LETTERS

Copy number polymorphism in *Fcgr3* predisposes to glomerulonephritis in rats and humans

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Identification of the genes underlying complex phenotypes and the definition of the evolutionary forces that have shaped eukaryotic genomes are among the current challenges in molecular genetics[1–3]. Variation in gene copy number is increasingly recognized as a source of inter-individual differences in genome sequence and has been proposed as a driving force for genome evolution and phenotypic variation[3–5]. Here we show that copy number variation of the orthologous rat and human *Fcgr3* genes is a determinant of susceptibility to immunologically mediated glomerulonephritis. Positional cloning identified loss of the newly described, rat-specific *Fcgr3* paralogue, Fcgr3-related sequence (*Fcgr3-rs*), as a determinant of macrophage overactivity and glomerulonephritis in Wistar Kyoto rats. In humans, low copy number of *FCGR3B*, an orthologue of rat *Fcgr3*, was associated with glomerulonephritis in the autoimmune disease systemic lupus erythematosus. The finding that gene copy number polymorphism predisposes to immunologically mediated renal disease in two mammalian species provides direct evidence for the importance of genome plasticity in the evolution of genetically complex phenotypes, including susceptibility to common human disease.

Glomerulonephritis is a major cause of kidney failure in humans, and is one of the most serious complications of autoimmune disorders such as systemic lupus erythematosus (SLE). In its most severe form, necrosis of mesangial and endothelial cells with accumulation of inflammatory and epithelial cells in Bowman's space give rise to the morphological appearance of glomerular 'crescents' or crescentic glomerulonephritis. Several rodent models of crescentic glomerulonephritis have been developed. The Wistar Kyoto (WKY) rat strain is uniquely susceptible to crescentic glomerulonephritis among rat strains tested, as demonstrated by susceptibility to experimentally induced nephrotoxic nephritis (NTN) (ref. 6 and H. Rennke, personal communication) and experimental autoimmune glomerulonephritis[7]. Glomerular injury and crescent formation in rat glomerulonephritis are macrophage-dependent[8], with morphological changes closely resembling those seen in human focal and segmental necrotizing glomerulonephritis[9]. The genetic complexity of glomerulonephritis in humans led us to study the genetics of glomerulonephritis in WKY rats.

Ten days after injection of nephrotoxic serum, WKY rats consistently developed NTN, whereas Lewis, Brown Norway and Wistar rats showed no crescent formation, minimal glomerular macrophage infiltration and no proteinuria (Supplementary Fig. 1 and data not shown). F_1 rats showed intermediate phenotypes for crescent formation, proteinuria and macrophage infiltration, and phenotypes in F_2 rats spanned the range of the parental strains (Supplementary Fig. 1). Crescent formation in F_2 rats was highly correlated with proteinuria ($r^2 = 0.66$; $P < 0.0001$) and with macrophage infiltration ($r^2 = 0.30$; $P < 0.0001$). Heritability was 0.96 for crescent formation, 0.80 for proteinuria and 0.52 for macrophage infiltration.

A genome screen for NTN susceptibility loci in F_2 rats revealed two major quantitative trait loci (QTLs) on chromosomes 13 and 16 (designated crescentic glomerulonephritis 1 (*Crgn1*) and 2 (*Crgn2*)), both of which were linked to crescent formation and proteinuria (logarithm of the odds (LOD) scores 7.4–9.1; Fig. 1a). Infiltration of macrophages was also strongly linked only to *Crgn1* (LOD 6.1; Fig. 1b, c). Several additional linkages (LOD scores 3–4) to crescent formation and proteinuria were detected on other chromosomes and were designated *Crgn3–7*. *Crgn1* and *Crgn2* accounted, respectively, for 21.8% and 16.8% of the genetic variance in crescent formation and 16.7% and 17.7% of the genetic variance in proteinuria. *Crgn1* accounted for 12.9% of the genetic variance in glomerular macrophage infiltration.

Several biological candidates were found in the *Crgn1* region of linkage including the genes encoding the activatory Fc receptor for IgG, *Fcgr3* (also known as FcγRIII), the inhibitory Fc receptor *Fcgr2* (FcγRII), and the common γ-subunit *Fcer1g* (FcRγ). We sequenced the coding region of *Fcer1g* and found no sequence variants. In *Fcgr2* we found a single nucleotide substitution, G388T, which changed codon 103 from arginine in Lewis rats to leucine in WKY rats. Sequence analysis of *Fcgr3* revealed evidence of a genomic rearrangement involving the *Fcgr3* locus and was therefore investigated further.

Polymerase chain reaction (PCR) amplification of *Fcgr3* exons from genomic DNA revealed single bands for exons 1–4 in Lewis and WKY rats, and a single band from WKY exon 5 but two discrete PCR products from Lewis exon 5 (Fig. 1d), suggesting duplication of exon 5 in Lewis genomic DNA, with loss of the shorter exon 5 from WKY genomic DNA. Direct sequence analysis of gel-purified products of exon 5 genomic DNA indicated the presence of a 226-base pair (bp) sequence in the longer PCR product (designated exon5_226+) that was absent in the shorter product (exon5_226−). The 226-bp sequence was situated in the 3′-untranslated region and contained a 153-bp short interspersed repetitive element (SINE).

PCR analysis of 27 divergent rat strains showed an identical pattern to Lewis, whereas spontaneously hypertensive rats (SHR) and stroke-prone SHR (SHRSP) showed the same pattern as WKY. Because WKY, SHR and SHRSP rats were derived in the mid-twentieth century from the same outbred Kyoto colony of Wistar rats[10], we determined the genotype of five additional strains derived

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from this colony. As with WKY, four of these also show loss of exon5_226− (Fig. 1e). The loss of exon5_226− from seven strains, all descended from the Kyoto Wistar colony, suggests that these strains have inherited this chromosomal segment identical-by-descent.

Because mesangial cell damage and glomerular necrosis are key features of NTN and are macrophage-dependent, we developed an *in vitro* assay of macrophage-mediated killing of antibody-coated glomerular mesangial cells as an intermediate phenotype of glomerular pathology in NTN. Macrophages from WKY rats showed markedly enhanced antibody-dependent cellular cytotoxicity (ADCC) compared with macrophages from Lewis rats (Fig. 2a). We further phenotyped Lewis, WKY and five of the other Kyoto-derived strains using a semi-automated, fluorescence-based assay of ADCC and found that five strains (WKY, SHR, WTC, WKYO, DON) had increased macrophage killing compared with Lewis, whereas one (IS/KYO) had macrophage activity comparable to Lewis (Fig. 2b). All of the strains with increased macrophage activity have lost exon5_226− from their genome. Haplotype analysis on chromosome 13 defined a 27-kilobase (kb) region, containing only one annotated gene (*Fcgr3*), that co-segregated with increased macrophage activity across these rat strains (Fig. 2c; see also Supplementary Information), excluding other genes in this region, including *Fcgr2*, as a cause of increased macrophage activity in WKY rats.

In addition to identification of the exon5_226+/− variant, sequence analysis of *Fcgr3* exon 5 revealed a single nucleotide deletion in the coding sequence at position 129 (ΔG129), found only in the shorter exon 5 (exon5_226−) (Fig. 3a). Reverse transcriptase PCR (RT–PCR) showed that the ΔG129-containing exon 5 is transcribed (Fig. 3b), and we designate the gene from which this transcription product is derived as *Fcgr3-rs*. No copies of the ΔG129 variant were found in 65 separate clone inserts amplified from WKY complementary DNA or genomic DNA, confirming loss of *Fcgr3-rs* from the WKY genome.

The ΔG129 variant results in a frameshift in the *Fcgr3-rs* coding sequence that predicts a novel cytoplasmic domain six amino acids longer than that encoded by *Fcgr3*. Sequence comparison showed that although Fcgr3-rs is highly similar to the other isoforms of Fcgr3 across the extracellular and transmembrane domains, the Fcgr3-rs cytoplasmic domain has no homology to other known proteins.

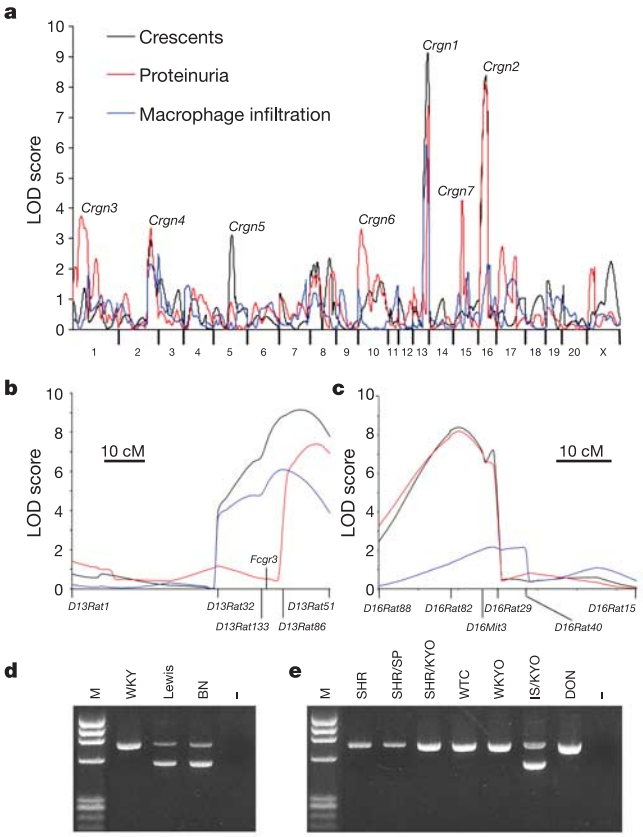

Figure 1 | Genome screen for NTN susceptibility loci, and duplication and loss of *Fcgr3* exon 5. **a**–**c**, Multi-point linkage plots showing location of susceptibility genes for crescent formation, proteinuria and macrophage infiltration on a whole genome plot (**a**), chromosome 13 (**b**) and chromosome 16 (**c**). cM, centimorgan. **d**, **e**, PCR amplification of *Fcgr3* exon 5 from Lewis and WKY rats with Brown Norway strain as reference, showing absence of exon5_226− in WKY (**d**), and in six out of seven other Kyoto-derived rat strains (**e**). −, negative control PCR; M, ΦX174 *Hae*III size marker.

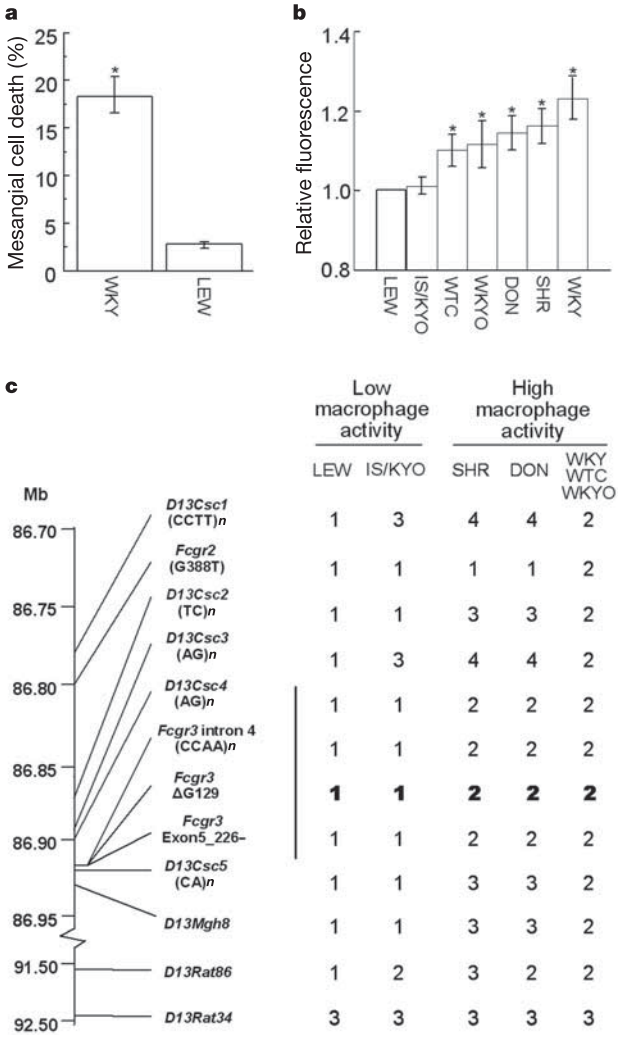

Figure 2 | Macrophage activity and haplotype analysis. **a**, **b**, Macrophage-mediated killing of antibody-coated mesangial cells by Lewis and WKY macrophages determined by visual assessment ($n = 5$ rats per strain; asterisk indicates $P < 0.001$) (**a**), and in different rat strains determined by release of fluorescence, normalized to Lewis macrophages ($n = 4$ rats per strain; asterisk indicates $P < 0.01$ compared to Lewis) (**b**). Data are mean ± s.e.m. **c**, Chromosome 13 haplotype. The vertical bar indicates the interval shared between rat strains with macrophage overactivity. No other marker genotypes co-segregated with macrophage overactivity within the *Crgn1* linkage region. High and low macrophage activity denote, respectively, strains with macrophage activity that is significantly increased, or not, compared to Lewis. Numbers denote classification of alleles: 1, Lewis; 2, WKY; 3, 4, other alleles. The *Fcgr3-rs* ΔG129 genotype is indicated in bold.

Southern analysis confirmed the presence of at least three copies of *Fcgr3*-like exon 5 sequences in Lewis and WKY genomic DNA and reaffirmed loss of *Fcgr3-rs* exon 5 from the WKY genome (Fig. 3c); clonotype analysis (Supplementary Fig. 2) indicated the presence in both Lewis and WKY rats of at least two distinct transcribed copies of exon5_ins226+. Additional Southern analysis of two *Fcgr3*-containing bacterial artificial chromosomes from the rat genome project (data not shown) showed identically sized restriction fragments to those shown in Fig. 3c, indicating that these genes reside in contiguous genomic DNA on chromosome 13.

We then carried out western analysis of macrophage lysates from Lewis and WKY rats using anti-Fcgr3-rs-specific antiserum. This detected a 66-kDa band in Lewis but not in WKY rats (Fig. 3d), confirming expression of Fcgr3-rs protein in Lewis macrophages and its deficiency in WKY.

To compare function of Fcgr3 and Fcgr3-rs, we stably transfected COS-1 cells with the common γ-subunit (*Fcer1g*) together with either the *Fcgr3* or *Fcgr3-rs* α-subunits. We also transfected COS-1 cells with the *Fcgr3* construct after modification by site-directed mutagenesis to delete the single nucleotide G129 from exon 5, yielding a chimaeric construct, *Fcgr3-ΔG*, that contains Fcgr3 extracellular and transmembrane domains with an Fcgr3-rs cytoplasmic domain. We tested the various stably transfected COS-1 cells for their potential to phagocytose opsonized sheep red blood cells (SRBCs). Similar expression of all *Fcgr3* constructs was confirmed by western analysis using an antibody to the Myc tag (data not shown), and cell surface expression was confirmed by flow cytometry (Supplementary Fig. 3). Cells transfected with both *Fcer1g* and *Fcgr3* showed levels of phagocytosis over tenfold greater than cells transfected with *Fcer1g* and either *Fcgr3-rs* or the *Fcgr3-ΔG* constructs (Fig. 3e). Co-transfection of *Fcgr3* with *Fcgr3-rs* showed a 70% inhibition of Fcgr3-mediated phagocytosis (Fig. 3f), indicating that loss of Fcgr3-rs-mediated inhibition of Fcgr3 is the likely mechanism for macrophage overactivity in WKY compared to Lewis rats.

Our previous studies of Fc receptor polymorphisms in northern European nuclear families with SLE showed unexpected mendelian errors for two polymorphisms in the *FCGR3B* gene in 14% of these families[11]. The association of *Fcgr3* copy number variation with immunologically mediated glomerulonephritis in the rat led us to test the hypothesis that copy number variation in human *FCGR3B* might explain these mendelian errors and that this could contribute to glomerulonephritis susceptibility.

We developed a quantitative PCR assay to measure *FCGR3B* gene copy number and applied this assay to 30 individuals from a subset of 8 of the nuclear families shown previously to have non-mendelian inheritance at *FCGR3B*. This showed significant variation in *FCGR3B* copy number that was consistent with an estimate by Southern analysis (Fig. 4a). *FCGR3B* copy number differed significantly in this sample from that expected for a single copy gene in a diploid genome ($P = 0.0004$; Supplementary Fig. 4).

We tested for an association between *FCGR3B* copy number and disease in SLE patients and in the subset of SLE patients with glomerulonephritis, referred to as lupus nephritis. Discordant sib pair analysis (one sib pair per family) showed no association between *FCGR3B* copy number and SLE ($n = 187$ sib pairs, Wilcoxon sign-rank test, $P_{2\text{-tailed}} = 0.083$, 95% confidence interval (CI) 0.082–0.085), but

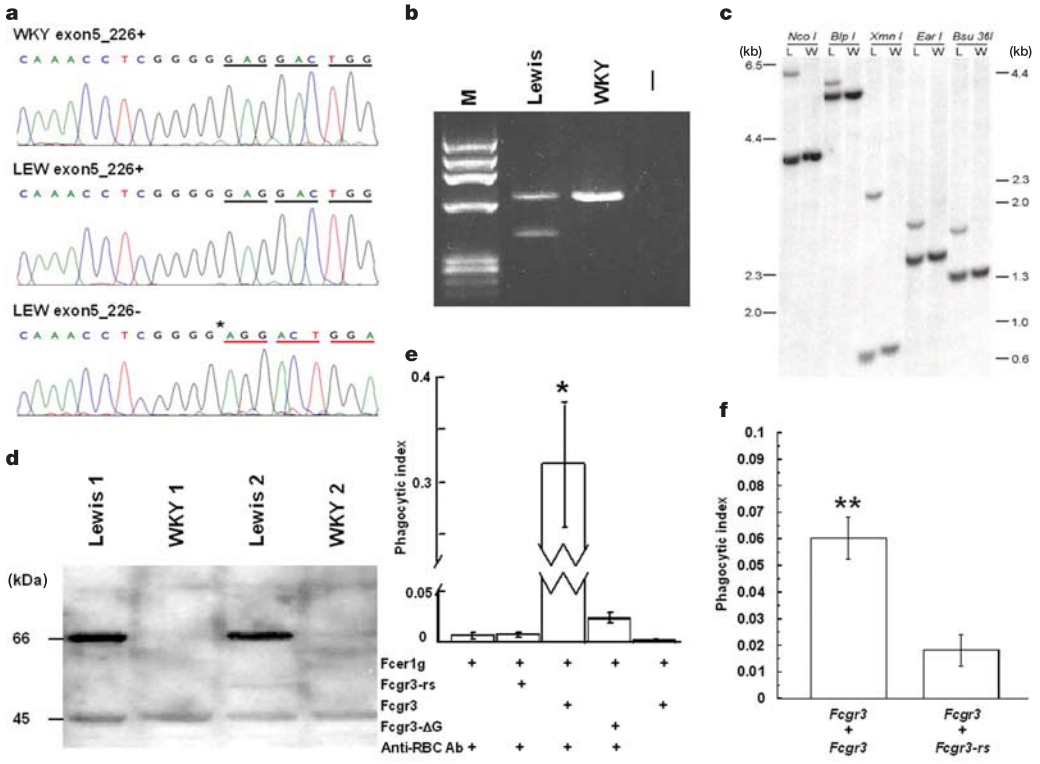

Figure 3 | Identification and functional characterization of *Fcgr3-rs*. **a**, Sequence analysis of exon 5 in Lewis and WKY rats showing a guanidine deletion at position 129 (ΔG129) in Lewis exon5_226−, denoted with an asterisk. The new reading frame is underlined in red. **b**, RT–PCR of Lewis and WKY RNA indicates expression of *Fcgr3-rs* exon5_226− in Lewis but not in WKY rats. M, ΦX174 *Hae*III size marker. **c**, Southern blot of the *Fcgr3* locus with genomic DNA from WKY (W) and Lewis (L) rats. All of the restriction enzymes cut within the exon 5 SINE-containing insertion, and do not cut within the probe sequence. *Xmn*I, *Ear*I and *Bsu*36I digests were loaded after the *Nco*I and *Blp*I digests and have separate size markers (kb). **d**, Western blot of peritoneal macrophage lysate using a custom antibody to the Fcgr3-rs cytoplasmic tail, showing presence of Fcgr3-rs in Lewis but not in WKY macrophages on replicate loadings. **e**, Phagocytosis of antibody-coated erythrocytes by COS-1 cells transfected with *Fcgr3* constructs. Asterisk, $P < 0.01$ compared to all other constructs. **f**, Co-transfection of *Fcgr3* with *Fcgr3-rs* showing inhibition of Fcgr3-mediated phagocytosis by Fcgr3-rs ($n = 8$ paired transfections). Double asterisk, $P < 0.002$. Data are mean ± s.e.m.

showed a weak association with lupus nephritis ($n = 61$ pairs, Wilcoxon sign-rank test, $P_{2\text{-tailed}} = 0.038$, 95% CI 0.037–0.039). Separate association analysis, carried out between all available lupus nephritis patients ($n = 63$) and unrelated, seronegative controls ($n = 141$) from the lupus cohort also showed association with lupus nephritis (Mann–Whitney *U*-test, $P = 0.001$, 95% CI 0.001–0.002; Fig. 4b). Logistic regression analysis, using age and gender as covariates, confirmed the association between *FCGR3B* copy number and lupus nephritis in this sample (χ^2 (3 degrees of freedom) = 13.5, $P = 0.004$; Supplementary Table 1A).

Because previous studies have shown an association between SLE and the *FCGR2A* and *FCGR3A* genes within the Fc receptor cluster on chromosome 1q23 (refs 12, 13), we carried out a sequential logistic regression analysis to test whether the effect of *FCGR3B* copy number on lupus nephritis was independent of *FCGR2A* and *FCGR3A*. Using data from 60 lupus nephritis patients and 109 unrelated, seronegative controls in this cohort for whom complete genotype data was available, the sequential logistic regression analysis model predicted *FCGR3B* copy number as being significantly and independently associated with lupus nephritis (χ^2 (3 degrees of freedom) = 13.4, $P = 0.004$), whereas the inclusion of *FCGR2A-G548A* and *FCGR3A-T559G* produced poorer models, indicating that the effects of *FCGR3A* and *FCGR2A* are very small compared with that observed for *FCGR3B* (Supplementary Table 1B). An analysis of *FCGR2A-G548A* and *FCGR3A-T559G* haplotypes in this group did not show any association with copy number at *FCGR3B* (Fisher's exact test, $P_{2\text{-tailed}} > 0.5$), providing further evidence that reduced copy number at *FCGR3B* is an independent risk factor for lupus nephritis.

Fc receptors, the genes of which are located in clusters across mammalian genomes, functionally link the humoral and cellular branches of the immune system and have a key role in activation and modulation of the immune response[14,15]. Our findings in rats that loss of Fcgr3-rs results in macrophage overactivity and glomerulonephritis susceptibility, and that Fcgr3-rs inhibits Fcgr3-mediated phagocytosis, suggest that Fcgr3-rs may be a potential therapeutic agent for autoimmune disease. Human FCGR3B is expressed mainly in neutrophils and is necessary for neutrophil tethering to immune complexes[15–17]. It is therefore plausible that reduced neutrophil expression of FCGR3B in patients with low *FCGR3B* copy number may lead to reduced glomerular clearance of immune complexes and susceptibility to autoimmune renal disease in patients with SLE, and possibly to other autoimmune disorders.

So far, there is little evidence that common, complex disease phenotypes can be caused by stably transmitted gene duplication/deletion or copy number polymorphisms[4]. Two examples that have been reported are *Cd36* deletion in insulin resistance in rats and *CCL3L1* copy number polymorphism in HIV-1/AIDS susceptibility in humans[18–20]. Although multiple genomic copies of *Fcgr3* have been suggested in the rat[21], and gene duplication and deletion in human *FCGR3B* have been reported in isolated cases or in families identified because of FCGR3B deficiency, their association with immune phenotypes has not been clear[22–24]. Our studies have demonstrated naturally occurring gene copy number variation within the syntenic *Fcgr3* clusters in rats and humans, and showed that loss of *Fcgr3-rs* in the rat and low *FCGR3B* copy number in humans are associated with susceptibility to immunologically mediated forms of glomerulonephritis in these two mammalian species. To our knowledge, this is the first demonstration in any species that gene copy number polymorphism predisposes to autoimmune disease. Our finding that copy number polymorphism at orthologous regions of diverse genomes is associated with immunologically related disease suggests that genome plasticity, manifested by gene duplication/deletion and copy number polymorphism, is a more common cause of genetically complex phenotypes than has hitherto been observed.

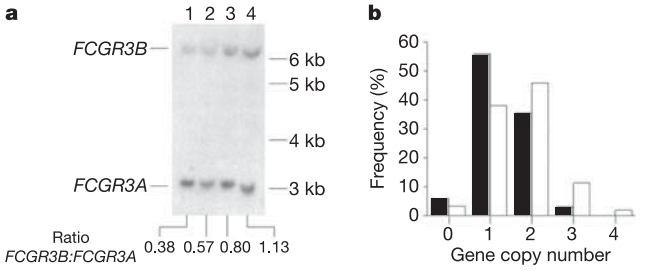

Figure 4 | Copy number polymorphism in human *FCGR3* and association with lupus nephritis. a, Southern blot of genomic DNA from four unrelated individuals, selected according to the quantitative PCR estimate of *FCGR3B* copy number, shown above the blot. The Southern probe was designed to cross-hybridize to *FCGR3A* and *FCGR3B* restriction fragments. *FCGR3B* band intensity, measured by densitometry, is normalized to *FCGR3A* band intensity, and shown below the plot as the ratio of *FCGR3B*:*FCGR3A*. Genomic DNA was digested with the restriction enzyme *Hpa*I. **b**, Histogram showing frequency distribution of *FCGR3B* copy number, per diploid genome, in lupus nephritis cases (filled columns) and unaffected controls (open columns).

METHODS

See Supplementary Information for detailed Methods.

Inbred strains and linkage studies. WKY/NCrlBR (abbreviated to WKY) and SHR/NCrlBR (SHR) rats were obtained from Charles River and Lew/HanHsd (Lewis) rats from Harlan. WTC, WKYO, DON and IS/KYO rats were obtained from T. Serikawa. Reciprocal crosses between WKY and Lewis (both *Rt1-l*) rats were used to generate 177 F_2 rats that were phenotyped for NTN and used in a genome screen for susceptibility genes with 128 polymorphic microsatellite markers. No phenotypic differences were found between the reciprocal crosses, which were combined for linkage studies. NTN phenotypes were assessed in 200–220 g male rats 10 days after intravenous injection of 0.1 ml of a rabbit antiserum to rat glomerular basement membrane[25]. Crescent formation, 24 h urinary protein and glomerular macrophage infiltration were assessed as described[26].

Direct sequencing and sequencing of cloned PCR products. RNA extraction from frozen tissues, cDNA preparation and PCR were undertaken as described[18], and clonotype analysis was performed using the high-fidelity polymerases *Pfu* (Promega) or *KOD1* (Novagen).

Southern and western analyses. Southern analyses were carried out as described[18]. For western analysis, 10 μg denatured protein from lysed thioglycollate-elicited macrophages or COS-1 cells was resolved by 4–12% gradient SDS–PAGE. Proteins were electro-blotted onto PVDF membranes (Invitrogen) and stained with antibodies against the Fcgr3-rs cytoplasmic domain (macrophages) or against Myc tag (COS-1 cells).

Antibody-directed cellular cytotoxicity. ADCC assays were carried out by visual assessment of cell death of antibody-coated mesangial cells and by a semi-automated dye release assay, as described[27]. Mesangial cells were derived from WKY kidneys. Similar results were found with Lewis mesangial cells. For the dye release assay, thioglycollate-elicited peritoneal macrophages were added to cultured mesangial cells loaded with calcein fluorescent dye in the presence of 10 μg ml^{-1} anti-Thy1.1 antibody. Calcein release into the supernatant was measured in a microplate fluorimeter. Differences between inbred strains were assessed by Student's *t*-test (1-tailed).

Studies of Fc receptor function in COS-1 cells. Constructs of *Fcer1g*, *Fcgr3*, *Fcgr3-rs* and *Fcgr3-ΔG* were created from PCR-amplified cDNA. A Myc tag was introduced into the amino terminus of all three *Fcgr3* α-subunit constructs to allow confirmation of expression by western blot. COS-1 cells stably transfected with *Fcer1g* were electroporated with *Fcgr3*, *Fcgr3-rs* or *Fcgr3-ΔG* and selected with G418 and hygromycin B. Fc-receptor-mediated function was assessed by measuring internalization into COS-1 cells of SRBCs coated with anti-SRBC antibody. Phagocytic index (RBCs per COS-1 cell) was calculated as described[28]. The effect of Fcgr3-rs on Fcgr3-mediated phagocytosis was determined by transfection of COS-1 cells that had been transfected with, and were expressing, *Fcer1g* and *Fcgr3*. Differences between groups were compared using Student's *t*-test (1-tailed).

Nucleotide and protein sequence analysis. Homologous sequences to rat *Fcgr3* and *Fcgr3-rs* exon 5 sequences were sought by searching the NCBI non-redundant and high-throughput genomic sequence databases using BLAST.

Quantification of *FCGR3B* copy number. Quantitative PCR was carried out

using SYBR Green Jumpstart *Taq* Readymix (Sigma) and analysed by the standard curve method. Oligonucleotides were designed to amplify specifically *FCGR3B* and to avoid paralogous or allelic sequence variants. *CD36* was used as a single-copy control. Data from *FCGR3B* and *CD36* were normalized to forkhead box P2 (*FOXP2*) to give an estimate of copy number. Sequence analysis was undertaken to confirm specificity of the *FCGR3B* PCR product.

Human genetics. DNA was available from 256 SLE nuclear families (consisting of parents and progeny) of northern European origin in which genotypes for *FCGR3B* had been previously determined[29]. SLE and lupus nephritis were defined according to ACR criteria[30]. This cohort consisted of a single SLE patient per family. Of the 256 SLE patients studied, 63 had nephritis and were available for analysis. Discordant sib pair analyses were performed for all families with available sib pairs (one sib pair per family) using the nonparametric Wilcoxon sign-rank test. We also compared the 63 lupus nephritis cases with unrelated controls ($n = 141$) (one per family) from within the SLE cohort. All controls used were seronegative for antinuclear antibody. The study was ethically approved under MREC 98/2/6.

A one-sample sign test of the median was used to determine whether gene copy number differed significantly from 2. To test for the significance of the difference between the distributions of the *FCGR3B* gene copy numbers we used the nonparametric Mann–Whitney *U*-test (2-tailed). For both the Wilcoxon sign-rank test and the Mann–Whitney *U*-test, 100,000 Monte Carlo simulations were performed to estimate exact *P* values and 95% confidence intervals. Logistic regression was performed using SPSS 12.0 with *FCGR3B* gene copy number and genotypes of *FCGR2A* and *FCGR3A* as predictor variables. Haplotypes for *FCGR2A-G548A* and *FCGR3A-T559G* were constructed as described[11].

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The sequence of *Fcgr3-rs* exon 5 has been deposited in GenBank under accession number AY561710. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to T.J.A. (t.aitman@csc.mrc.ac.uk) or H.T.C. (t.h.cook@imperial.ac.uk).