# Copy number variation of the activating *FCGR2C* gene predisposes to idiopathic thrombocytopenic purpura

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**Gene copy number variation (CNV) and single nucleotide polymorphisms (SNPs) count as important sources for interindividual differences, including differential responsiveness to infection or predisposition to autoimmune disease as a result of unbalanced immunity. By developing an *FCGR*-specific multiplex ligation-dependent probe amplification assay, we were able to study a notoriously complex and highly homologous region in the human genome and demonstrate extensive variation in the *FCGR2* and *FCGR3* gene clusters, including previously unrecognized CNV. As indicated by the prevalence of an open reading frame of *FCGR2C*, Fcγ receptor (FcγR) type IIc is expressed in 18% of healthy individuals and is strongly associated with the hematological autoimmune disease idiopathic thrombocytopenic purpura (ITP) (present in 34.4% of ITP patients; OR 2.4 (1.3-4.5), $P < .009$). FcγRIIc acts as an activating IgG receptor that exerts antibody-mediated cellular cytotoxicity by immune cells. Therefore, we propose that the activating *FCGR2C*-ORF genotype predisposes to ITP by altering the balance of activating and inhibitory FcγR on immune cells. (Blood. 2008;111:1029-1038)**



## Introduction

Human Fcγ receptors are glycoproteins that bind the Fc portion of IgG. Fcγ receptors are encoded on chromosome 1q23-24. Depending on their expression on effector cells, FcγRs exert different effects.[1]

The low-affinity FcγRs have been implicated in immune-complex–mediated auto-inflammation and are encoded by *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A,* and *FCGR3B*. The current paradigm in FcγR biology states that cell activation is balanced by activating and inhibitory FcγRs. Loss of inhibitory FcγRIIb expression, altered function due to single nucleotide polymorphisms (SNPs), or overrepresentation of activating FcγRs results in unbalanced immunity and subsequent auto-inflammation.

Genetic polymorphisms affecting IgG-subclass binding exist in *FCGR2A*[2], *FCGR3A,*[3,4] and *FCGR3B*.[5] A functional polymorphism has been identified in FcγRIIa.[2] FcγRIIa-H131 binds human IgG2, whereas FcγRIIa-R131 does not.[6] In FcγRIIIa, a polymorphism at amino acid 158, a valine or phenylalanine, has been identified. As a result, FcγRIIIa-V158 has a higher affinity for IgG1 and IgG3 than FcγRIIIa-F158.[3,4] Allelic variation in FcγRIIIb is composed of differences in 4 amino acids, referred to as human neutrophil antigen 1 (HNA1a [NA1]) and HNA1b [NA2]).[7] FcγRIIIb-HNA1a internalizes IgG1- or IgG3-opsonized particles more efficiently than does FcγRIIIb-HNA1b.[5]

Also, the negative signaling through FcγRIIb is subject to genetic variation. An SNP in exon 5 of *FCGR2B*, which changes isoleucine to threonine at residue 232 (I232T) in the transmembrane domain, alters receptor signaling through the inhibitory FcγRIIb.[8-10] In addition, functional polymorphisms in the promoter region of the *FCGR2B* gene were shown to be linked to transcriptional activity.[11] Close to *FCGR2B*, the *FCGR2C* gene is located, which is believed to be a pseudogene. Very likely, *FCGR2C* results from an unequal crossover event between the 5′ part of the genomic sequence of *FCGR2B* and the 3′ part of *FCGR2A*.[12]

Recent papers[13,14] have described the identification of large deletions and duplications of DNA fragments when genomes of healthy individuals were compared. The identification and characterization of copy number variation (CNV) show that in addition to single nucleotide differences, genomes of unrelated individuals have large regions of thousands to millions of nucleotides that are different. From a functional perspective, gene copy number differences can contribute to variation in gene expression, at the transcript and/or protein level. Gene-expression studies have shown that subtle differences in expression levels of genes can have significant consequences, as reported for *DEFB4*[15] and *CCL3L1*.[16]

CNV in the human FcγR gene cluster is not a new phenomenon. Deletion and duplication of the *FCGR3B* gene has been described by our group,[17-19] in linkage with a deletion/duplication of the *FCGR2C* gene.[18] Recently, Aitman et al observed an association between low copy number of *FCGR3B* and glomerulonephritis in the autoimmune disease systemic lupus erythematosus (SLE).[20]

The contribution of Fcγ receptor genes to the genetic susceptibility of autoimmune/inflammatory diseases is very complex.

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Genetic variation, SNPs and CNV, within the Fcγ receptor cluster could collectively be responsible for a "susceptibility phenotype," altering the balance between activating and inhibitory receptor signaling.

To study the genetic variation of this complex gene cluster, we developed a multiplex ligation-dependent probe amplification (MLPA) assay that enabled us to study the genetic variation in the low-affinity FcγR genes (*FCGR2A, FCGR2B, FCGR2C, FCGR3A,* and *FCGR3B*) in one assay. We used this MLPA assay to study a cohort of idiopathic thrombocytopenic purpura (ITP) patients in a case-control study.

ITP is a disease characterized by thrombocytopenia with otherwise normal cell lineages and no other explanation for the isolated thrombocytopenia. Destruction of autoantibody-sensitized platelets by FcγR-bearing phagocytic cells in the reticuloendothelial system plays an important role, although the exact pathophysiology of this autoimmune disorder is not precisely known.[21] The role of Fc receptors is underscored by the fact that intravenous immunoglobulin (IVIg) treatment and splenectomy (removal of the platelet-destructing organ) are effective treatment options.[21]

Despite its proven beneficial effect, the exact mechanism of action of IVIg in ITP is not known. Several models have been proposed,[22] including blockade of the Fc receptor for IgG,[23,24] increased surface expression of FcγRIIB,[25] and the acute interaction of IVIg with activating FcγRs on dendritic cells.[26]

In children, the disease is often self-limiting within a 6-month period. In about 20% of cases, the thrombocytopenia sustains.[27,28] In adult ITP, the disease is more often chronic, and an association with a prior infection has not been observed.

Previous studies concerning polymorphisms in the *FCGR* gene cluster in ITP patients show conflicting results. While confirming the previously observed overrepresentation of the SNP in *FCGR3A* encoding the FcγRIIIa-158V variant in pediatric ITP, we demonstrate that the copy number of an open reading frame (ORF) form of the *FCGR2C* gene results in the expression of a functionally activating FcγRIIc. The activating *FCGR2C*-ORF allele is determined by an SNP in exon 3 that changes the common stop codon in the pseudogene *FCGR2C* into a glutamine. In this study, we show that *FCGR2C* gene variation represents a significant genetic parameter associated with ITP.

## Methods

### Subjects

DNA of 116 patients with ITP was available for analysis. Informed consent was obtained from all parents of patients younger than12 and from all patients older than 12. Diagnosis and treatment were according to the guidelines of the American Society of Hematology and the United Kingdom practice for management of acute childhood ITP.[29-31] Healthy adult white volunteers (n = 100) served as controls. These donors were not known to have hematological disorders of any kind in the past or at present. The study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam and was performed in accordance with the Declaration of Helsinki.

### Multiplex ligation-dependent probe amplification

MLPA was developed as an alternative for the Southern blot (RLFP) method to detect deletions and duplications of genes (or part of genes) but can be adapted to detect SNPs as well in one multiplex assay with 50 different targets (J. Schouten, MRC-Holland, Amsterdam, The Netherlands).

MLPA probes were designed specifically for the *FCGR2A, FCGR2B, FCGR2C, FCGR3A,* and *FCGR3B* genes. As the *FCGR* gene cluster is very homologous, this was not possible for all sites within a given gene. At least 3 probes per gene were designed to cover every single gene. In this way, CNV and partial insertions/deletions can be studied. Separate probes were designed to study known (functional) SNPs in *FCGR2A* (131H/R), *FCGR2B* (232I/T), promoter of *FCGR2B* and *FCGR2C* (−386 G/C), *FCGR3A* (158V/F), and *FCGR3B* (HNA1a/HNA1b/HNA1c). For an overview of the location and the specific target sequences of the probes, see Figure 1 and Table S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Several DNA samples of previously well-typed individuals were used for validation of the MLPA assay with respect to CNV and SNPs.[17,18,27]

Probes were constructed in collaboration with MRC-Holland and embedded in a standard MLPA kit. Because of the homology between certain probes, some probes had to be divided over 2 separate mixes to prevent competition. The MLPA assay was performed according to the first description by Schouten et al.[32]

In brief, 5 μL of DNA (20 ng/μL) was denatured at 98°C for 5 minutes and then cooled to 25°C in a thermal cycler with heated lid; 1.5 μL probe mix and 1.5 μL buffer were added to each sample and incubated for 1 minute at 95°C, followed by 16 hours at 60°C. Then, 32 μL of ligase-65 mix was added to each sample while at 54°C, followed by an incubation of 15 minutes at 54°C and 5 minutes at 98°C. This ligation mixture was diluted 4 times. Then, 10 μL polymerase mix was added, containing one single primer pair. Directly after adding the polymerase mix, the polymerase chain reaction (PCR) was started. PCR conditions were 36 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 60 seconds at 72°C, followed by 20 minutes at 72°C. After the PCR reaction, 1 μL of the PCR reaction was mixed with 0.5 μL CXR 60-400 (Promega, Madison, WI) internal size standards and 8.5 μL deionized formamide, and the mixture was incubated for 10 minutes at 90°C. The products were then separated by electrophoresis on an ABI-3130XL (Applied Biosystems, Foster City, CA).

The program Genemarker (version 1.30) was used to analyze the samples (Soft Genetics, State College, PA). Since MLPA is a relative quantification assay, one sample has to be assigned as reference sample. The normalized height (or the area) of the probe amplification product of the unknown sample was divided by the normalized height of the probe amplification product of the reference. A ratio between 0.8 and 1.2 was considered normal, below 0.8 was considered as loss in copy number, and above 1.2 as gain of copy number. For information concerning the control probes used in the standard MLPA, we refer to MRC-Holland (Amsterdam, The Netherlands).

### FCGR2B/C promoter haplotypes

Due to the high homology between the promoter of the *FCGR2B* gene and that of the *FCGR2C* gene at position −386 G > C (rs3219018), it was not possible to design a specific MLPA probe exclusively for one of these genes. A gene-specific long-range PCR was performed on those samples carrying the uncommon *FCGR2B/C* variant −386C as detected by the MLPA assay. For this purpose, the expand long template PCR system (Roche Applied Science, Mannheim, Germany) was used according to the manufacturer's instructions. In brief, a 15-kb fragment was amplified with a nonspecific *FCGR2B/C* sense primer (5′-GCCATCCTGACATACCTCCT-3′), annealing in the promoter region and an *FCGR2B*-specific antisense primer (5′-CCCAACTTTGTCAGCCTCATC-3′) or the *FCGR2C*-specific antisense primer (5′-CTCAAATTGGGCAGCCTTCAC-3′), both annealing in exon 7. The PCR conditions were 94°C for 2 minutes, 10 cycles of 94°C for 10 seconds, 60°C for 30 seconds, 68°C for 12 seconds, followed by 20 cycles of 94°C for 15 seconds, 60 °C for 30 seconds, 68°C for 12 seconds, with an elongation of each cycle with 20 seconds at 68°C, followed by a final elongation at 72°C for 7 minutes. PCR products were purified by GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham, United Kingdom) and sequenced with BigDye terminator cycle sequencing (v 1.1) (Applied Biosystems) with the sense primer used in the PCR reaction.

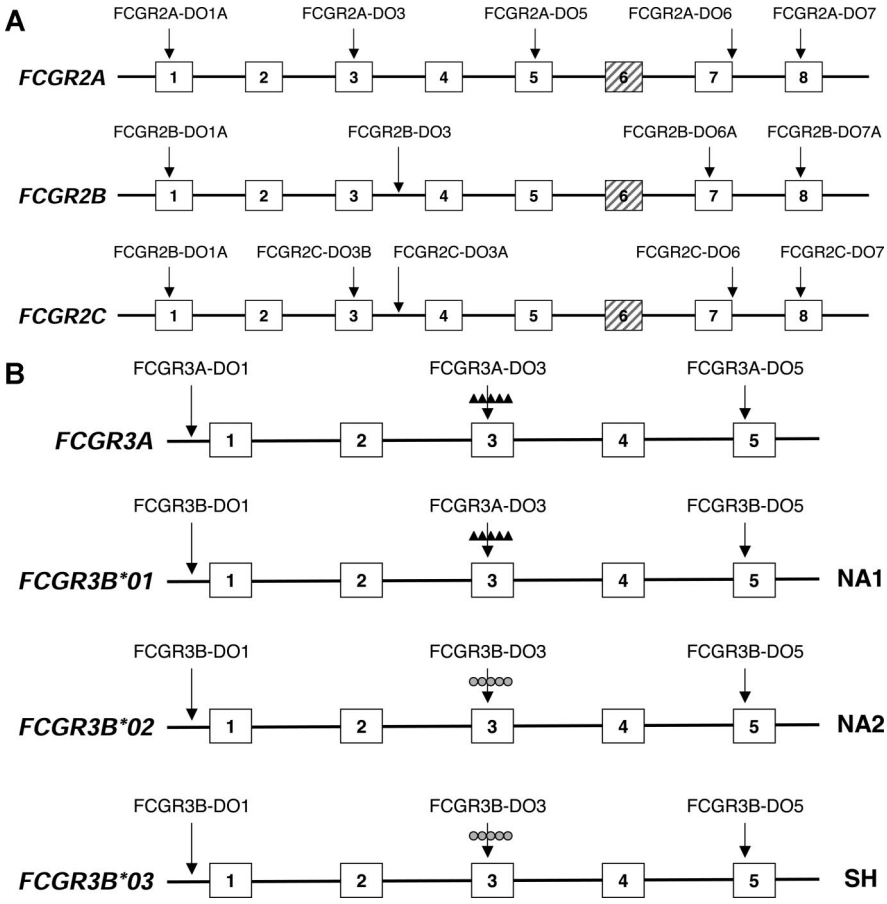


**Figure 1. Location of MLPA probes within the *FCGR2* and *FCGR3* gene clusters.** (A) Location of MLPA probes within the *FCGR2* gene cluster. Several probes within each separate gene, being either specific for *FCGR2A, FCGR2B,* or *FCGR2C* were used to determine CNV and SNPs. Shown are only the probes to determine CNV as indicated by the arrows. For a more detailed probe description, including the SNP probes, we refer to Table S1. (B) Location of MLPA probes within the *FCGR3* gene cluster. Several probes within each separate gene were used to determine CNV and the SNPs. Note that the probe that recognizes *FCGR3B*-HNA1a is the same that recognizes *FCGR3A.* For a more detailed probe description, we refer to Table S1.

### Real-time quantitative PCR

Intron-spanning primers specific for FcγRIIc were designed, forward primer 5′-ATCATTGTGGCTGTGGTCACTGG-3′ and reverse primer 5′-CTTTCTGATGGCAATCATTTGACG-3′, and β-glucuronidase was used as a reference gene. Amplification by PCR was performed on a LightCycler instrument (Roche, Almere, The Netherlands) and analyzed with software version 3.5. The reaction was performed with Lightcycler FastStart DNA Master$^{PLUS}$ SYBR Green I (Roche Diagnostics, Indianapolis, IN), as has been previously described in detail.[33]

### Monoclonal antibodies and reagents

The following monoclonal antibodies (mAbs) against human Fcγ receptors (FcγR) were used: CD16 (anti-FcγRIII, IgG1 isotype, clone 3G8; Sanquin, Amsterdam, The Netherlands); CD16-APC-Cy7 (anti-FcγRIII, IgG1 isotype, clone 3G8; BD Pharmingen, Alphen a/d Rijn, The Netherlands), CD32 (anti-FcγRII, IgG1 isotype, clone KB61; Sanbio, Uden, The Netherlands), and CD32-FITC (anti-FcγRII, IgG1 isotype, clone KB61; DakoCytomation, Glostrup, Denmark). For the detection of NK cells, CD56-APC (isotype IgG1, clone B159; BD Pharmingen) was used. Relevant isotype controls were IgG1-FITC (clone 203; Sanquin), IgG2b-FITC (clone GC198; Sanquin), IgG1-APC (clone MOPC-21; BD Pharmingen), and IgG1-APC-Cy7 (clone X40; BD Pharmingen). Expression of these markers was analyzed by tricolor flow cytometry on a FACS LSRII machine (BD Biosciences) using BD FACS Diva Software (version 5.0.1; BD Biosciences, San Jose, CA).

### Cell lines

The FcγR-bearing P815 murine mastocytoma cells were a generous gift of Dr R. Mous (AMC, Amsterdam, The Netherlands) and were cultured in Iscove modified Dulbecco medium (IMDM; Biowhittaker Europe, Verviers, Belgium) supplemented with 10%, v/v, fetal calf serum (FCS; Bodinco, Alkmaar, The Netherlands), 100 U/mL penicillin (Gibco, Paisley, United Kingdom), and 100 μg/mL streptomycin (Gibco).

### Isolation of neutrophils and PBMCs

Heparinized venous blood was collected from healthy donors and patients after obtaining informed consent and was separated over a Percoll gradient (GE Healthcare) into PBMCs as interphase and granulocytes in the pellet as described before.[33] Purity and viability of both cell fractions were more than 95%, as determined by flow cytometry and trypan blue exclusion, respectively. PBL (peripheral blood lymphocytes) were obtained from PBMCs by allowing the monocytes to adhere for 1 hour on the plastic surface of a culture flask. In some experiments for mRNA isolation from selective cell types, further purification by MiniMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed with the relevant MoAbs for positive selection. In these cases, purity of cell fractions was more than 99%.

### In vitro activation of neutrophils and PBMCs

Neutrophils and PBMCs were isolated as described above and cultured in 24-well plates at a density of $10^6$ cells/mL. Wells contained medium alone (control cells), 50 U/mL rhGM-CSF (generous gift from Dr Lucien Aarden, Sanquin Research, Amsterdam, The Netherlands), 5 ng/mL TNFα (PeproTech EC, London, United Kingdom), or 20 ng/mL LPS (Sigma-Aldrich, St Louis, MO) supplemented with rhLBP (LPS-binding protein, Boehringer Ingelheim, Germany). Each condition was as applied in triplicate. After 4 hours, samples were taken for quantification of FcγRIIc mRNA.

### In vitro activation of NK cells

PBLs were isolated as described above and cultured in 24-well plates at a density of $3.5 \times 10^5$ cells/mL. Wells contained either medium alone (control cells), 50 U/mL rhIL-2 (Strathmann Biotec, Hannover, Germany), or 10 ng/mL rhIL-15 (R&D Systems, Abingdon, United Kingdom). Each condition was as applied in duplicate. At days 0, 2, and 4, samples were taken for analysis by flow cytometry of their FcγR expression. Samples taken at day 2 were also assessed for cytotoxicity.

**Table 1. Copy number variation in the *FCGR2* and *FCGR3* genes**

| | Controls | ITP patients | Adult ITP | Children ITP |
|---|---|---|---|---|
| ***FCGR2C*** | | | | |
| 1 | 7 (7) | 6 (5.2) | 0 (0) | 6 (8.3) |
| 2 | 82 (82) | 103 (88.8) | 41 (93.1) | 62 (86.1) |
| 3 | 11 (11) | 7 (6.0) | 3 (6.8) | 4 (5.6) |
| ***FCGR3A*** | | | | |
| 1 | 1 (1) | 4 (3.4) | 2 (4.5) | 2 (2.8) |
| 2 | 96 (96) | 108 (93.1) | 39 (88.6) | 69 (95.8) |
| 3 | 3 (3) | 4 (3.4) | 3 (6.8) | 1 (1.4) |
| ***FCGR3B*** | | | | |
| 1 | 7 (7) | 6 (5.2) | 0 (0) | 6 (8.3) |
| 2 | 82 (82) | 103 (88.8) | 41 (93.1) | 62 (86.1) |
| 3 | 11 (11) | 7 (6.0) | 3 (6.8) | 4 (5.6) |

Individuals with 1, 2, or 3 copies of a gene were observed. No significant difference existed in frequencies between controls and ITP patients. Data are no. (%).

### Redirected antibody-dependent cytotoxicity assay

The cytotoxicity of unstimulated and IL-12– or IL-15–stimulated NK cells was assessed by incubating NK cells in the presence of FcγR-bearing P815 cells as targets. Target cells were plated in 96-well round-bottom plates at $10^4$/well. PBLs were mixed with target cells at E:T ratios of 50:1, 25:1, 12:1, and 6:1, in the presence or absence of either 5 μg/mL anti-FcγRII (clone KB61) or 5 μg/mL anti-FcγRIII (clone 3G8). After 4 hours of incubation at 37°C at 5% $CO_2$, cells were harvested, and 7-amino-actinomycin (7-AAD; Invitrogen, Leiden, The Netherlands) was added to determine cell death by flow cytometry. The percentage of specific cell death was calculated by subtracting the percentage of spontaneous cell death from experimental samples.

### Statistics

Genotyping data were analyzed by Fisher exact tests, a $P$ value of less than .05 was considered statistically significant. Results of the in vitro data are expressed as mean plus or minus SEM. Where applicable, Student $t$ test or one-way ANOVA was used. For comparison of the various time points, a 2-way ANOVA was performed.

## Results

### Distribution of FCGR gene CNV in healthy controls

Genetic variation in the *FCGR* gene cluster was studied with a newly developed MLPA assay (Figure 1 and Table S1). In total, 100 healthy white controls were evaluated. We observed no CNV for the *FCGR2A* and *FCGR2B* genes. In contrast, both the *FCGR3A* and the *FCGR3B* genes showed variation in copy number (Table 1). As has been reported previously by our group,[17] CNV in *FCGR3B* is directly linked to similar variation in the *FCGR2C* gene number. Here, we confirm this linkage by use of MLPA.

We found a CNV in *FCGR3A* that was not linked to the gene copy number of the other *FCGR* genes studied here. Furthermore, the number of *FCGR3A* genes corresponded with the number of FcγRIIIa molecules on immune cells (manuscript in preparation), as has been described previously for *FCGR3B* gene copy variation.[17] The CNV for the *FCGR3A* gene was observed in 3 individuals with 3 gene copies and in 1 individual with only a single *FCGR3A* gene copy (Figure 2).

### Distribution of FCGR gene SNPs in healthy controls

Regarding the distribution of the most common SNPs within these genes, that is, FcγRIIa-131H/R, FcγRIIb-232I/T, FcγRIIIa-158V/F, and the FcγRIIIb HNA1a/HNA1b/HNA1c, we observed allele frequencies that were close to the frequencies reported before in the white population (Table 2).[8,34,35]

For the FcγRIIIb polymorphisms, we found that our MLPA assay is superior to tests previously described for genotyping. When conventional genotyping results of the *FCGR3B*1* (HNA1a), *FCGR3B*2* (HNA1b), *FCGR3B*3* (HNA1c) alleles were compared with the results obtained with MLPA, all observed differences could be explained by variation in the copy number. For instance, an individual formerly predicted to be *FCGR3B*1, FCGR3B*1* (HNA1a/HNA1a) could now be correctly typed as *FCGR3B*1/−* (HNA1a/null). The *FCGR3B*3* (HNA1c) variant was observed in 4 (4%) of control individuals.

In the control group, 25% of the donors were heterozygous for the SNP at position −386 (G > C) in the promoter region of the *FCGR2B/C* genes (Table 3). In 100 donors, we did not observe the homozygous −386C variant. Gene-specific long-range PCR was performed on the DNA samples carrying the *FCGR2B/C* −386G/C variant to discriminate between the promoters of either the *FCGR2B* or *FCGR2C* gene, because it was not possible to design a discriminating gene-specific probe for our MLPA assay.

In the *FCGR2C* gene, an SNP in exon 3 converts a glutamine in the ORF to the most commonly found stop codon. In the control group, 82% of the individuals were homozygous for the STOP codon (*FCGR2C* $^{stop}$, *FCGR2C* $^{stop/stop}$, or *FCGR2C* $^{stop/stop/stop}$), which is close to what has been reported previously.[36]

### Genetic variation in ITP

In total, 116 white ITP patients were evaluated, divided into ITP of childhood-onset (< 16 years at time of diagnosis; n = 72; male, 48.6%) and ITP of adult-onset (> 16 years at time of diagnosis; n = 44; male, 15.9%). Genetic analysis was performed by the same MLPA and PCR assays as used in controls.

First, variation in copy number was not associated with susceptibility to ITP in our cohort. As in the control group, we found no CNV in *FCGR2A* and *FCGR2B*. In the ITP group, 3 copies of the *FCGR3B* allele were found in 7 patients and 1 single *FCGR3B* allele in 6 patients, respectively. In all patients, these single or triple copy alleles were linked to an identical variation in the copy number of the *FCGR2C* gene. These results are similar to what was found in the control group. Hence, we did not observe an association between the CNV of *FCGR3B* and susceptibility to ITP (Table 1). In 4 patients, a loss of copy number for *FCGR3A* was observed, and also in 4 patients a gain in copy number was observed, resulting in 1* *FCGR3A* allele and 3* *FCGR3A* alleles, respectively (Figure 2).

Second, genotyping results of the FcγRIIa-131H/R and the FcγRIIb-232I/T SNPs also showed no significant differences in genotype or allele frequencies when ITP samples were compared with the control samples (Table 2). The allele frequencies of the *FCGR3A* SNP for the FcγRIIIa-158F and FcγRIIIa-158V variants were 55.2% and 44.8%, respectively, for the ITP patients. The allele frequencies differed significantly between ITP patients and healthy controls ($P$ = .003; Table 2). When adult-onset and pediatric-onset ITP patients were separately analyzed, only for the children a significant overrepresentation of the FcγRIIIa-158V was observed ($P$ < .001; Table 2), as shown before in pediatric ITP.[27,28] Allele frequency in adult-onset ITP patients was comparable to the control samples. Any genotypic variation of the polymorphism FcγRIIIa-158V/F resulting from CNV was separately confirmed by quantitative PCR (LightCycler method; data not shown).

With MLPA, also a significant difference in genotype ($P$ = .01) frequency for the *FCGR2B/C* promoter polymorphism at position

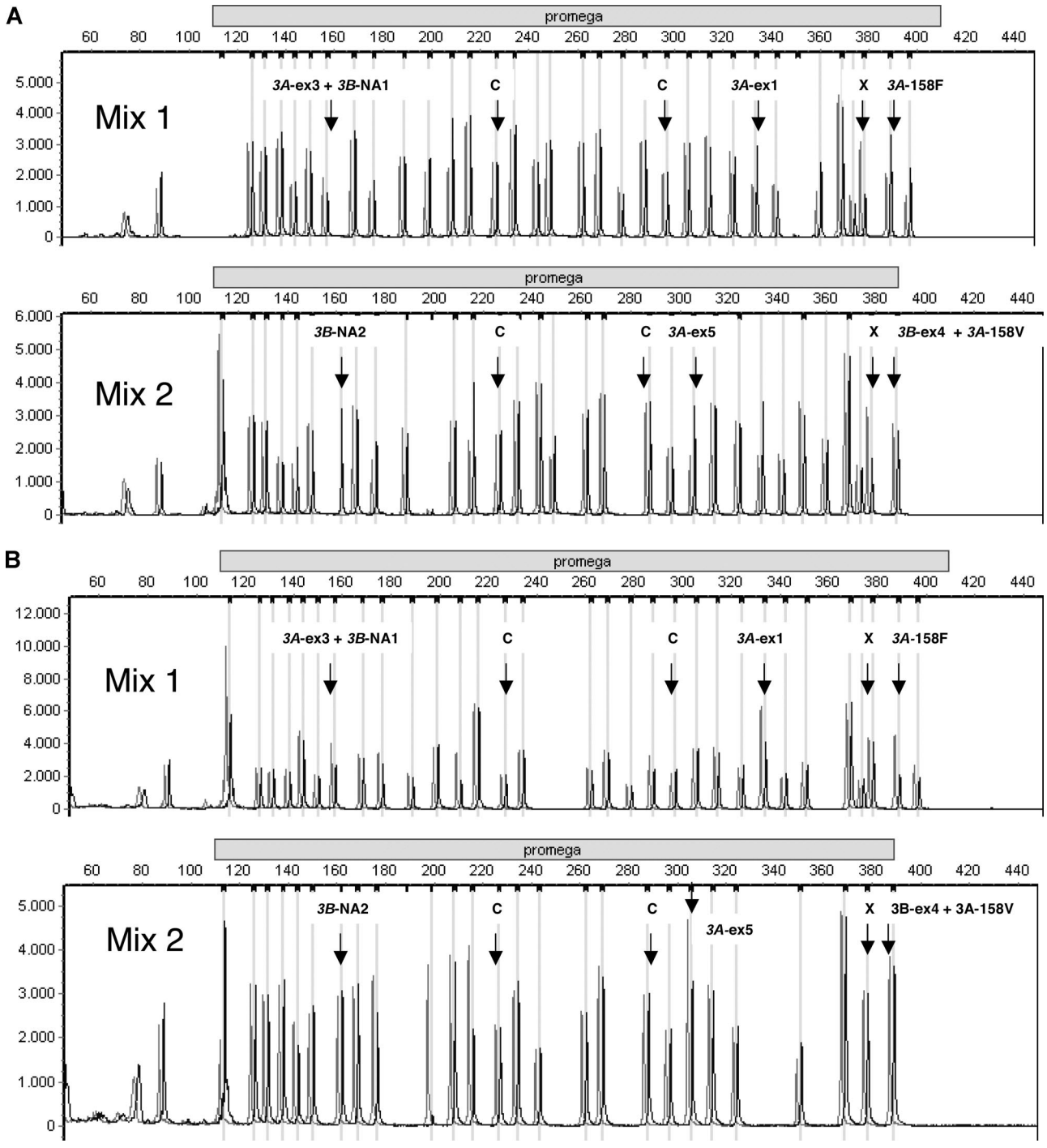


**Figure 2. CNV in FCGR3A.** (A) MLPA electropherogram. Individual A with only 1 allele FCGR3A (gray line) compared with individual B with 2 alleles of *FCGR3A* (black line). FCGR3A-specific probes are indicated with an arrow. As an example, 2 of the control probes are indicated (C). One of the probes is specific for the X-chromosome (X). Individual A: Female, FCGR3B-Na1Na1, FCGR3A-158F. Individual B: Male, FCGR3B-Na2Na2, FCGR3A-158FF. (B) MLPA electropherogram. Individual C with 3 alleles FCGR3A (gray line) compared with individual D with 2 alleles of *FCGR3A* (black line). FCGR3A-specific probes are indicated with an arrow. As an example, 2 of the control probes are indicated (C). One of the probes is specific for the X-chromosome (X). Individual C: Female, FCGR3B-Na2Na2, FCGR3A-158VFF. Individual D: Female, FCGR3B-Na2Na2, FCGR3A-158VF. Because of the homology between certain probes, probes had to be divided over 2 separate mixes to prevent competition.

−386 G/C was observed (Table 3). The genotype −386 CC was rare, as it was only observed in one patient.

Regarding the *FCGR2C* gene, a significant overrepresentation of the ORF in the ITP group was observed ($P = .009$; Table 3), with 82% being $FCGR2C^{stop}$, $FCGR2C^{stop/stop}$, or $FCGR2C^{stop/stop/stop}$ in the control cohort versus 65.5% in the ITP group. Moreover, 3 ITP patients were found to carry the homozygous $FCGR2C^{ORF/ORF}$ genotype. W observed that 86.2% of the individuals carrying an *FCGR2C*-ORF allele were also heterozygous for the *FCGR2B/C* −386 promoter polymorphism (data not shown). Individuals carrying an *FCGR2C*-ORF allele and a heterozygous *FCGR2B/C* −386 promoter polymorphism were all observed to have the promoter haplotype *FCGR2C* −386C/−120T, previously designated 2B.2,[37] as was analyzed with specific long-range PCR (Table 4). When FcγRIIIa-158V data were combined with the *FCGR2C*-ORF allele and promoter haplotype *FCGR2C* −386C/−120T, we did not observe an absolute linkage (data not shown).

**Table 2. Genotyping results of SNPs in the *FCGR2* and *FCGR3* genes**

| | Controls | All ITP | Adult ITP | Children ITP |
|---|---|---|---|---|
| ***FCGR2A*** | | | | |
| Genotype frequency | | | | |
| 131RR | 20 (20) | 27 (23.3) | 8 (18.2) | 19 (26.3) |
| 131HR | 52 (52) | 54 (46.6) | 26 (59.1) | 28 (38.9) |
| 131HH | 28 (28) | 35 (30.2) | 10 (22.7) | 25 (34.7) |
| Allele frequency | | | | |
| 131R | 92 (46) | 108 (46.6) | 42 (47.7) | 66 (45.8) |
| 131H | 108 (54) | 124 (53.4)* | 46 (52.3)* | 78 (54.2)* |
| ***FCGR2B*** | | | | |
| Genotype frequency | | | | |
| 232II | 81 (81) | 97 (83.6) | 37 (84.1) | 60 (83.3) |
| 232IT | 15 (15) | 15 (12.9) | 5 (11.4) | 10 (13.9) |
| 232TT | 4 (4) | 4 (3.4) | 2 (4.5) | 2 (2.8) |
| Allele frequency | | | | |
| 232I | 177 (88.5) | 209 (90.1) | 79 (89.8) | 130 (90.2) |
| 232T | 23 (11.5) | 23 (9.9)* | 9 (10.2)* | 14 (9.7)* |
| ***FCGR3A*** | | | | |
| Genotype frequency | | | | |
| 158F | 0 (0) | 3 (2.6) | 2 (4.5) | 1 (1.4) |
| 158FF | 48 (48) | 30 (25.9) | 15 (34.1) | 15 (20.8) |
| 158FFF | 1 (1) | 2 (1.7) | 2 (4.5) | 0 (0) |
| 158V | 1 (1) | 1 (0.9) | 0 (0) | 1 (1.4) |
| 158VV | 7 (7) | 23 (19.8) | 8 (18.2) | 15 (20.8) |
| 158VVV | 1 (1) | 0 (0) | 0 (0) | 0 (0) |
| 158VF | 41 (41) | 55 (47.4) | 16 (36.4) | 39 (54.1) |
| 158VVF | 1 (1) | 0 (0) | 0 (0) | 0 (0) |
| 158VFF | 0 (0) | 2 (1.7) | 1 (2.3) | 1 (1.4) |
| Allele frequency | | | | |
| 158F | 141 (69.5) | 128 (55.2) | 56 (62.9) | 72 (50.3) |
| 158V | 62 (30.5) | 104 (44.8)† | 33 (37.1)‡ | 71 (49.7)§ |
| ***FCGR3B*** | | | | |
| Genotype frequency | | | | |
| HNA1a | 4 (4) | 4 (3.4) | 0 (0) | 4 (5.6) |
| HNA1aHNA1a | 13 (13) | 13 (11.2) | 5 (11.4) | 8 (11.1) |
| HNA1aHNA1aHNA1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| HNA1b | 3 (3) | 2 (1.7) | 0 (0) | 2 (2.8) |
| HNA1bHNA1b | 34 (34) | 55 (47.4) | 22 (50.0) | 33 (45.8) |
| HNA1bHNA1bHNA1b | 1 (1) | 0 (0) | 0 (0) | 0 (0) |
| HNA1aHNA1b | 35 (35) | 35 (30.2) | 14 (31.8) | 21 (29.2) |
| HNA1aHNA1aHNA1b | 2 (2) | 1 (0.9) | 0 (0) | 1 (1.4) |
| HNA1aHNA1bHNA1b | 8 (8) | 6 (5.2) | 3 (6.8) | 3 (4.2) |
| Allele frequency | | | | |
| HNA1a | 77 (37.7) | 73 (31.3) | 27 (29.7) | 46 (32.4) |
| HNA1b | 127 (62.3) | 160 (68.7)* | 64 (70.3)* | 96 (67.6)* |
| HNA1c (SH) | 4 (4) | 5 (4.3)* | 1 (2.3)* | 4 (5.6)* |

Due to CNV, the 3-allelic variation was observed for *FCGR3A*-158VF and *FCGR3B*-HNA1aHNA1b. Data are no. (%). Significance levels are indicated by symbols.
*$P$ is not significant.
†$P$ is .003.
‡$P$ is .3.
§$P$ is less than .001.

### *FCGR2C* splice variants

To date, 5 splice variants of FcγRIIc have been reported, 2 of which result in a membrane-anchored receptor, that is, FcγRIIc1 encoded by the full transcript, and FcγRIIc3 reported to lack exon 7.[38,39] We hypothesized that alternative splicing of the FcγRIIc transcript is restricted to the *FCGR2C*-stop allele. To investigate this, we cloned the FcγRIIc transcripts from a single individual with an $FCGR2C^{ORF/ORF}$ genotype and from 3 individuals with an $FCGR2C^{ORF/stop}$ genotype into a bacterial expression vector and obtained the sequence of at least 20 clones per individual. Upon sequencing of all these clones, we found that individuals genotyped as $FCGR2C^{ORF/ORF}$ or $FCGR2C^{ORF/stop}$ only express FcγRIIc1 (data not shown). Various studies have reported about a polymorphism at the splice donor site of the intron following the C2 exon (exon7).[12,38,40] In general, we observed the nucleotides AT at the splice border of $FCGR2C^{stop/stop}$ individuals and only in rare cases a heterozygous GT/AT (detectable with probe 2C-DO6 and 2A-DO6), which will, however, not be expressed due to the Stop codon. In case of an *FCGR2C*-ORF allele, the reverse was true. We mainly observed a GT at the splice border, but in some cases we did observe AT. This could imply that FcγRIIc1 may not be the only functional splice variant expressed. Further studies will have to be done to investigate the function of the transmembrane protein of transcripts with both the ORF and the AT splice border.

### Distribution of *FCGR2C* expression

We explored the mRNA expression of FcγRII isoforms in neutrophils, monocytes, NK cells, T cells, and B cells from healthy

**Table 3. Frequency of nonspecific promotor polymorphism −386 G > C and frequency of stop codon versus ORF in exon 3 of *FCGR2C***

| | Controls | All ITP | Adult ITP | Children ITP |
|---|---|---|---|---|
| **Promotor 2B/C −386** | | | | |
| GG | 75 (75) | 68 (58.6) | 25 (56.8) | 43 (59.7) |
| CG | 25 (25) | 47 (40.5) | 18 (40.9) | 29 (40.2) |
| CC | 0 (0) | 1 (0.9)*† | 1 (2.3)†‡ | 0 (0)†§ |
| **FCGR2C exon 3** | | | | |
| STOP | 6 (6) | 4 (3.4) | 0 (0) | 4 (5.6) |
| STOP/STOP | 66 (66) | 68 (58.6) | 28 (63.6) | 40 (55.6) |
| STOP/STOP/STOP | 10 (10) | 4 (3.4) | 1 (2.3) | 3 (4.2) |
| ORF | 1 (1) | 2 (1.7) | 0 (0) | 2 (2.8) |
| ORF/ORF | 0 (0) | 3 (2.6) | 2 (4.5) | 1 (1.4) |
| ORF/STOP | 16 (16) | 32 (27.6) | 11 (25) | 21 (29.2) |
| ORF/ORF/STOP | 0 (0) | 1 (0.9) | 1 (2.3) | 0 (0) |
| ORF/STOP/STOP | 1 (1) | 2 (1.7)¶‖ | 1 (2.3)§‖ | 1 (1.4)‖# |
| **FCGR2C exon 3 (allele frequency)** | | | | |
| STOP | 186 (91.2) | 189 (81.1) | 73 (80.2) | 116 (81.7) |
| ORF | 18 (8.8) | 44 (18.9)** | 18 (19.8)* | 26 (18.3)* |

Data are no. (%).
*$P$ is .01.
†GG vs CG + CC.
‡$P$ is .03.
§$P$ is .05.
¶$P$ is .009.
‖STOP + STOP/STOP + STOP/STOP/STOP vs ORF + ORF/ORF + ORF/STOP + ORF/ORF/STOP + ORF/STOP/STOP.
#$P$ is .02.
**$P$ is .004.

volunteers. Transcripts for FcγRIIa, FcγRIIb2 (lacking exon 6 of *FCGR2B*), and FcγRIIc, but not FcγRIIb1 (containing exon 6 of *FCGR2B*), were found in both neutrophils and monocytes, while B cells contained only FcγRIIb1.[33] T cells did not express any of the FcγRII isoforms, whereas NK cells solely expressed FcγRIIc. This is in line with previous findings (Figure 3A).[41]

### Expression of FcγRIIc on NK cells

*FCGR2A, FCGR2B,* and *FCGR2C* have 92% to 96% sequence homology. There are no monoclonal antibodies available that can truly distinguish between FcγRIIa, FcγRIIb, and FcγRIIc, rendering it difficult to quantify the protein expression on cells. However, we found that NK cells only expressed mRNA of the FcγRIIc isoform. For this reason, we examined the expression of FcγRII on NK cells of individuals with the $FCGR2C^{ORF/stop}$ genotype and the $FCGR2C^{stop/stop}$ genotype. We found that the presence of an *FCGR2C*-ORF allele correlated with FcγRII expression on NK cells, whereas the absence of a functional allele corresponded with the total absence of any FcγRII on NK cells (Figure 3B and Table 4). Thus, FcγRIIc is present on NK cells.

### In vitro activation of NK cells

The FcγRIIc expression on NK cells was measured 3 times at various time intervals (range, 2–5 months) in 4 individuals with the $FCGR2C^{ORF/stop}$ genotype. Variable expression levels of FcγRIIc occurred, irrespective of the constant *FCGR2B/C* promoter haplotype 2B.2[37] in all individuals tested. Minor fluctuation was observed over time (MFI ± SEM: 1707 ± 211).

Subsequent testing in NK-cell cultures with the NK-cell activators IL-2 or IL-15 showed the ability to modulate the FcγR expression on NK cells. In line with earlier reports,[42] we found that both IL-2 and IL-15 up-regulated the surface expression of FcγRIIIa on NK cells. In contrast, FcγRIIc expression was down-regulated during culture, although IL-15 significantly rescued the loss of FcγRIIc expression compared with either IL-2 or medium (Figure 3C).

Because surface expression of FcγRIIc on neutrophils and monocytes cannot be monitored, the regulation of FcγRIIc mRNA in these cells was tested after incubating neutrophils as well as PBMCs with the inflammatory activators LPS, GM-CSF, or TNFα. In particular, GM-CSF induced a strong up-regulation of FcγRIIc mRNA in neutrophils obtained from individuals with an $FCGR2C^{ORF/stop}$ genotype, whereas this was found to a significantly lower extent in individuals with an $FCGR2C^{stop/stop}$ genotype ($P < .01$; Figure 3D).

### Redirected antibody-dependent cellular cytotoxicity

To assess whether FcγRIIc expression on innate immune cells was functionally active, we tested the capability of the NK cells to kill

**Table 4. Correlation of FCGR2C-ORF with FcγRIIc expression on NK cells and with promoter haplotype *FCGR2C* 2B.2**

| | Expression | | MLPA | | Gene-specific PCR | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | NK cells | MFI | *FCGR2C* stop/ORF | *2B/C* −386 | *2B* −386 | *2B* −120 | *2C* −386 | *2C* −120 |
| 1 | No | nd | stop/stop | W | — | — | — | — |
| 2 | No | nd | stop/stop | W | — | — | — | — |
| 3 | No | nd | stop/stop | W | — | — | — | — |
| 4 | No | nd | stop/stop | W | — | — | — | — |
| 5 | No | nd | stop/stop | W | — | — | — | — |
| 6 | No | nd | stop/stop | W | — | — | — | — |
| 7 | No | nd | stop/stop | W | — | — | — | — |
| 8 | No | nd | stop/stop | W | — | — | — | — |
| 9 | No | nd | stop/stop | W | — | — | — | — |
| 10 | No | nd | stop/stop | W | — | — | — | — |
| 11 | Yes | 590 | ORF/stop | He | GC | TA | GC | TT |
| 12 | Yes | 1188 | ORF/stop | He | GC | TA | GC | TT |
| 13 | Yes | 1648 | ORF/stop | He | GC | TA | GC | TT |
| 14 | Yes | 2602 | ORF/stop | He | GC | TA | GC | TT |
| 15 | Yes | 2123 | ORF/stop | He | GC | TA | GC | TT |
| 16 | Yes | 1109 | ORF/stop | He | GC | TA | GC | TT |
| 17 | Yes | 2380 | ORF/stop | He | GC | TA | GC | TT |
| 18 | Yes | 1243 | ORF/stop | He | GC | TA | GC | TT |
| 19 | Yes | 1394 | ORF/stop | He | CC | AA | GC | TT |
| 20 | Yes | 1629 | ORF/stop | He | GC | TA | GC | TT |

MFI indicates mean fluorescence units; nd, not determined; W, wild-type; —, not tested because wild-type in MLPA; and He, heterozygous.

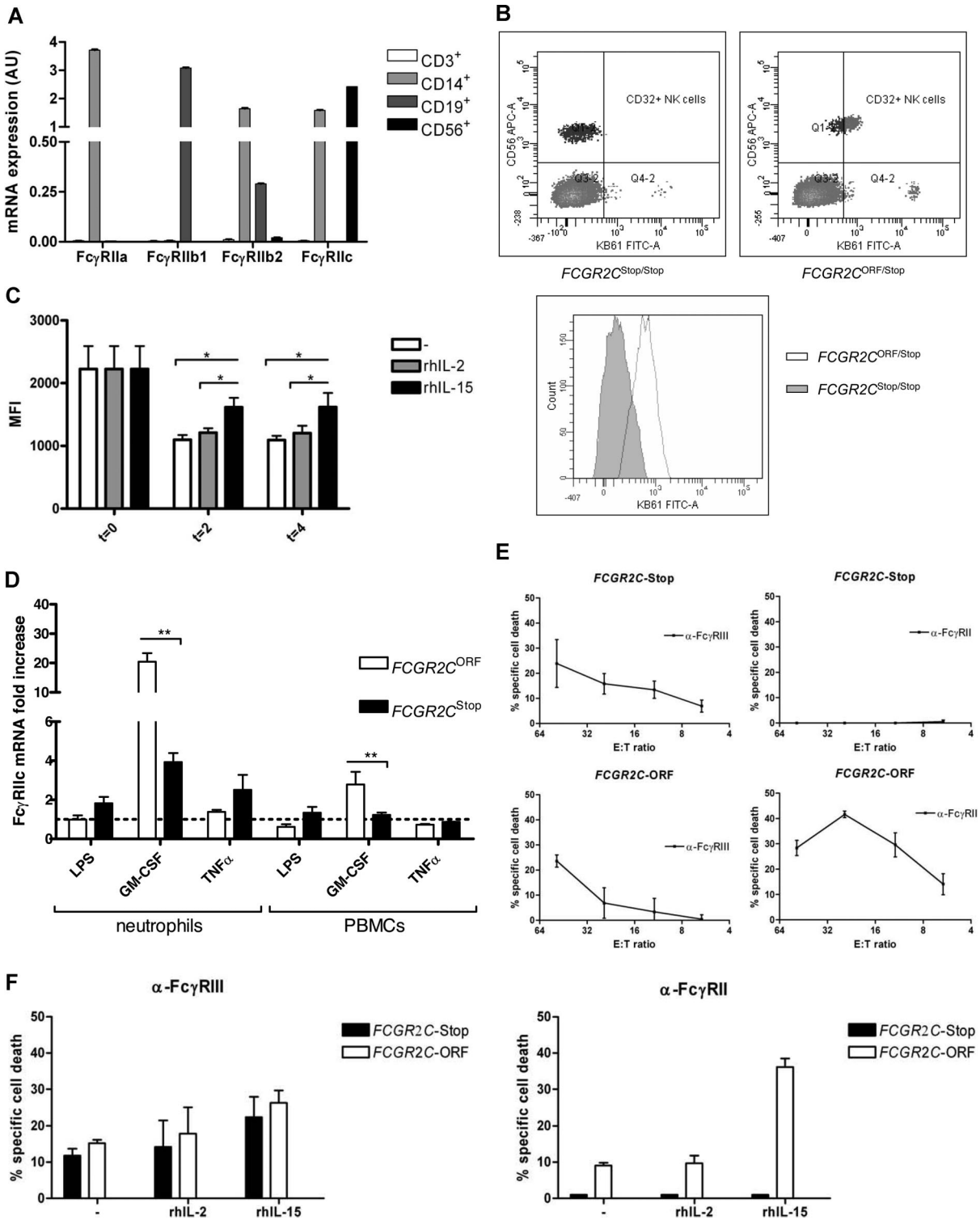


**Figure 3. FcγRIIc expression and function.** (A) Distribution of FcγRIIc mRNA expression in leucocytes. T cells $CD3^+$, monocytes, and neutrophils $CD14^+$, B cells $CD19^+$, NK cells $CD56^+$. (B) FcγRII expression on NK cells is limited to the *FCGR2C*-ORF genotype. Blood cells were incubated with CD56 and CD32. Lymphocytes were gated on the basis of their forward scatter/side scatter pattern. NK-cell population was determined as CD56-positive lymphocytes. (C) FcγRIIc expression on NK cells is modulated by IL-15 but not by IL-2. PBMCs were isolated and cultured as described in "In vitro activation of NK cells." Expression of CD32 on $CD56^+$ NK cells was measured by flow cytometry with CD32, at the indicated time-points. There was a significant higher expression on days 2 and 4 on the IL-15–stimulated NK cells ($P = .01$, n = 5). Data are expressed as mean plus or minus SEM. (D) FcγRIIc mRNA is strongly up-regulated by GM-CSF on cells of *FCGR2C*-ORF donors. Neutrophils and PBMCs were isolated and cultured for 4 hours with the indicated stimuli as described in "In vitro activation of neutrophils and PBMCs." FcγRIIc mRNA was measured by quantitative RT-PCR. GM-CSF strongly up-regulated FcγRIIc mRNA in neutrophils and, to a lesser extent, in PBMCs of *FCGR2C*-ORF genotyped donors, but not in *FCGR2C*-Stop donors ($P = .0001$ and $P = .01$, respectively; n = 5-8). Data are expressed as mean plus or minus SEM. (E) rADCC. PBLs were isolated as described in "Isolation of neutrophils and PBMCs," and FcγRIIc functionality was assessed by rADCC. Cells from both *FCGR2C*-Stop and *FCGR2C*-ORF genotyped donors killed anti-FcγRIII–coated targets with similar kinetics (left panels). In contrast, only cells from *FCGR2C*-ORF genotyped donors were capable of killing anti-FcγRII–coated targets (right panels) (n = 4). Data are expressed as mean plus or minus SEM. (F) rADCC with stimulated cells. PBLs were obtained and subsequently cultured for 2 days with or without IL-2 or IL-15. Thereafter, the cells were harvested and used in a rADCC. Cells from both *FCGR2C*-Stop and *FCGR2C*-ORF genotyped donors killed anti-FcγRIII–coated targets (left panel). In both cases IL-15 and, to a lesser extent, IL-2 enhanced specific lysis of the anti-FcγRIII–coated targets (n = 3). In contrast, only cells from *FCGR2C*-ORF genotyped donors were capable of killing anti-FcγRII–coated targets (right panel). Here again, IL-15 strongly enhanced the specific cell lysis (n = 3). Data are expressed as mean plus or minus SEM.

antibody-coated target cells. To selectively target either FcγRIIc or FcγRIIIa on NK cells, we used the rADCC test with the FcγR-bearing murine mastocytoma P815 cell line loaded with anti-FcγRII or anti-FcγRIII antibody.

We found that NK cells of an *FCGR2C*$^{ORF/stop}$ genotype donor were able to kill both anti-FcγRII and anti-FcγRIII–loaded target cells (Figure 3E). In contrast, NK cells of an *FCGR2C*$^{stop/stop}$ genotype donor were able to kill only anti-FcγRIII–loaded target cells. When the expression of FcγRIIc was reduced by culturing NK cells in the presence of activating IL-2 or IL-15 cytokines, the effect of FcγRIIc crosslinking in the rADCC assay showed even more pronounced killing capacity in IL-2– or IL-15–activated NK cells (Figure 3F).

## Discussion

Genetic variation in immunologically relevant genes results in differential responsiveness to infection but might subsequently also predispose to autoimmune disease as a result of unbalanced immunity. We investigated the role of the *FCGR* gene cluster on the predisposition to ITP by using a novel MPLA assay to analyze the relevant SNPs as well as CNV in *FCGR2A, FCGR2B, FCGR2C, FCGR3A,* and *FCGR3B.*

By using MLPA, we were able to genotype more accurately than has been possible before, because now CNV and SNP analysis can be interpreted in one test system. *FCGR2A* and *FCGR2B* did not show gene copy number variation, in contrast to the other genes studied. We demonstrate a variation in the copy number for the *FCGR3A* gene, which, to our knowledge, has never been reported before. The relevance of CNV for the activating *FCGR2C* allele was indicated by the fact that in 91% of the white control population we found an SNP in exon 3 that results in a stop codon determining the presence of a pseudogene. However, the remaining 9% of the population expresses a functionally activating FcγRIIc on their immune cells. Although the presence of a pseudogene or ORF is determined by an SNP, this variation behaves like a copy number polymorphism with respect to the activating *FCGR2C* gene. Regarding the existence of an unbalanced immunity in autoinflammation, we found a significant overrepresentation of the *FCGR2C*-ORF allele in the ITP population (19%).

The relevance of this observation on the *FCGR2C*-ORF allele for ITP was subsequently shown by in vitro studies. We found expression of FcγRIIc mRNA in neutrophils, monocytes, and NK cells, but not in T and B cells. Neutrophils and monocytes also express other isoforms of FcγRII[33,43] and, although they have a key role as phagocytes in ITP, they are not ideal candidates to study the expression and function of FcγRIIc. NK cells express FcγRIIc but no FcγRIIa or FcγRIIb, and these cells were therefore used as a functional read-out for FcγRIIc, however, no direct link with the pathogenesis of ITP can be made. Only individuals carrying an *FCGR2C*-ORF allele were found to express FcγRIIc protein on NK cells (Figure 3B). We furthermore demonstrated that NK cells bearing FcγRIIc can mediate antibody-dependent cellular cytotoxicity (ADCC) to antibody-coated targets, demonstrating that FcγRIIc is indeed an activating IgG receptor molecule on immune cells. Although expressed to a lower extent than FcγRIIIa, FcγRIIc seems to mediate more killing than FcγRIIIa (Figure 3E). This might be due to the fact that FcγRIIc contains its own signaling motif, whereas FcγRIIIa is dependent on the γ-ζ heterodimer for signaling.

When investigating the role of FcγRIIc under inflammatory conditions, we found that IL-15 (but not IL-2) modulated FcγRIIc expression on NK cells, and GM-CSF strongly up-regulated FcγRIIc mRNA in phagocytes. Again, this was only observed in individuals carrying an *FCGR2C*-ORF allele. FcγRIIc might play a vital role during inflammatory conditions, mediating enhanced uptake of immune complexes or killing of antibody-coated target cells. This may lead to increased platelet destruction in ITP, in which these cytokines are present in sera of patients.[44,45]

In conclusion, we found that *FCGR2C*-ORF predisposes to ITP. We have shown that FcγRIIc is expressed by phagocytes and NK cells and that this enhances effector function toward antibody-coated targets. Furthermore, we have shown that under inflammatory conditions (GM-CSF or IL-15), FcγRIIc function is even further enhanced. Thus, taken together, these results suggest that *FCGR2C* is a variably expressed gene highly relevant for immunity that may contribute to susceptibility and severity of infections and autoimmune disease. We believe that this novel *FCGR*-specific MPLA will be applicable to study other autoimmune/inflammatory diseases more extensively than has been possible before.

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## Authorship

Contribution: W.B.B. and E.v.M. designed and performed research, analyzed data, and wrote the paper. M.B., M.P., H.R.K., and T.W.K. provided patient material. J.G. performed research, M.de B. provided vital analytical tools, and D.R. designed parts of the research. M.B., M.de H., and H.R.K. provided intellectual input, and T.W.K. designed the overall study, supervised the research, and helped write the paper.

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