Copy number of *FCGR3B*, which is associated with systemic lupus erythematosus, correlates with protein expression and immune complex uptake

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Copy number (CN) variation (CNV) has been shown to be common in regions of the genome coding for immune-related genes, and thus impacts upon polygenic autoimmunity. Low CN of *FCGR3B* has recently been associated with systemic lupus erythematosus (SLE). FcγRIIIb is a glycosylphosphatidylinositol-linked, low affinity receptor for IgG found predominantly on human neutrophils. We present novel data demonstrating that both in a family with FcγRIIIb-deficiency and in the normal population, *FCGR3B* CNV correlates with protein expression, with neutrophil uptake of and adherence to immune complexes, and with soluble serum FcγRIIIb. Reduced FcγRIIIb expression is thus likely to contribute to the impaired clearance of immune complexes, which is a feature of SLE, explaining the association between low *FCGR3B* CNV and SLE that we have confirmed in a Caucasian population. In contrast, antineutrophil cytoplasmic antibody–associated systemic vasculitis (AASV), a disease not associated with immune complex deposition, is associated with high *FCGR3B* CN. Thus, we define a role for *FCGR3B* CNV in immune complex clearance, a function that may explain why low *FCGR3B* CNV is associated with SLE, but not AASV. This is the first report of an association between disease-related gene CNV and variation in protein expression and function that may contribute to autoimmune disease susceptibility.

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A substantial amount of the human genome is subject to copy number (CN) variation (CNV); 12% of it lies within CN-variable regions (1). These regions contain submicroscopic segments of DNA, ranging in size from kilobases to megabases, which may be deleted in some individuals but duplicated, perhaps several times, in others. CNV has been defined as a DNA segment that is 1 kb or larger and is present at variable CN in comparison with a reference genome (2). The implications of such widespread genetic CNV on phenotypic variation are as yet uncertain, but some CN variants are clearly associated with disease. CNV may directly cause disease when the genomic rearrangements disrupt genes vital for normal development; e.g., both microdeletions and microduplications at chromosome 17p11.2 result in syndromes characterized by developmental delay and mental retardation (3). CNV has also been associated with complex disease traits, e.g., susceptibility to HIV infection,

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psoriasis, and systemic lupus erythematosus (SLE), diseases in which multiple genetic and environmental factors play a role in pathogenesis (4–8). As the number of genetic association studies linking CNV and common, complex disease traits increases, it will be necessary to determine the effect of CNV on both gene expression and cellular function to explain how CNV affects disease pathogenesis. This is particularly important in view of several potential problems with CN assays, which often produce results that are continuously distributed across the population, rather than falling into discrete "bins" associated with CN. Difficulties in CN assignation could thus lead to false associations (7). In addition, CNV in one gene may be in linkage disequilibrium with CNV and/or single-nucleotide polymorphisms in other genes, making it difficult to determine by genetic analysis alone which variant is causal. Correlation of expression and function with CN assays can therefore help validate the CN assay itself, in addition to explaining the disease association.

CN variability can affect gene expression: in a recent study by Stranger et al., CNV accounted for 18% of variation in gene expression in cell lines from individuals in the HapMap project (9). Because many genes that control the immune system are found in CNV regions (1), it is not surprising that CNV should determine differences in immune system activation between individuals, and thus susceptibility to immune-mediated disease. CNV in two genes in different chromosomal locations (*C4* on chromosome 6 [6] and *FCGR3B* on chromosome 1 [5]) have been independently associated with susceptibility to SLE, a complex polygenic autoimmune disease characterized by autoantibody production, immune complex deposition, and inflammatory damage to multiple organ systems.

FcγRIIIb is one member of a family of low-affinity Fcγ receptors that are widely expressed on cells of the immune system, binding IgG in immune-complexed rather than soluble form. They are encoded by a cluster of genes found on distal chromosome 1 and can be activatory (FcγRIIa, FcγRIIIa, FcγRIIIb, and FcγRIIc) or inhibitory (FcγRIIb) (10). FcγRIIIb is found only in humans and is unique among this family of receptors in that its expression is largely restricted to neutrophils and it is linked to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (11). It has no cytoplasmic domain, and therefore is thought to signal through association with complement receptor 3 (12), FcγRIIa (13), or lipid rafts (14). FcγRIIIb can also be released from the surface of neutrophils upon activation (15). There are two common polymorphic forms of *FCGR3B* (*FCGR3B*1* and *FCGR3B*2*), which differ by five nucleotides (141, 147, 227, 277, and 349) within exon 3, resulting in a 4-aa substitution in its membrane-distal, immunoglobulin-like extracellular domain. This gives rise to two isoforms termed FγRIIIb-HNA1a and FcγRIIIb-HNA1b (previously known as NA1 and NA2, respectively) (16). The FcγRIIIb-HNA1b (NA2) polymorphism has been associated with SLE (17), whereas the FcγRIIIb-HNA1a (NA1) polymorphism has been associated with antineutrophil cytoplasmic antibody–associated systemic vasculitis (AASV) (18).

The function of FcγRIIIb has been difficult to elucidate, as there is no mouse orthologue. Studies using ligation or blocking of FcγRIIIb and those removing all GPI-linked proteins from the neutrophil surface have often given conflicting results, which is perhaps not surprising in view of the different methods used. Thus, there is debate about whether FcγRIIIb binds immune-complexed IgG under static (19) or only under flow (20) conditions. One study suggests that FcγRIIIb binding mediates phagocytosis (21), whereas others suggest it is involved in surface binding only (19, 22). FcγRIIIb ligation may play a role in generation of the neutrophil respiratory burst (23), but, again, this is controversial (24). Approximately 0.1% of Europeans do not express FcγRIIIb (25, 26). Such FcγRIIIb-deficient individuals have been described in the context of neonatal neutropenia (25) and in a single case of SLE (27). In contrast to antibody-blocking studies (21), an in vitro study of cells from FcγRIIIb-deficient individuals failed to show a functional deficit, leading to the suggestion that an increase in surface expression of FcγRI may compensate (28). Thus, the function of FcγRIIIb remains poorly defined, presumably because of nonphysiological effects of the blocking agents used in some studies and the fact that CNV has not been taken into account. Further studies in FcγRIIIb-deficient subjects are the best way to define function and resolve these controversies.

In this study, we first confirmed the observation that SLE was associated with low *FCGR3B* CN in Caucasians (5), though we did not find such an association with SLE in a Chinese population. To determine the mechanism underlying this association, we investigated the functional effects of *FCGR3B* CNV. Using neutrophils from an individual deficient in the receptor and from members of her family, we showed that *FCGR3B* CN correlates with cell surface expression, levels of soluble FcγRIIIb, and neutrophil adherence to, and uptake of, immune complexes. We found the same relationship between *FCGR3B* CN, protein expression, and function exists in the general population. We then went on to investigate *FCGR3B* CNV in AASV. AASV is a systemic autoimmune disease characterized by inflammation and necrosis of the microvasculature. The pathology is mediated by FcγR-mediated activation of neutrophils rather than immune complex deposition as in SLE. Unlike SLE, we found that AASV was associated with increased *FCGR3B* CN. By demonstrating the functional effects of reduced *FCGR3B* CN on protein expression and immune complex handling, we have provided an explanation for the association of low *FCGR3B* CN with SLE, but not with AASV.

RESULTS AND DISCUSSION

Association of low *FCGR3B* CN with SLE in a UK, but not Hong Kong, population

We sought to replicate the previously reported association between low *FCGR3B* CN and SLE (26). We assessed *FCGR3B* CN using a quantitative PCR method modified after that described by Fanciulli et al. (26), which compares the number of cycles required for amplification of *FCGR3B* to reach

threshold, compared with the number of cycles required for amplification of a control gene that does not show CNV; in this case, *CD36*. *FCGR3B/CD36* ratios as determined by this assay are not integers, as might have been expected, but form a near continuous distribution, which is a well recognized feature of CN assays (7, 8). We therefore compared cases and controls on each plate using a Student's *t* test, and then performed a Student's *t* test stratified by the two plates of samples. In total, 171 samples from Caucasian patients with SLE were compared with 176 ethnically matched control samples. We found lower CN was significantly associated with SLE in Caucasians (P = 0.027; Fig. 1 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072413/DC1), but such an association was not seen in a Chinese SLE cohort from Hong Kong, comprising 159 cases and 150 controls (Fig. 1 B and Fig. S1). This lack of association held true when the patients with lupus nephritis (n = 57) were analyzed independently (Fig. S2). *FCGR3B* CN distributions did not differ significantly between the control Caucasian and Chinese populations (Fig. S3). SLE is more common, more severe, of earlier onset, and has some distinct clinical features in East Asians compared with Europeans. These clinical differences are independent of environment and likely to be genetically determined, thus it is to be expected that SLE will have different genetic influences in different ethnic groups (29); e.g., a transmembrane polymorphism in neighboring *FCGR2B* is associated with SLE in Asian (30), but not European or African-American, populations (31).

Correlation between *FCGR3B* CN, protein expression, and function in a family

To define the function of FcγRIIIb, and whether this function was influenced by CNV, we first studied CN, expression, and function using a family with *FCGR3B* deficiency. As part of a comprehensive program of expression profiling of purified cell subsets in autoimmune disease, we identified an individual with SLE who had no *FCGR3* mRNA in neutrophils, but normal levels of *FCGR3* mRNA in monocytes (Supplemental clinical information, available at http://www.jem.org/cgi/content/full/jem.20072413/DC1, and Fig. 2 A). The probes used on our array do not differentiate between *FCGR3A* and *B*, but the expression pattern was consistent with absent *FCGR3B*. Flow cytometric analysis of the proband confirmed the lack of neutrophil cell surface FcγRIIIb. Surface expression of FcγRIIIb showed a clear trimodal pattern, consistent with Mendelian inheritance of an *FCGR3B*-null allele (Fig. 2, B and C). Fig. 2 C shows a flow cytometry plot for six family members labeled A–F showing the clear differences in protein expression in the proband (A), her son, daughter, and brother with a single *FCGR3B*-null allele (B–D), and her husband and other brother (E and F) with two copies of *FCGR3B*. *FCGR3B* CN, as measured by quantitative real-time PCR (qPCR), was lower in individuals B, C, and D than in individuals E and F (Fig. 2 D). *FCGR3B* CN, as determined by qPCR, correlated with CN inferred from protein expression and heredity in family members (Fig. 2 E). Further PCR analysis demonstrated that the genetic defect spared *FCGR3A* and *FCGR2B* and included *HSPA7* and the *FCGR2C* (Fig. 2 F), consistent with previous observations in *FCGR3B*-null individuals (25), although the CN variable region at this locus may show more complexity and variability in the general population. The genomic defect seen in this family was confirmed at the protein level; FcγRI, FcγRIIa, and FcγRIIb were expressed on the cell surface of neutrophils, and FcγRIIIa was expressed on monocytes (Fig. S4). CD59 was also present on neutrophils (excluding a general defect in GPI linkage as an explanation for reduced FcγRIIIb; Fig. 2 G).

We next analyzed neutrophils from family members with known *FCGR3B* CN to define its function. Superoxide anion production in response to several stimuli thought to be FcR independent, such as formyl-Met-Leu-Phe (fMLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), and PMA, was not affected by *FCGR3B* CN (Fig. 3 A). To assess the ability of neutrophils to localize to immune complexes, we analyzed the adherence of neutrophils flowed over surfaces coated with IgG, and demonstrated that adherence was proportional to *FCGR3B* CN (Fig. 3 B). Immune complex adherence to, and uptake by, neutrophils also increased with increasing CN (Fig. 3, C and D). Reduced neutrophil function was not associated with reduced expression of other FcγRs; indeed, a slight increase in expression of FcγRI and FcγRIIa was observed in the FcγRIIIb-deficient proband (Fig. S4). There was also a correlation between CN, as measured by qPCR, and soluble circulating FcγRIIIb (Fig. 3 E), which is released upon neutrophil activation (15).

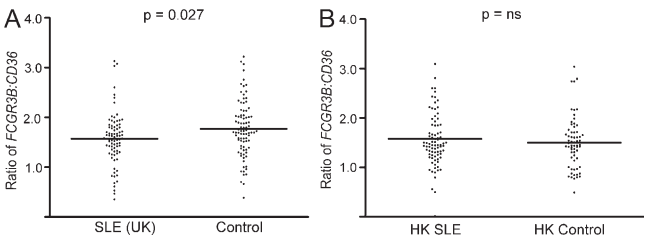

Figure 1. Association of low *FCGR3B* CN with UK, but not Hong Kong, SLE. qPCR was used to determine *FCGR3B/CD36* ratios in normal controls and patients with SLE. In each case, results from 1 (representative of 2) qPCR plate are shown (all of the raw data are shown in Fig. S1, A and B). The horizontal bar indicates the mean. The P values shown indicate comparison of all cases and controls using a stratified Student's *t* test. (A) UK SLE patients, n = 171; UK controls, n = 176. (B) Hong Kong SLE patients, n = 159; Hong Kong controls, n = 150.

FCGR3B CN correlates with protein expression and function in the normal population

We wished to determine if this association of *FCGR3B* CN with expression and function was seen in normal populations. We used qPCR to determine CN in 33 healthy individuals. Although we have shown in the family, and by comparison with the HapMap data, that *FCGR3B* CN is proportional to results from the qPCR assay, definitive assignment of CN in

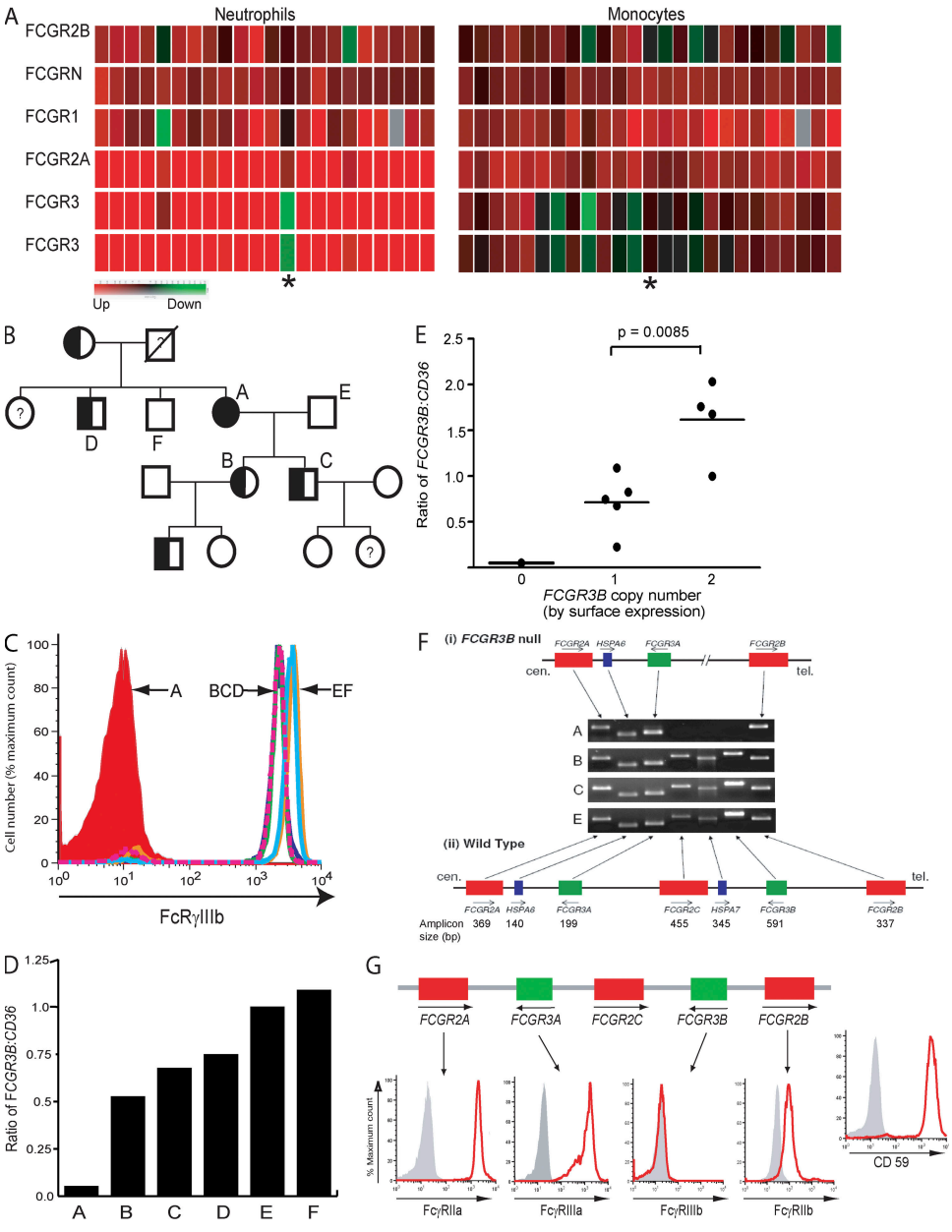

Figure 2. Correlation between *FCGR3B* CN and protein expression in a family. (A) Array of gene expression patterns (mRNA) for FCGR genes in neutrophils and monocytes of individuals with SLE. Each row corresponds to a gene, and each column to an individual. Red indicates increased expression compared with PBMC reference; green represents reduced expression. The patient (A in panel B) with no *FCGR3B* expression is marked with an asterisk. (B) Family tree of the *FCGR3B*-deficient patient A, showing Mendelian inheritance of the null allele. CN was determined using flow cytometry. (C) Flow cytometry of neutrophils stained for PE-labeled antibody to FcγRIIIb demonstrates reduced surface expression on cells from individuals B, C, and D (with a single *FCGR3B* copy) compared with individuals E and F, who have two *FCGR3B* copies. Geometric mean fluorescences were 7, 2,265, 2,241, 2,303, 3,484, and 3,730 for A–F, respectively. (D) Gene dosage of *FCGR3B* relative to *CD36*, determined by qPCR, for patient A (no *FCGR3B*), her daughter patient B, her son patient C, and her brother patient D (with a single *FCGR3B* copy), as well as for her husband patient E and her other brother patient F (with two copies of *FCGR3B*). (E) qPCR was performed on DNA from all family members whose FcγRIIIb expression had been determined by flow cytometry. Gene dosages of *FCGR3B* relative to *CD36* (by qPCR) were significantly higher in those individuals who by flow cytometry were shown to have greater surface expression of FcγRIIIb. The horizontal bar indicates the mean. (F) Delineation of the extent of the deletion in patient A and family members B, C, and E using PCR; *FCGR3B*, *HSPA7*, and *FCGR2C* are absent. (G) A similar delineation using flow cytometry in patient A. FcγRIIa (neutrophils), FcγRIIb (neutrophils shown, confirmed on B cells, and not depicted) and FcγRIIIa (NK cells) are present (isotype control shaded gray), but FcγRIIIb (neutrophils) is absent. CD59 is expressed on neutrophils, thus GPI linkage is intact.

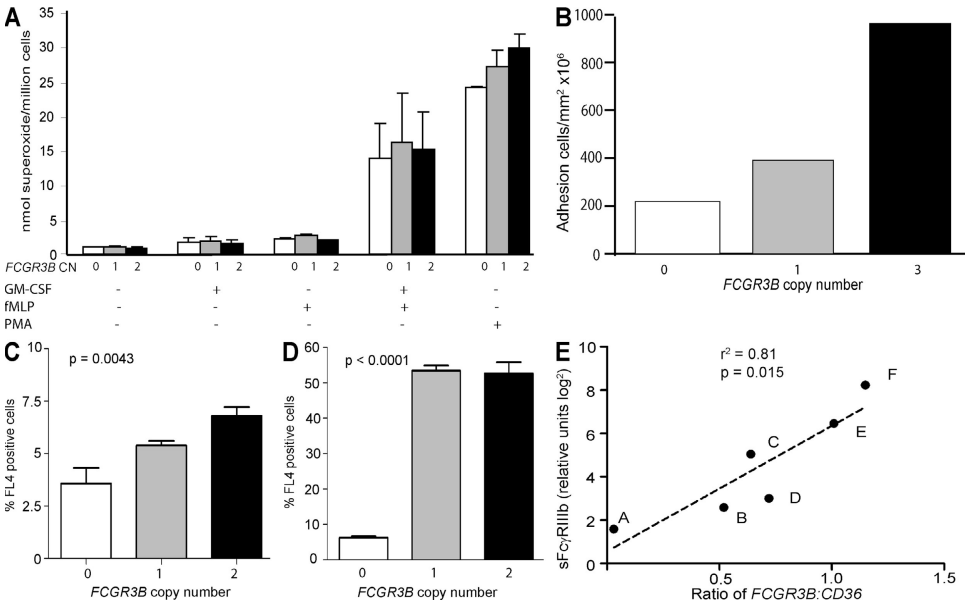

Figure 3. Correlation between *FCGR3B* CN and neutrophil function in a family. (A) No significant differences in superoxide anion production between family members with 0, 1, and 2 copies of *FCGR3B* were observed after Fc receptor–independent stimulation with GM-CSF, fMLP, or PMA (two-way ANOVA, P = ns). Mean and SEM from two independent experiments are shown. (B) Quantification of neutrophil adhesion to IgG-coated glass slides after 4 min of flow at a shear stress of 0.1 Pa in the *FCGR3B*-null individual and her single-copy daughter relative to the three CN control. (C) Percentages of neutrophils with bound, antibody-opsonized, Alexafluor-labeled ovalbumin after 10 min at 4°C, for family members with 0, 1, and 2 copies of *FCGR3B*. Mean and SEM are shown for triplicate repeats of one experiment that is representative of two. (D) Percentages of neutrophils with bound and internalized, antibody-opsonized, Alexafluor-labeled ovalbumin after 10 min at 37°C, for family members with 0, 1, and 2 copies of *FCGR3B*. Mean and SEM are shown for triplicate repeats of one experiment that is representative of two (the same experiment is shown in C). P values in C and D refer to one-way ANOVA with a post test for linear trend. (E) sFcγRIIIb in serum, as measured by ELISA, increases with *FCGR3B* CN in patient A's family (letters correspond to individuals shown in Fig. 1 B).

the general population is difficult (7, 8). We therefore selected individuals whose *FCGR3B/CD36* ratios were clearly low (lowest five values), intermediate, or high (highest five values), and using neutrophils from these individuals showed that CNV was associated with FcγRIIIb expression (Fig. 4, A and B, and Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20072413/DC1). As in the index family, there was a clear correlation between CN and neutrophil adherence to immunoglobulin-coated surfaces under flow conditions, with high CN individuals having four times the rate of adherence of those with low *FCGR3B* CN (Fig. 4, C and D, and Supplemental videos). Once adherence had occurred, spreading of the neutrophils on the surface appeared independent of *FCGR3B* CN (Table S1). Immune complex uptake was also reduced in those with low CN (Fig. 4 E), whether or not complement was present (not depicted). As in the family, levels of circulating soluble FcγRIIIb correlated with *FCGR3B* CN as determined by qPCR (Fig. S6 A). Thus, the association between *FCGR3B* CN, protein expression, and function seen in the index family could be extrapolated to the population at large.

SLE has long been known to be associated with defects in immune complex clearance, and SLE lesions are invariably associated with immune complex deposition (32). The functional data presented here, in both an FcγRIIIb-deficient individual and her family and in normal volunteers with different CN, show clearly that reduced expression of FcγRIIIb is associated with reduced immune complex uptake and with reduced neutrophil adhesion to immune complex–bearing surfaces. We have also confirmed a genetic association between SLE and low CN. Our data would suggest that the mechanism underlying this association is a failure of neutrophil trafficking to inflammatory lesions and reduced ability to ingest immune complexes once there, thereby reducing immune complex clearance and predisposing to SLE. Hence "normal" levels of FcγRIIIb (both neutrophil-surface and soluble) may be important in protection against SLE. Our results thus provide a mechanism explaining the genetic association of low *FCGR3B* CN and SLE.

Association of high *FCGR3B* CN with AASV in three UK cohorts

Unlike SLE, AASV is not associated with immune complex deposition. Rather, whereas neutrophils are not thought to play a major role in inflammatory damage in SLE, it is clear that Fc receptor-mediated neutrophil activation is important in AASV pathogenesis (33). Thus, it might be expected that increased *FCGR3B* CN would be associated with AASV. We therefore analyzed *FCGR3B* CN in 286 ethnically matched controls and 556 patients derived from the following 3 independent

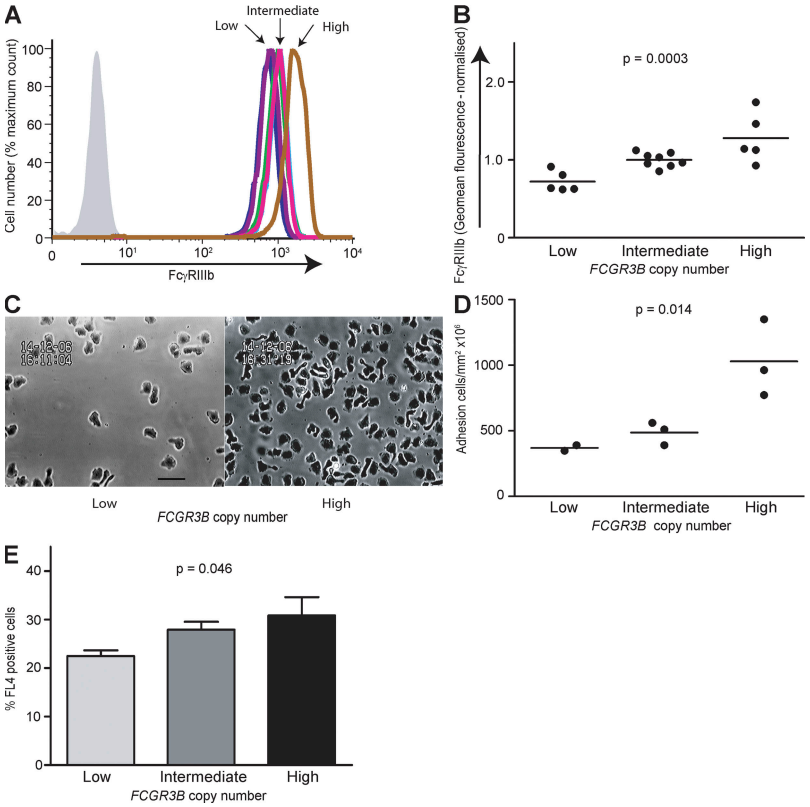

Figure 4. *FCGR3B* CN is proportional to gene expression and function in the normal population. Surface expression of FcγRIIIb increases with increasing *FCGR3B* CN. (A) Flow cytometry plot of neutrophils stained with PE-labeled anti-FcγRIIIb in one experiment that is representative of three. (B) Expression of FcγRIIIb (geometric mean fluorescence normalized in three separate experiments to the mean of intermediate CN individuals) correlates with gene dosage of *FCGR3B*. P value refers to one-way ANOVA with a post test for linear trend. The horizontal bar indicates the mean. (C) Neutrophil adhesion to IgG-coated glass slides after 4 min of flow at a shear stress of 0.1 Pa in an individual with low *FCGR3B* compared with that of an individual with high *FCGR3B*. Bar, 20 µm. (D) Neutrophil adhesion to bound IgG under flow conditions increases with *FCGR3B* CN (after 4 min of flow at a shear stress of 0.1 Pa) in two experiments, which were pooled. P value refers to one-way ANOVA with a post test for linear trend. The horizontal bar indicates the mean. (E) Uptake of immune complexes in individuals with low, intermediate, or high *FCGR3B*. Percentages of neutrophils positive for FL4 after 10 min incubation with antibody-opsonized, Alexafluor-labeled ovalbumin at 37°C are greater in individuals with more *FCGR3B*. Mean and SEM are shown for three to six individuals per group from two experiments. P value from nonparametric one way ANOVA (Kruskal-Wallis test).

AASV cohorts: a UK cohort with biopsy-proven, ANCA-associated renal vasculitis (cohort 1, $n = 347$), an independent ANCA-associated vasculitis cohort (cohort 2, $n = 136$), and a vasculitis cohort from Birmingham, UK (cohort 3, $n = 73$). Further details of these cohorts are described in the online supplemental material. Cases and controls were plated together and compared using a Student's *t* test as described above and in the Materials and methods. No association between disease and low CN was seen in any of the three cohorts; in fact, AASV was associated with high CN when cases from all cohorts were compared with controls ($P = 10^{-8}$ calculated using a Student's *t* test stratified by plate of samples; Fig. 5, A–C, and Fig. S1). To substantiate the observation that *FCGR3B* CN distribution differed between SLE and AASV, we compared samples from both groups directly on a single plate (Fig. 5 D). Patients with SLE showed a significantly lower mean *FCGR3B/CD36* ratio than patients with AASV ($P = 0.0028$; Fig. 5 D). We then measured levels of soluble circulating FcγRIIIb in patients with SLE and AASV at both disease onset and after therapy (patients described in the Supplemental clinical information and Table S2, available at http://www.jem.org/cgi/content/full/jem.20072413/DC1). In the 43 patients with AASV, soluble FcγRIIIb levels at diagnosis correlated with *FCGR3B/CD36* ratio, and levels were significantly higher than both controls and the 15 lupus patients (Fig. S6, B and C).

An early report suggested that low, rather than high, *FCGR3B* CN is associated with AASV (26). The most likely reason for this is that that study compared groups after CN assignation, despite the continuous nature of the data. This is known to risk producing misleading results (7, 8), is likely to explain the variation in CN distribution observed between ethnically similar control groups (26), and may be part of the reason why low *FCGR3B* CN was found to be associated with lupus nephritis alone in one study (5), but not others ([26] and this study). To ensure the accuracy of our data, we

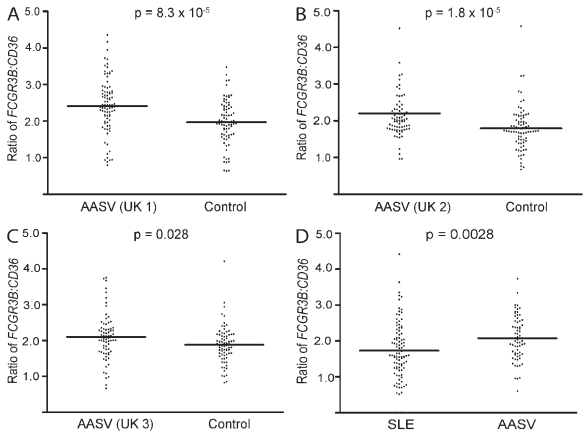

Figure 5. Association of high *FCGR3B* CN with AASV. qPCR was used to determine *FCGR3B/CD36* ratios in normal controls and cohorts of patients with AASV (cohorts described in Supplemental materials and methods). In each case, results from one representative qPCR plate are shown (all of the raw data are shown in Fig. S1 C). The horizontal bar indicates the mean. P values shown for each cohort indicate comparison of all cases and controls using a stratified Student's *t* test. (A–C) AASV versus control in three independent UK cohorts. (D) Single plate of SLE and AASV, compared using a Student's *t* test. Supplemental materials and methods and Fig. S1 C are available at http://www.jem.org/cgi/content/full/jem.20072413/DC1.

used three independent cohorts (including one overlapping with [26]) and larger numbers of patients, and we compared raw values using a stratified Student's *t* test rather than arbitrary "binning" (as discussed in the Materials and methods). In addition, we correlated results with a family of known *FCGR3B* CN and with DNA from individuals with CN determined independently from the HapMap data. Finally, we correlated our assay with FcγRIIIb surface expression and function (Fig. 4, A and B, and Fig. S5), and with soluble FcγRIIIb titers (Fig. S6). We are therefore confident AASV is associated with increased *FCGR3B* CN. Nonetheless, all of the results in this field will need to be reassessed as more sophisticated CN assays are developed in the future.

The associations of high *FCGR3B* CN with AASV and low CN with SLE is consistent with what is known of the pathophysiology of these diseases. In AASV, unlike SLE, inflammatory lesions are characterized by neutrophil infiltration, but not immune complex deposition (33). ANCA is thought to mediate its pathogenic effect by activating neutrophils in an Fcγ receptor–dependent fashion (34) and should therefore be exacerbated by increased *FCGR3B* CN. In addition, AASV is associated with *FCGR3B*1,* the isoform of FcγRIIIb that confers increased phagocytosis, (16, 18), whereas SLE is associated with *FCGR3B*2,* which is associated with reduced phagocytosis (16, 30, 35), providing further evidence that the receptor plays a different role in each disease.

CNV is an important cause of genetic variation in populations, and has recently been implicated in complex disease causation. The known associations with disease will increase as whole-genome association studies are analyzed for CNV. These associations will need to be investigated to determine the mechanism by which CNV contributes to disease. In this study, we use a family with *FCGR3B* deficiency to demonstrate not only the physiological function of the receptor, but also the effect of *FCGR3B* CNV on protein expression and function. By extending these studies into the general population, we show for the first time the association between CNV, expression, and function of a disease-related gene, providing a functional explanation for the genetic association between low *FCGR3B* CN and SLE.

MATERIALS AND METHODS

FCGR3B CN determination

qPCR. *FCGR3B* CN was measured using qPCR modified from that previously described (5), with the exception that *FCGR3B* values were normalized to *CD36* rather than *FOXP2*. In brief, 10 μl PCR reactions, using 2.5 ng of genomic DNA as template, were performed using SYBR green (Quantifast; QIAGEN) and an ABI 7900HT real-time PCR system with a 384-well module (ABI). Cycling conditions were 95°C for 5 min, and then 40 cycles of 95°C for 10 s, followed by 60°C for 30 s. All samples were measured in duplicate, and for each sample the difference between replicate Ct values was determined. The mean difference across all samples was then determined, and any samples with a difference in values exceeding the mean plus two standard deviations were removed from the dataset.

The number of cycles required for amplification of *FCGR3B* to reach threshold (the Ct value) was then compared with the number of cycles required for amplification of *CD36*, a gene that does not show CNV. The ratio of *FCGR3B* Ct/*CD36* Ct is therefore a measure of *FCGR3B* CN. Two difficulties posed by the assay had to be circumvented as follows. First, we found that the *FCGR3B/CD36* ratios were not integers, as might be expected from the principle of CNV. This is a well recognized problem with CN assays (7). Indeed, when we downloaded array comparative hybridization data for probes encompassing the *FCGR3B* locus (from 270 HapMap individuals), we saw a spread of log intensities similar to the pattern seen using the qPCR assay (Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20072413/DC1). We obtained DNA from 14 of these individuals from the Coriell Institute (Camden, NJ), and found a close correlation between aCGH data and qPCR ratios of *FCGR3B/CD36* (Fig. S8 B). The second potential problem we found was that, although the distribution of *FCGR3B/CD36* ratios for a given set of samples is remarkably constant upon repeat measurement, the absolute values may vary on repetition (Fig. S8 B). We therefore plated control and diseased samples together. We compared cases and controls on each plate using a Student's *t* test, and then performed a Student's *t* test stratified by both plates of samples. We were able to do this because the means of the *FCGR3B/CD36* ratios for SLE cases were lower than control means on both plates of samples used, and the means of the AASV samples were greater than controls on all seven plates assayed. Fig. S1 shows the raw data for all plates of SLE and AASV cases and controls.

Microarray analysis

Blood samples were obtained from 15 SLE patients with active disease and 10 age- and sex-matched normal controls. Neutrophils and monocytes were isolated by magnetic cell sorting, total RNA was extracted, and microarray hybridizations were performed as previously described (36), except that pooled targets were resuspended in 60 μl hybridization buffer and hybridized on a SlideBooster hybridization station (Advalytix). All samples were hybridized to custom spotted oligonucleotide microarrays in duplicate, using a dye-swap strategy, against a common reference RNA prepared from pooled PBMC RNA obtained from seven normal controls. Raw image data were extracted using Koadarray v2.4 (Koada Technology); probes were listed

as present if they had a spot confidence value >0.3 in at least 1 channel. Background subtracted intensity values for all probes considered present were imported into GeneSpring v7.2 and Lowess normalized before further analysis. Microarray data have been deposited in ArrayExpress with the accession no. E-TABM-463.

Genomic PCR

DNA was extracted from peripheral whole blood using the QIAamp Mini blood kit (QIAGEN) as per the manufacturers' protocol. For gene-specific PCRs, optimum specificity was obtained by performing annealing temperature gradients on each primer pair. All PCRs were performed in 1× Promega PCR buffer (including 1.5 mM $MgCl_2$) with Taq polymerase. Gene specificity was confirmed by sequencing of all amplicons. Primer sequences are shown in Table S3.

Functional assays

Immune complex adhesion and uptake assays. Whole blood was diluted in PBS with Ca^{2+}/Mg^{2+} to give a neutrophil concentration of 10^6 cells/ml. To make the immune complexes, Alexafluor-labeled ovalbumin (Invitrogen) was opsonized with rabbit polyclonal antiovalbumin antibody (Sigma-Aldrich) at 37°C for 1 h. Neutrophils were incubated with ovalbumin, in opsonized or nonopsonized form, for 10 min, at either 37°C or 4°C. Erythrocytes were then lysed with BD FACS Lysing Solution. The percentage of neutrophils positive for Alexafluor-labeled ovalbumin was assessed by flow cytometry, as described previously.

Neutrophil adhesion assay. Neutrophils were isolated from whole blood by two-step gradient centrifugation as previously described (37). The adhesion of flowing neutrophils to IgG-coated microslides and the subsequent behavior of the adherent cells was measured as previously described (37).

Superoxide assay. Respiratory burst activity was determined by means of the superoxide dismutase–inhibitable reduction of cytochrome *c*, as previously described (38).

Flow cytometry

The antibodies used in this study were: anti-FcγRIIIb (clone 3G8; BD Biosciences), anti-FcγRIIa (clone FLI8.26; BD Biosciences), anti-FcγRIIb (MacroGenics) (39), anti-FcγRI (clone 10.1; Invitrogen), anti-CD56 (clone MEM88; Invitrogen), and anti-CD59 (clone Mem-43; Invitrogen). Isotype controls were IgG2a (clone G155-178), IgG1κ (clones MOPC-21), and IgG2bκ (clone MPC-11). 100-µl aliquots of whole blood were incubated with 10 µl Fc block (Miltenyi Biotec) and 10 µl of appropriate labeled antibodies for 20 min at room temperature. Erythrocytes were lysed using BD FACS Lysing Solution, and surface expression assessed by flow cytometry on a FACSCalibur (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc.).

sFcγRIIIb ELISA

Microtitre plates (MaxiSorp; Nunc) were coated with 0.3 µg/ml anti-CD16 mAb (clone DJ130c; Dako) overnight at 4°C. Plates were washed three times with PBS/0.05% Tween-20 and blocked for 2 h at room temperature with PBS/10% FCS. Serum samples were diluted in PBS, added to the plate, and incubated for 4 h at room temperature. Plates were washed and incubated for 2 h at room temperature with biotinylated anti-CD16 mAb (clone 3G8). After a further wash, plates were revealed with streptavidin-HRP followed by TMB (Sigma-Aldrich) according to manufacturer's instructions. Absorbance at 450 nm was measured after 15 min on an OPTImax tunable microplate reader.

Statistical analyses

The statistical significance of data characterizing the function of FcγRIIIb was determined using either a one- or two-way analysis of variance test. When comparing *FCGR3B/CD36* ratios between cases and controls on an individual plate, an unpaired *t* test with Welch's correction was performed. All statistical tests were performed using either the Prism software (GraphPad) or the R statistical system (www.r-project.org). P values <0.05 were considered significant. Further details regarding individual statistical tests performed can be found in the appropriate figure legend.

Research ethics

This study was approved by the Cambridge Local Research Ethics Committee, the Leeds East Research Ethics Committee, the Oxford Multi-center Research Ethics Committee, and the Institutional Review Board of the University of Hong Kong/Hospital Authority.

Online supplemental material

The Supplemental materials and methods contain additional methodology. The Supplemental clinical information contains clinical details for the *FCGR3B*-deficient individual and her family, as well as clinical information on the SLE patients whose microarray data are shown in Fig. 1, and the AASV patients whose soluble FcγRIIIb levels are shown in Fig. S6. Table S1 shows the percentage of cells that undergo spreading after adhesion is unrelated to *FCGR3B* CN. Table S2 is a summary of clinical features of SLE and AASV patients whose soluble FcγRIIIb levels are shown in Fig. S6. Table S3 shows gene-specific primer sequences used for Fig. 1 E. Fig. S1 shows qPCR raw data for all plates of samples analyzed. Fig. S2 shows that, in a Chinese population from Hong Kong, *FCGR3B* CN does not differ significantly in patients with SLE nephritis compared with healthy controls. Fig. S3 shows that *FCGR3B* CN does not differ significantly in healthy Chinese individuals from Hong Kong compared with UK Caucasians. Fig. S4 Cell surface expression by flow cytometry of FcγRIIa (A), FcγRI (B) and FcγRIIb (C) on neutrophils and FcγRIIIa (D) on monocytes of patient A (in red) and two individuals known to have *FCGR3B*. Fig. S5 shows that the finding that *FCGR3B* CN is proportional to gene expression is reproducible. Fig. S6 shows correlation of *FCGR3B* CN and soluble FcγRIIIb. Fig. S7 shows array comparative genomic hybridization data across the *FCGR3B* locus on chromosome 1q22-23. Fig. S8 shows the *FCGR3B/CD36* ratio detected by qPCR correlates with aCGH data and is reproducible. Videos 1–4 show neutrophil adhesion and spreading to IgG-coated glass slides during 4 min of flow at a shear stress of 0.1 Pa. Videos are shown for representative individuals with low, intermediate, and high *FCGR3B* CN. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20072413/DC1.

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