2B.4 consists of –386C and –120A. Exact location of these promoter polymorphisms was determined with a gene-specific long-range PCR for *FCGR2B* and *FCGR2C* as described previously [9]. Since –120A only occurred in the promoter of *FCGR2B* (supplementary Table S1, available at *Rheumatology* Online), promoter haplotypes were constructed from MLPA data as follows: any C at –386 accompanied by an A at –120 was designated as a 2B.4, and was allocated to *FCGR2B*. For all seven

individuals with 2B.4 haplotypes in *FCGR2B* studied for expression analysis, this was confirmed with the gene-specific long-range PCR for *FCGR2B* (supplementary Table S1, available at *Rheumatology* Online, and data not shown).

Flow cytometry

Whole blood leukocytes were isolated from heparin blood by lysis of red blood cells with an isotonic ammonium chloride buffer. Expression levels of different FcγRIIs on various types of leukocytes of 136 healthy Dutch Caucasian donors were determined by flow cytometry. The following mAbs were used to detect leukocytes subsets: anti-CD3-Pe-Cy7 clone SK7 (T cells), anti-CD14-Pe-Cy7 clone M5E2 (monocytes), anti-CD19-APC clone HIB19 (B cells) and anti-CD56-APC clone B159 (NK cells), all from BD Pharmingen (San Diego, CA, USA). Neutrophils were selected on the forward scatter (FSC)/side scatter (SSC) pattern. FcγR expression was measured with anti-FcγRIII-FITC clone 3G8 (BD Pharmingen), and anti-FcγRIIb/c clone 2B6, Alexa Fluor 488 labelled (a generous gift from MacroGenics, Rockville, MD, USA). This clone binds FcγRIIb and FcγRIIc equally well [22], but does not bind FcγRIIa [36]. To ensure specific detection of FcγRIIb, only individuals without the *FCGR2C*-ORF genotype (who cannot express FcγRIIc) (n = 105) were included for the analysis of FcγRIIb expression levels. Non-specific binding and background fluorescence were corrected for by subtracting the median fluorescence intensity of relevant isotype controls with the same fluorescent labels from the median fluorescence intensity of the staining antibodies 3G8 and 2B6. Some individuals were analysed more than once at different time points with similar results, and means are shown for these individuals. Cells were analysed on a FACS CANTO II machine (BD).

Statistical analysis

To determine susceptibility to SLE, the allele frequencies and copy number of copy number regions (CNRs) between SLE patients and healthy controls were compared using Fisher's exact test and logistic regression. Odds ratios (ORs), 95% CIs and P-values were calculated. To determine which factors were independently associated with an increased susceptibility to SLE, all polymorphisms with a $P < 0.1$ in the single regression model were put into a multiple logistic regression model. The number of CNRs or allele copies per CNR or variant, respectively, was counted. These counts were treated as a nominal class variable.

Logistic regression analysis was also used to associate allele frequencies and copy number of CNRs with clinical manifestations of SLE within the SLE patients. ORs and CIs of clinical manifestations were determined for *FCGR2A*-131R, *FCGR2A*-27W, *FCGR3A*-158V, *FCGR3B*-NA1 and *FCGR2B*-232T. *FCGR2C*-ORF and *FCGR2B*-2B.4 were interpreted as either present (regardless of the amount of copies) or absent. This way, ORs and 95% CIs were calculated. Differences in expression of FcγRs obtained from flow cytometry for all groups were tested using ANOVA or the Kruskall–Wallis test in the case of a non-Gaussian distribution. Differences between two groups were subsequently tested with Student's *t*-test. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM, Armonk, NY, USA). Figures were made with GraphPad Prism software version 6.04 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

We determined the CNVs and SNPs of the low-affinity FcγR genes *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A* and *FCGR3B* with MLPA in 266 Caucasian SLE patients and 919 healthy controls. Allele frequencies of healthy controls between countries were very similar and no statistical differences were observed ($P > 0.05$) (data not shown). Demographic and cumulative clinical features of the SLE patients are shown in supplementary Table S2, available at *Rheumatology* Online. In the 186 patients of the VUmc and UMCG lupus cohorts, nephrological manifestations (i.e. presence of proteinuria > 0.5 g/24 h and/or cell casts) were observed in 71 (38.6%) patients, of which 49 (26.6%) were biopsy proven. All 82 patients from the Dutch Lupus Nephritis Study had a history of nephrological manifestations and biopsy-proven LN.

Susceptibility to SLE

Table 1 shows the frequencies of tested CNVs and SNPs in SLE patients and healthy controls. In all patients and controls, CNV always occurred as a combination of *FCGR2C* with either *FCGR3A* or *FCGR3B*, in three distinct CNV regions (CNRs) as previously defined (Fig. 1) [7]. The CNRs were analysed as such for susceptibility to SLE and this revealed a significant association with low copy number of CNR1 (*FCGR2C* and *FCGR3B*) (Table 2). Copy number changes in CNR2 or CNR3 were not significantly associated with susceptibility to SLE. For the SNPs, increased susceptibility to SLE was found for *FCGR2A*-131R and a trend for the 2B.4 haplotype of *FCGR2B* (Table 1). *FCGR2A*-27W, *FCGR3A*-158V, *FCGR2C*-ORF, *FCGR3B*-NA1, *FCGR3B*-SH and *FCGR2B*-232I were not associated with susceptibility to SLE (Table 1). In a multivariable regression analysis, *FCGR2A*-131R, CNR1 and the 2B.4 haplotype were all independently and significantly associated with an increased susceptibility to SLE (Table 2).

Effect of *FCGR3B* CNV and the −386C/G and −120A/T SNPs in the promoter of *FCGR2B* on expression levels

Two of the three significantly associated variants conceivably have an impact on the expression levels of FcγRs. In order to test this relationship, the expression levels of different FcγRs on leukocytes were determined in a large group (n = 136) of our Dutch Caucasian healthy controls, using flow cytometry. Hereby, the strong correlation of *FCGR3B* copy number with expression of FcγRIIIb on neutrophils was confirmed (Fig. 2), indicating that CNV

TABLE 1 Copy numbers and allele frequencies of FcγR II and III genes in 919 healthy controls and 266 SLE patients

Variant	Healthy controls, n (%)						SLE patients, n (%)					P-value
	0	1	2	3	4		0	1	2	3	4	
Copy number region												
CNR1	1 (0.1)	60 (6.5)	766 (83.4)	84 (9.1)	8 (0.9)		0 (0)	33 (12.4)	205 (77.1)	28 (10.5)	0 (0)	**0.0099**
CNR2	0 (0)	11 (1.2)	867 (94.3)	40 (4.4)	1 (0.1)		0 (0)	1 (0.4)	256 (96.2)	9 (3.4)	0 (0)	0.5709
CNR3	0 (0)	0 (0)	917 (99.8)	2 (0.2)	0 (0)		0 (0)	0 (0)	265 (99.6)	1 (0.4)	0 (0)	0.6497
Allele frequencies						MAF						MAF
FCGR2A-131R	269 (29.3)	463 (50.4)	187 (20.3)	0 (0)	0 (0)	0.455	56 (21.1)	133 (50.0)	76 (28.6)	0 (0)	0 (0)	0.538	**0.0034**
FCGR2A-27W	713 (77.6)	194 (21.1)	12 (1.3)	0 (0)	0 (0)	0.119	211 (79.3)	51 (19.2)	4 (1.5)	0 (0)	0 (0)	0.111	0.7690
FCGR3A-158V	385 (41.9)	403 (43.9)	129 (14.0)	2 (0.2)	0 (0)	0.363	116 (43.6)	111 (41.7)	39 (14.7)	0 (0)	0 (0)	0.355	0.8886
FCGR2C-ORF	719 (78.2)	185 (20.1)	14 (1.5)	1 (0.1)	0 (0)	0.117	207 (77.8)	53 (19.9)	6 (2.3)	0 (0)	0 (0)	0.122	0.7517
FCGR3B-NA1	373 (40.6)	428 (46.6)	116 (12.6)	2 (0.2)	0 (0)	0.362	97 (36.5)	141 (53.0)	28 (10.5)	0 (0)	0 (0)	0.370	0.2809
FCGR3B-SH	873 (95.0)	46 (5.0)	0 (0)	0 (0)	0 (0)	0.025	254 (95.5)	12 (4.5)	0 (0)	0 (0)	0 (0)	0.023	0.8721
FCGR2B-232T	720 (78.3)	181 (19.7)	18 (2.0)	0 (0)	0 (0)	0.118	216 (81.2)	46 (17.3)	4 (1.5)	0 (0)	0 (0)	0.102	0.6210
FCGR2B haplotype 2B.4	748 (81.4)	157 (17.1)	14 (1.5)	0 (0)	0 (0)	0.101	203 (76.3)	61 (22.9)	2 (0.8)	0 (0)	0 (0)	0.122	0.0809

Text in bold indicates significant values. CNR: copy number region; FcγR: Fc gamma receptor; MAF: minor allele frequency.

of *FCGR3B* results in a clear gene-dosage effect. When determining the effect of the promoter haplotype 2B.4 on expression levels of FcγRIIb, it is important to consider that monoclonal antibodies that recognize the extracellular domain of FcγRIIb will also recognize FcγRIIc. This is due to the fact that the extracellular part of these receptors is identical. Individuals with an *FCGR2C*-ORF will express FcγRIIc on NK cells, neutrophils and monocytes [22], and possibly B cells [37]. The analysis of expressions levels of FcγRIIb should therefore be performed in *FCGR2C*-Stop donors only. Five healthy *FCGR2C*-Stop donors heterozygous for 2B.4 and two homozygous for 2B.4 were tested in an expression analysis. This revealed that the 2B.4 haplotype causes expression of FcγRIIb on neutrophils in the steady state, whereas in individuals with the 2B.1 haplotype, its expression is virtually absent (Fig. 3A). A similar effect was observed in monocytes (Fig. 3B), but on B cells, which express high levels of FcγRIIb, no difference between the different promoter haplotypes was observed (Fig. 1D). The 2B.4 haplotype did not cause expression of FcγRIIb on T cells or NK cells (Fig. 1C and E).

Susceptibility to LN

We then tested within the patient cohort for associations of *FCGR2/3* polymorphisms with susceptibility to renal disease in SLE. This revealed that the presence of the *FCGR2B* promoter haplotype 2B.4 was statically significant and negatively associated with a clinical history of nephrological manifestations or biopsy-proven LN (Table 3). Only two patients were homozygous for the 2B.4 haplotype and neither had a history of nephrological manifestations or biopsy-proven LN. Other SNPs and CNVs were not significantly associated with renal manifestations or SLE. Out of 131 patients with biopsy-proven LN, 119 (90.8%) had a predominantly class III or IV LN, 9 (6.9%) had class V LN and 2 (1.5%) had class II LN. One patient had a biopsy-proven LN of which the class could not be retrieved by chart review. We did not observe any significant differences between the tested allele polymorphisms and classes III and IV LN compared with the other LN classes (data not shown).

Finally, ORs and CIs of disease manifestations other than renal were calculated for the different polymorphisms (supplementary Table S3, available at *Rheumatology* Online). In the presence of many clinical manifestations, a relatively small study population and multiple testing, no clear associations were found.

Discussion

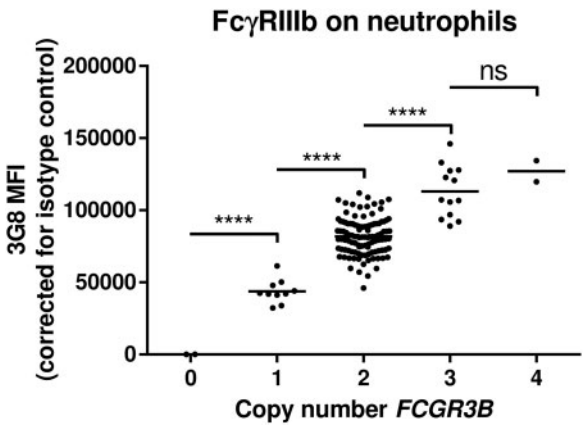
The main findings of our study are an independent and significant association with susceptibility to SLE of *FCGR2A*-131R, copy number of CNR1 (including *FCGR3B*) and the 2B.4 haplotype in the promoter of *FCGR2B*, which encodes the inhibitory FcγRIIb. Furthermore, a negative association between the 2B.4 haplotype of FcγRIIb and renal disease was shown. Thirdly, and for the first time, *FCGR2C*-ORF in SLE was

Table 2 Odds ratios and 95% CIs for susceptibility to SLE for three FcγR polymorphisms, based on single and multiple logistic regression models

Variant	Contrast	Single model		Multiple model	
		OR (95% CI)	P-value	OR (95% CI)	P-value
CNR1	Overall		0.007		**0.010**
	0 *vs* 2	0.00 (0.00, ∞)	0.992	0.00 (0.00 – ∞)	0.992
	1 *vs* 2	2.06 (1.31, 3.24)	0.002	2.04 (1.29, 3.23)	**0.002**
	3 *vs* 2	1.25 (0.79, 1.97)	0.336	1.15 (0.72, 1.8)	0.565
	4 *vs* 2	0.00 (0.00, ∞)	0.978	0.00 (0.00, ∞)	0.978
FCGR2A-131H/R	Overall		0.004		**0.003**
	HR *vs* HH	1.38 (0.98, 1.96)	0.067	1.43 (1.00, 2.03)	**0.047**
	RR *vs* HH	1.95 (1.32, 2.89)	0.001	2.00 (1.33, 2.99)	**0.001**
FCGR2B haplotype 2B.4	Overall		0.066		**0.026**
	2B.1/2B.4 *vs* 2B.1/2B.1	1.44 (1.03, 2.01)	0.033	1.59 (1.13, 2.24)	**0.008**
	2B.4/2B.4 *vs* 2B.1/2B.1	0.53 (0.12, 2.34)	0.401	0.69 (0.15, 3.10)	0.629

Text in bold indicates significant values. CNR: copy number region; FCGR: Fc gamma receptor; OR: odds ratio.

Fig. 2 Copy number of *FCGR3B* determines expression levels of FcγRIIIb on human neutrophils



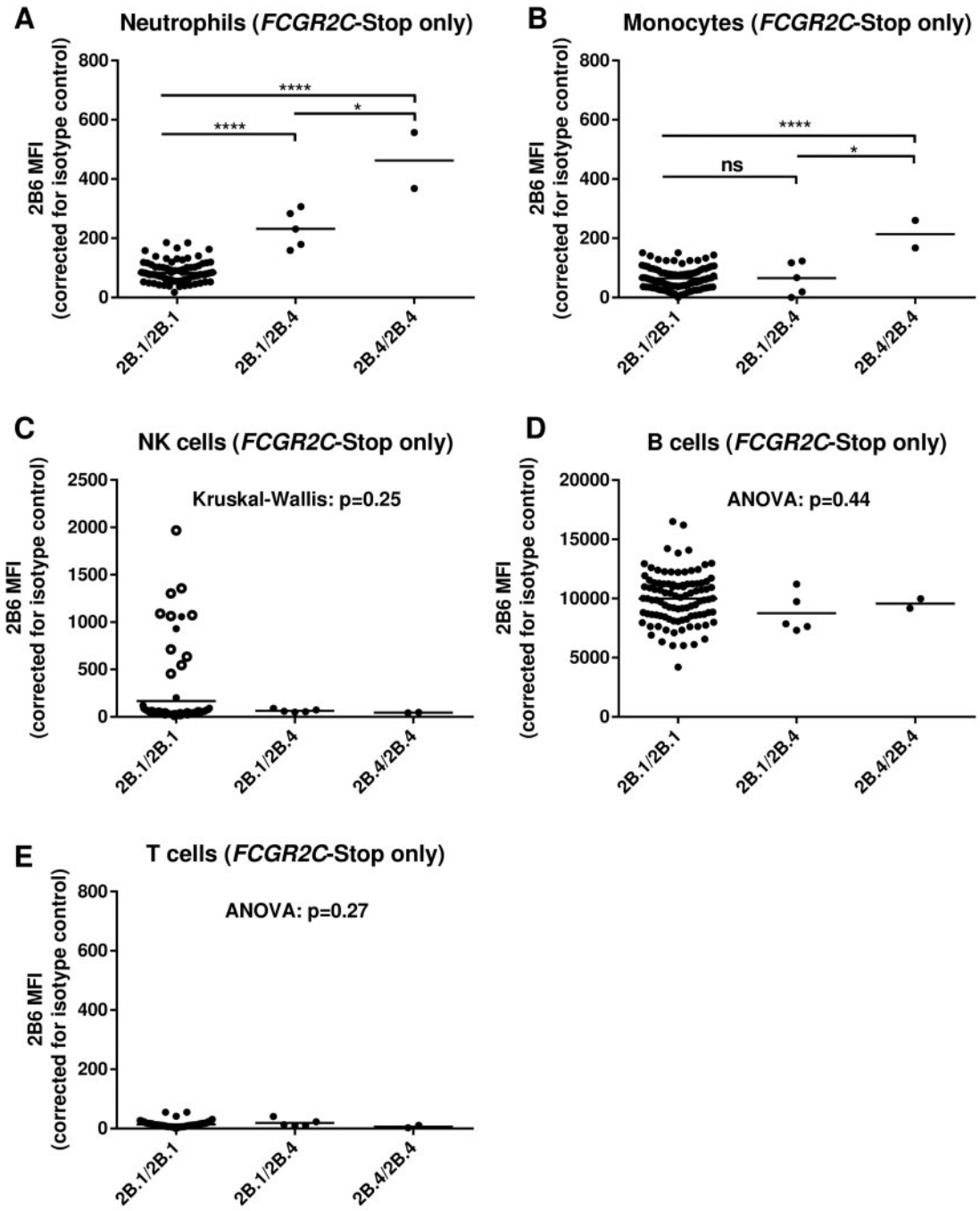
Individuals were stratified according to their *FCGR3B* copy number; expression levels of FcγRIIIb on neutrophils are shown for the different groups. Individuals with 0 copies of FCGR3B, n = 2; individuals with 1 copy, n = 10; individuals with 2 copies, n = 109; individuals with 3 copies, n = 13; individuals with 4 copies, n = 2. ns: non-significant; ****P < 0.0001; MFI: median fluorescence intensity.

investigated and an association with increased susceptibility to SLE was not found.

The strength of the present study is that it is the first to investigate the most relevant and functional SNPs and CNVs of FcγRII and FcγRIII polymorphisms in one and the same study population, enabling the determination of the individual contribution of each polymorphism in multivariable analysis. This analysis has not been done before as extensively as in the current cohort study. Previous studies and meta-analyses focused on a single SNP or CNV in relationship to SLE and/or LN susceptibility. Furthermore, detailed data of the SLE patients were available, including the results of the gold standard for LN, which is renal biopsy.

Regarding susceptibility to SLE with *FCGR2A*-131R, two meta-analyses also demonstrated *FCGR2A*-131R to be a susceptible genotype in patients of European descent [16, 17]. In addition, a meta-analysis showed low copy number of *FCGR3B* to be a susceptibility genotype for SLE in a meta-analysis [38]. However, our data now show that CNV of *FCGR3B* occurs solely as a combined deletion of different genes, collectively termed CNR1, and therefore, the interpretation of a low copy number of *FCGR3B* may not be as straightforward as it seems. Deletion of CNR1 in theory has four effects that could contribute to SLE susceptibility, as it contains three genes (*FCGR2C*, *HSPA7* and *FCGR3B*) with CNV, and additionally leads to the ectopic expression of FcγRIIb on NK cells [22]. However, CNV of *FCGR2C* in itself cannot account for susceptibility to SLE, being in most cases a pseudogene (and only the number of copies of the *FCGR2C*-ORF variant is associated with functional differences). Similarly, *HSPA7* is a non-expressed pseudogene. On the other hand, individuals with a deletion of CNR1 all had ectopic expression of FcγRIIb on NK cells, confirming our earlier finding in a larger group [22]. Indeed, the ectopic expression of FcγRIIb on NK cells was recently suggested as a potential explanation for the increased susceptibility to SLE [39], although not supported by any experimental evidence. The gene dosage effect of copy number of *FCGR3B* on FcγRIIIb expression therefore seems to be the more prominent and logical explanation for the susceptibility to SLE, as it may lead to impaired clearance of immune complexes from the circulation [5, 6]. Finally, the third risk factor identified in our susceptibility study was the promoter haplotype 2B.4, which confirms the findings of two earlier studies [25, 26]. The effects of this promoter haplotype that have been reported thus far are contradictory. One group showed an increase of FcγRIIb on B cells as well

FIG. 3 Effect of the promoter haplotype 2B.4 in *FCGR2B* on expression of FcγRIIb on various leukocytes



Individuals were stratified according to their *FCGR2B* promoter haplotype (haplotype 2B.1 consists of –386G and –120T (nucleotide positions relative to start codon), haplotype 2B.4 consists of –386C and –120A). 2B.1/2B.1: individuals homozygous for the 2B.1 haplotype, n = 98. 2B.1/2B.4: individuals heterozygous for the 2B.1 and 2B.4 haplotype, n = 5. 2B.4/2B.4: individuals homozygous for the 2B.4 haplotype, n = 2. Expression levels are shown for circulating neutrophils (**A**), monocytes (**B**), NK cells (**C**), B cells (**D**) and T cells (**E**). In (**C**), individuals with a deletion of CNR1 are shown as open circles. ns: non-significant; *$P < 0.05$; ****$P < 0.0001$; MFI: median fluorescence intensity.

TABLE 3 Odds ratios of FcγR allele frequencies of 266 SLE patients in relation to LN according to the revised ACR criteria or biopsy proven

Clinical variables	*FCGR2A* 131-R *vs* H OR (95% CI)	*FCGR2A* 27W *vs* Q OR (95% CI)	*FCGR3A* 158V *vs* F OR (95% CI)	*FCGR2C* ORF yes *vs* no OR (95% CI)	*FCGR3B* NA1 *vs* NA2 OR (95% CI)	*FCGR2B* 2B.4 *vs* 2B.1 OR (95% CI)
Nephrological manifestations	1.11 (0.79, 1.57)	0.74 (0.43, 1.27)	0.90 (0.63, 1.28)	0.71 (0.40, 1.26)	0.82 (0.57, 1.17)	**0.54 (0.31, 0.96)***
Biopsy-proven LN	1.20 (0.86, 1.70)	0.73 (0.42, 1.27)	0.92 (0.65, 1.31)	0.88 (0.48, 1.63)	0.81 (0.57, 1.16)	**0.51 (0.28, 0.91)***

*$P < 0.05$. Text in bold indicates significant values. OR: odds ratio.

as monocytes and neutrophils when 2B.4 was present [13, 25], whereas another study demonstrated an opposite effect, arguing that the 2B.4 haplotype resulted in a decrease of FcγRIIb on B cells [26]. A potential bias that may have resulted in these discordant findings is expression of FcγRIIc, which is highly homologous to FcγRIIb. Because these two receptors are identical in the extracellular domains, monoclonal antibodies detecting surface expression will not be able to distinguish between them. Although FcγRIIc can only be expressed in the subset of individuals (<15%) carrying the *FCGR2C*-ORF, this may have led to a substantial bias because the 2B.4 haplotype and the *FCGR2C*-ORF often co-occur [23]. For our analysis, we circumvented this problem by selecting only individuals without the *FCGR2C*-ORF for an expression analysis in a large group of individuals, now showing conclusive evidence for the fact that only the 2B.4 haplotype leads to *de novo* expression of FcγRIIb on myeloid cells. We did not find evidence for any effect (be it an increase or a decrease) on the expression of FcγRIIb on circulating B cells by the 2B.4 haplotype.

Another novel finding of the present study is the negative association of the *FCGR2B* promoter haplotype 2B.4 with LN (in both clinically and biopsy-proven LN). Whereas the 2B.4 promoter in SLE contributes to susceptibility to SLE, it may also protect against the development of LN. Besides a negative association of the 2B.4 haplotype with LN, we also observed a negative association with specific auto-antibodies (immunological manifestations according to ACR criteria; supplementary Table S3, available at *Rheumatology* Online), including anti-dsDNA. Previous studies have shown a clear association between the presence of anti-dsDNA and LN [40]. Recent studies have shown neutrophil extracellular traps (NETs) to be of pathophysiological importance in SLE [41]. NETs are able to trap invading microbes and subsequently eliminate them by NETosis. NETs contain DNA and histones, which could be a potential source for autoantigens. Furthermore, degradation of NETs seems to be impaired in SLE and is associated with increased titres of anti-dsDNA and LN [42, 43]. A potential hypothesis for our negative association between 2B.4 haplotype and LN could be that increased FcγRIIb expression on neutrophils increases the threshold for NET formation, thereby decreasing the levels of anti-dsDNA and the chance for development of LN. Together with the *FCGR3B* gene-dosage effect and low FcγRIIIb expression on neutrophils, this inflammatory cell type may well contribute to the pathogenesis of SLE.

Other FcγR polymorphisms were not associated with LN in the present study. Regarding *FCGR3A*-158, one meta-analysis reported an association with LN for the *FCGR3A*-158FF genotype [18], which was not found in a more recent meta-analysis [14]. Two earlier studies reported an association between low copy number (<2) of *FCGR3B* and LN compared with no renal involvement in 107 and 204 SLE patients, respectively [27, 28]. In a larger study amongst 536 SLE patients of whom 161 had LN, this association of *FCGR3B* with LN was not observed [20].

A potential limitation of our study is that the MLPA probes for the 2B.4 promoter haplotype cannot distinguish between *FCGR2B* and *FCGR2C*. To solve this problem, we determined the exact location of the 2B.4 promoter haplotype in a large group of individuals and showed that the 2B.4 promoter exclusively occurred in *FCGR2B*, confirming that our approach of allocating this haplotype to *FCGR2B* is correct.

In conclusion, our study demonstrates the pertinent and independent role of low-affinity FcγR polymorphisms in the susceptibility to SLE and one of its most serious clinical manifestations, LN. The polymorphic variants associated with SLE lead to less avid binding of pro-inflammatory receptors or increased expression of anti-inflammatory receptors, thereby supporting defective waste disposal as a key mechanism in the pathogenesis of SLE.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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