Comprehensive approach for identification of functional FCGR2C alleles resulting in protein expression as a determinant for predicting predisposition to autoimmunity

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Abstract
The balance of activating and inhibitory signals from the low affinity Fc gamma receptors modulates immune responses triggered by IgG antibody-immune complexes. In homeostasis, this leads to antigen clearance, while in autoimmune diseases to unwanted immune response. Besides the activating receptors FcγRIIa, FcγRIIla, and the inhibitory FcγRIIb receptor, a third activating receptor, FcγRIIc, was shown to be expressed on several immune cell types, however, only in the presence of a functional FCGR2C-ORF allele. FcγRIIc expression is associated with autoimmune diseases such as idiopathic thrombocytopenic purpura, systemic lupus erythematosus or systemic sclerosis. Thus, the determination of the functional FCGR2C gene resulting in protein expression on immune cells becomes highly relevant, particularly in the context of unwanted immune responses through inadvertent FcγRIIc activation by molecules targeting stimulation of the inhibitory receptor FcγRIIb, currently pursued by several pharmaceutical companies. The high degree of homology within the FCGR2/3 gene cluster complicates development of an accurate method for identification of FcγRIIc expression. Here we describe a comprehensive approach to characterize genetic status of the FCGR2C gene locus consisting of cDNA sequencing, SNaPshot genotyping and low-coverage next-generation sequencing. This might enable Mendelian randomization hypothesis testing across autoimmune diseases to personalize therapies and enhance treatment outcomes.

KEYWORDS
copy number variation, FCGR2C expression, low affinity immunoglobulin receptors, SNP, systemic lupus erythematosus
1 | INTRODUCTION

Human cells of the hematopoietic lineage express a spectrum of Fc gamma receptors (FcγRs), which bind the Fc region of immunoglobulin G (IgG) and lead to immune response initiated by the antibody-mediated recognition of a specific antigen. While the high affinity of the interaction allows FcγRI receptor to bind monomeric IgG, the low affinity FcγRs bind only immune complexed IgG by functional avidity of the individual low affinity interactions. Binding of immune complexes leads to crosslinking of FcγR receptors and the induction of effector functions such as antibody-dependent cell-mediated cytotoxicity, phagocytosis and regulation of B-cell functions.1,2 Thus, transduced activating or inhibitory signals balance antibody-mediated immune cell responses and play an important role in antigen clearance under homeostatic conditions or exacerbation of inflammation under pathogenic autoimmune conditions accompanied by chronic antigen exposure. There are several low affinity activating receptors, FcγRIIa, FcγRIIa and FcγRIIc, and only one inhibitory receptor, FcγRIIb. The activating FcγRIIa and FcγRIIc receptors are encoded by FCGRA2A and FCGRC2, respectively, and contain immunoreceptor tyrosine-based activation motifs (ITAMs), while the FcγRIIb receptor (encoded by FCGRB2) contains cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM). The high degree of homology between the FCGR2 genes and their co-location in a gene cluster on chromosome 1p23.3 indicates that FCGRC2 originated from an ancestral segmental duplication. Consequently, FCGRC2 locus regions comprising exons 1–6 and 7–8 are highly homologous to FCGRB2 and FCGRA2A, respectively. Thus, FcγRIIb protein bares properties of the inhibitory FcγRIIb within its extracellular ligand-binding part, while within its intracellular signaling part it is homologous to activating FcγRIIa. FCGRC2 expression was detected in NK cells, monocytes, neutrophils and B-cells, where it can counterbalance inhibitory signals from the co-expressed FcγRIIb and lead to an augmented immune response.3,4 In contrast to the initial finding that FcγRIIC expression correlates with better prognosis in rheumatoid arthritis patients,5 recently, a body of evidence is accumulating to support a direct correlation between FcγRIIC expression and predisposition for autoimmune diseases such as ITP, SLE, systemic sclerosis and more severe malaria phenotypes, as well as an association with augmented humoral immune responses.3,6–8 Thus, the determination of the functional FCGRC2 gene resulting in protein expression on the cell surface of immune cells becomes highly relevant when considering the development of therapies for immune complex-mediated autoimmune disorders by means of stimulation of the inhibitory receptor FcγRIIb, currently pursued by several pharmaceutical companies.

For both FcγRII and FcγRIII receptors, several functionally important single-nucleotide polymorphisms (SNPs) and copy-number variations (CNVs) have been shown to result in differences in expression levels, functional properties, as well as causing a cumulative amount of transmitted signals upon recognition of IgG-containing immune complexes. The function of FcγRIIB is significantly affected by the rs1050501 SNP NM_001002274.2:c.695T > C in exon 3 of FCGRC2 results in a change from glutamine to a STOP codon and therefore loss of protein expression.11 Thus, the expression of FcγRIIC critically depends on the presence of an FCGRC2-ORF allele.11 Previous studies have estimated FCGRC2 expression prevalence between 15% - 45%,12,13 however, due to the technical challenges associated with the genotyping of this highly homologous gene cluster, more precise estimations are lacking. The FCGRC2/3 gene complex displays high CNV with four known CNV regions, which are caused by various types of non-allelic homologous recombination particularly affecting FCGRC2 and resulting in different gene-dosage effects.6,14 In a number of FCGRC2-STOP donors, a deletion event within the locus has led to FcγRIIB expression on NK cells, which -otherwise do not express it. In these cases, the FCGRC2 deletion is hypothesized to place FCGRB2 under the regulation of upstream regulatory elements that control FCGRC2 expression.4 Lack of FcγRIIC protein expression in individuals encoding for non-classical (n.c.) FCGRC2-ORF can also occur. In such case, a SNP (G > A) at the start of intron 7 gives rise to a premature STOP codon.

At present, several therapies for autoantibody-mediated diseases are being developed that exploit activation of the inhibitory FcγRIIB. In such cases, inadvertent stimulation of activating FcγRIIC due to sequence homology might result in disease escalation rather than remission. Thus, it is important to develop accurate methods for determination of functional FCGRC2 alleles leading to protein expression, as well as CNV assessment. In this study, we have developed two independent approaches, which, when combined, ensure accurate determination of FCGRC2 allele(s). Using these approaches, we detected functional FCGRC2-ORF allele in 24% of 151 individuals with different ethnicity and health status and have demonstrated that it led to FcγRIIC protein expression on the cell surface, thus verifying the validity of the described methods. The determination of the functional status of the FCGRC2 gene provides an opportunity for more detailed population studies that would not only deepen our understanding of the interplay of the FcγRII receptors resulting in balanced immune responses, but also lead to improved therapeutic options due to a better stratification of patients suffering from immune complex-mediated autoimmune disorders.

2 | MATERIAL AND METHODS

2.1 | Subjects

Samples from healthy adult volunteers (n = 135) and systemic lupus erythematous (SLE) patients (n = 16) from different ethnicities were analyzed (Table 1). The majority of healthy donor samples were obtained from local donors (n = 68), mainly of Caucasian origin, through a general practitioner (GP) in Vienna, Austria. Remaining samples from healthy donors and SLE patients were obtained from commercial sources, Tebu-bio (n = 31), Amsbio (n = 9), Tissue solutions (n = 30), Cellsystem (n = 9), Stemcell (n = 3) and invent
**TABLE 1** Overview of the genotyping results from healthy donor and SLE patient samples

| Ethnicity      | Healthy                          | ORF/STOP | Total  |
|----------------|----------------------------------|----------|--------|
| African-American | 105 (78)                        | 24 (18)  | 135 (100) |
| Asian          | 6 (4)                            | 7        |        |
| Caucasian      | 63 (74)                          | 17 (20)  | 85 (100) |
| Hispanic       | 21                               | 6        | 27     |
| Mixed          | 2                                |          | 2      |
| **Total**      | **116 (77)**                     | **27 (18)** | **151 (100)** |

| Ethnicity      | SLE                              | ORF/STOP | Total  |
|----------------|----------------------------------|----------|--------|
| African-American | 11 (69)                         | 3 (19)   | 16 (100) |
| Caucasian      | 8                               | 3 (23)   | 13 (100) |
| Hispanic       | 1                               |          | 1      |
| ND             | 1                                |          | 1      |
| **Total**      | **116 (77)**                     | **27 (18)** | **151 (100)** |

Number of individuals with respective genotype is displayed. Frequency of genotype (% of total) is shown in brackets for populations where relevant number of samples was collected. SLE, systemic lupus erythematosus; ORF, ORF/STOP and STOP – samples encoding only FCGR2C–ORF, both FCGR2C–ORF and -STOP, and only STOP alleles, respectively.

Diagnostica (n = 1). The information on ethnicity was provided by the donors themselves, no additional investigations were undertaken. All analyses were performed under full compliance of the local Ethics Commission’s approval (Ethics approval EK1180/2016, Ethics Commission of the Medical University Vienna, Vienna, Austria). A double-blinded coding system was employed for donor pseudonymity, as stipulated within the guidelines of the Local Ethics Commission.

### 2.2 Messenger RNA isolation, RT-PCR and FCGR2C cDNA sequencing

For messenger RNA isolation, fresh whole blood in PAXgene Blood RNA Tubes (IVD) (BD, Bioscience) or frozen PBMCs were used. Isolation of mRNA from fresh whole blood was performed by using PAXgene Blood RNA Kit – PreAnalytiX (QIAGEN), while mRNA from frozen PBMCs was isolated by using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Subsequently, first-strand cDNA was synthesized with AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies) according to the manufacturer’s protocol.

PCR amplification was performed in a 25 µL reaction containing 1x PCR Master Mix (Phusion Flash High-Fidelity PCR Master Mix, Thermo Scientific), 0.5 pmol/µL of primer Pf and primer Pr, respectively, and was carried out in a Mastercycler ep Gradient S (Eppendorf). Cycling conditions were: 3 min at 98 °C, followed by 45 cycles of 15 s denaturation at 98 °C, 15 s annealing at 60 °C and 45 s extension at 72 °C with a final extension of 2 min at 72 °C. FCGR2C Stop region sequencing of the 880 bp fragment was done at Eurofins genomics, Austria. Both strands were sequenced via the Sanger method with the above-mentioned PCR primers. Sequence information for all primers used in this study can be found in an online Supplementary table 2.

### 2.3 SNaPShot SNP genotyping

Genomic DNA samples were amplified using Accu Prime Taq high fidelity polymerase (Life technologies) in using 50 ng template over 50 cycles and temperature profile of 20 s denaturation followed by annealing at 58 °C and 7 min extension at 68 °C. DNA samples not yielding prominent products were reamplified using a semi-nested PCR approach: In a first PCR, 50 ng template DNA was amplified over 45 cycles using primers Forward and Reverse. 2.5 µL of the first PCR was used as a template for 45 cycles using primers Forward-nested and Reverse (Figure 3B, Supplementary table 2). Semi-nested PCR products were purified and subjected to SNaPshot® SNP genotyping.

Duplex SNP genotyping was performed with SNaPshot® Multiplex System (Life technologies): 0.4 µM rs223_rev and 0.8 µM rs223_for primers were used for SNP genotyping according to the manufacturer’s protocol (Supplementary table 2). SNaPshot products were treated with 1 µL Shrimp Alkaline Phosphatase (Affymetrix, 1 U/µL) for 30 min at 37 °C, following denaturation for 15 min, 75 °C. One microliter of phosphatized SnaPshot product was added to 11 µL formamide/standard mix (600 µL formamide was supplemented with 0.3 µL Size standard (GeneScan™ 120 LIZ™ dye). After denaturation for 2 min at 94 °C, SNaPshot® SNP genotyping was performed on an ABI PRISM® 310 Genetic Analyzer using POP-4TM optimized polymer and analyzed using GeneMapper Software.

### 2.4 Flow cytometry

Flow cytometry analysis was performed using 100 µL of whole blood. The sample was incubated for 15 min at room temperature in the dark with a mix of fluorescently labeled antibodies: CD14 Alexa Fluor 700
(clone 61D3), CD19 APC-eFluor 780 (clone SJ25C1), CD3 eFluor 450 (clone UCHT1), CD56 (NCAM) APC (clone CMSSB) all eBioscience, and CD45 PerCP (clone HI30), Isotype PE (REA 293) all obtained from Biologend. FCγRIIb BV650 (clone 2B6) and FCγRIIb/c PE (clone SM201) were labeled at Biolegend. FCγRIIA FITC (clone IV.3) was from Stem cell Technologies. The cells were then lysed twice with 1 mL RBC Lysis Buffer for 10 min and washed with 1 mL PBS. Cell viability was investigated by staining with fixable viability dye (eFluor 506, eBioscience) at 4°C for 30 min in the dark. After washing in 1x PBS with 1% BSA, cells were transferred to a 96-well plate and acquired with a BD LSR Fortessa. Analysis was performed with FlowJo software version 10.4.2. The CD56+ NK cells were analyzed by gating on CD19−CD14−CD3−CD45+ lymphocytes upon electronic removal of duplicate events.

2.5 Low coverage whole-genome sequencing (WGS)

Quantity and quality of genomic DNA were assessed by Qubit™ 2.0 Fluorometric Quantitation system (Life Technologies) and agarose gel electrophoresis, respectively. Libraries were prepared from 1 μg input material using the TruSeq™ DNA PCR-Free HT Library Prep Kit (Illumina) with IDT for Illumina - TruSeq™ DNA UD Indexes. Briefly, genomic DNA was sheared using a Covaris® Focused-ultrasonicator instrument (Covaris), DNA fragments were cleaned, end-repaired and 3′ A-tailed, followed by ligation of the sequencing adapters. After quality control, individual libraries were diluted, equimolarly pooled and sequenced on HiSeq 4000 platform (Illumina) using 150 bp paired-read chemistry.

2.6 CNV analysis

The paired-end short reads were aligned to the human reference genome (GRCh37) by using BWA’s (v0.7.12) mem command. The Picard tools software (v1.8) (http://broadinstitute.github.io/picard) was used for sorting and indexing the aligned short reads and marking PCR duplicates. Copy number variations were detected by using CNVkit (v0.9.1). This analysis relies on generating expected read counts in each genomic window (~10,000 bp) from a set of control samples and calculating the log2 ratios of the read counts from the affected samples relative to the control samples. We used four control samples to generate the expected normal read counts for each genomic window. The batch pipeline recommended in the CNVkit manual (http://cnnkit.readthedocs.io/en/stable/pipeline.html#batch) was utilized for generating the reference values, the calculation of the log2 ratios of the samples and the segmentation of the log2 ratios.

2.7 Genetic association analysis

The association between genotype and disease status was analyzed using R version 4.04. The SLE population was compared with both the healthy controls from the current study, as well as healthy control data from the study performed by Nagelkerke and colleagues. Data from this study was selected because a relatively large number of healthy controls were available with high quality FCGR2C genotype results. As SLE predominately affects females, the analysis using healthy controls from the current study was limited to female population. Since the Nagelkerke and colleagues did not report the data by gender, the comparison with this data included both males and females. For each comparison, a simple logistic regression model was fitted, relating the FCGR2C-ORF genotype to the disease status. The p-value for association was computed from the likelihood ratio test, and a 95% confidence interval for the odds ratio was computed from the profile likelihood method.

3 RESULTS

3.1 Molecular approach for the determination of the genetic status of the FCGR2C gene locus

The FCGR2C gene originated from a recent evolutionary unequal cross-over event between the 5′ part of FCGR2B and the 3′ part of the FCGR2A gene, hence, the very high homology between the 3 genes. In particular, there is only one amino acid difference (encoded in exon 5) compared to FCGR2B and only one amino acid difference (encoded in exon 8) compared to FCGR2A. Besides the 99% homology between FCGR2C and respective parts of FCGR2A and FCGR2B genes (Figure 1A), all three genes of the FCGR2 family share their highest homology within exons 3−5 (>96%, NCBI BLASTN 2.7.0+) (Supplementary Figure 1). This high degree of sequence homology in combination with the genomic variability of the FCGR2/3 gene cluster, reflected in the high degree of copy number variation, hampers the precise and efficient determination of the prevalence of FCGR2C gene expression. We here report a systematic approach to reliably determine the genetic status of the FCGR2C gene locus, which consists of messenger RNA sequencing for identification of the FCGR2C-ORF and other SNPs within the FCGR2C gene, SNaPshot genotyping the FCGR2C-ORF SNP for determining zygosity and validation of the obtained genetic results by performing flow cytometry analysis of the FcyRIIb/c expression on NK cells. For samples displaying inconsistent SNP and protein expression findings, the method using standard, short-read, low-coverage, next-generation sequencing was established to detect CNVs, as such cases were reported previously. The overall workflow for determination of the genetic status of the FCGR2C gene is outlined in Figure 1B.

3.2 FCGR2C genotyping by sequencing of FCGR2C mRNA-specific PCR products

To determine the status of the rs759550223 SNP in FCGR2C gene, NRM_047648.1.n.268T > C, exon 3, sequencing of FCGR2C-specific PCR products was performed (Figure 2). To this end, a PCR was designed to

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**Translated from a technical scientific text**

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3.2 FCGR2C genotyping by sequencing of FCGR2C mRNA-specific PCR products

To determine the status of the rs759550223 SNP in FCGR2C gene, NRM_047648.1.n.268T > C, exon 3, sequencing of FCGR2C-specific PCR products was performed (Figure 2). To this end, a PCR was designed to
amplify FCGR2C sequence from the 3' end of exon 1 to the second ITAM motif-encoding sequence in exon 8 using cDNA as a template. In detail, the forward primer spanning exon 1 and 2 was designed to bind both the FCGR2B and the FCGR2C cDNA sequence and was screened for common SNPs in Ensembl. The specificity for FCGR2C cDNA isomers was enabled by a reverse primer designed within the second ITAM motif-encoding sequence in exon 8 of the FCGR2B transcript (horizontally striped box). Non-classical (n.c.) form of exon 7 (vertically striped box) in FCGR2C gene indicates an existence of SNP G > A in intron 7 leading to a loss of exon 7 due to frameshift and premature STOP. ITIM and ITAM motifs are indicated by a black bar. S1: signal sequence 1; S2: signal sequence 2; EC1: extracellular 1; EC2: extracellular 2; TM: transmembrane; SP: spliced; C2: cytoplasmic 2; C3: cytoplasmic 3; ITIM: immunoreceptor tyrosine-based inhibitory motif; ITAM: immunoreceptor tyrosine-based activation motif. Diagram is not drawn to scale. B) A two-step genotyping workflow for functional determination of the FCGR2C gene.

The experimental design of the bidirectional sequencing of a FCGR2C cDNA--specific PCR covering a sequence from the 3' end of exon 1 up to the second ITAM encoding sequence in exon 8 of the FCGR2C mRNA also permitted the further investigation of other individual SNPs. The transmembrane SNP NM_001002274.2:c.695T > A; c.695C > T; p.I232T) in transmembrane domain leading to altered cell membrane aggregation properties of FcγR2B is indicated by a white star. Exon 6 (open box) is spliced in all FCGR2 mRNA isomers except for FCGR2B1/B3 isomers of the FCGR2B transcript (horizontally striped box). Non-classical (n.c.) form of exon 7 (vertically striped box) in FCGR2C gene indicates an existence of SNP G > A in intron 7 leading to a loss of exon 7 due to frameshift and premature STOP. ITIM and ITAM motifs are indicated by a black bar. S1: signal sequence 1; S2: signal sequence 2; EC1: extracellular 1; EC2: extracellular 2; TM: transmembrane; SP: spliced; C2: cytoplasmic 2; C3: cytoplasmic 3; ITIM: immunoreceptor tyrosine-based inhibitory motif; ITAM: immunoreceptor tyrosine-based activation motif. Diagram is not drawn to scale. B) A two-step genotyping workflow for functional determination of the FCGR2C gene.
FIGURE 2  Identification of FCGR2C-ORF transcript with coding capacity. A. Overview of method used for the identification of FCGR2C-ORF transcripts. Pf: forward primer; Pr: reverse primer. B. cDNA sequence alignment of regions selected for FCGR2C-specific PCR. The selected region spans junction of exons 1 and 2 (top) and is located within exon 8 (bottom) for forward and reverse primer selection, respectively. Identical bases are shaded grey. Sequences within forward (Pf) and reverse (Pr) primers that are homologous to the template are marked in bold-italics. ITAM motifs are underlined. C. Gel electrophoresis analysis of the FCGR2C-specific PCR products from donors Fc_1328, Fc_5989 and Fc_8504. M: size marker. D. Targeted sequencing of the rs759550223 variant in the three representative donors from panel C. The reference sequence from the ENSEMBL database, which represents the sequence of the FCGR2C-STOP allele. Identical bases are shaded grey. E. Representative Sanger sequence chromatograms for FCGR2C-STOP and -ORF alleles. rs759550223 SNP is indicated by an arrow. F. Identification of additional SNPs in FCGR2C-ORF divergent from FCGR2C-STOP alleles. SNPs rs775494223 (NR_047648.1:n.288A>G; same sense; c.189 G > A) and rs111603147 (NR_047648.1:n.347C>A; missense; c.248 C > T) are indicated by arrows. Alignment of ENSEMBL FCGR2A, B and C gene sequences within exon 3 with FCGR2C-ORF donor sequence.
screened individuals revealed that in addition to the known SNP NR_047648.1:n.268T > C (p.Gln90Ter, rs759550223), two additional SNPs, NR_047648.1:n.288A > G (same sense, rs775494223) and NR_047648.1:n.347C > A (p.Pro83Gln, rs111603147), were observed within exon 3 (Figure 2F), which are also present in the FCGR2B sequence. Interestingly, all 35 sequenced FCGR2C-ORF alleles displayed homology to FCGR2B for the two additionally identified SNPs in exon 3. In contrast, all 94 FCGR2C-STOP alleles that were sequenced displayed SNPs NR_047648.1:n.268T > C, NR_047648.1:n.288A > G and NR_047648.1:n.347C > A in exon 3, which is divergent from the FCGR2B sequence. Using this approach, 35 out of the 129 screened individuals were identified as FCGR2C-ORF allele carrying donors.

3.3 | FCGR2C genotyping by SNaPshot on FCGR2C-gene specific PCR products

The cDNA sequencing approach described above identifies functional FCGR2C alleles, however, it cannot determine the composition of the FCGR2C alleles within an individual. For that purpose, a SNaPshot single base extension genotyping method was developed. To ensure FCGR2C specificity while avoiding false-positive signals from the homologous FCGR2B exon 3 sequence, an FCGR2C gene-specific semi-nested long-range PCR was established as the basis for the genotyping primers (Figure 3C, top panel). Extension of SNaPshot primers by PCR product was used as a template for binding of SNaPshot extension primers (Figure 3B). This FCGR2C-specific PCR amplification steps were achieved by using 2 common FCGR2B/2C forward primers located within the promoter region and within intron 2, respectively, while the FCGR2A/2C-binding reverse primer was placed within intron 6. The reverse primer was not able to bind FCGR2B due to an insertion of 31 bp in the primer binding region (Figure 3B). This FCGR2C-specific PCR product was used as a template for binding of SNaPshot extension primers (Figure 3C, top panel). Extension of SNaPshot primers by binding of ddTTP on FCGR2C-STOP alleles and ddCTP to FCGR2C-ORF alleles resulted in a distinct chromatogram pattern (Figure 3C, bottom panel). This genotyping analysis revealed the presence of FCGR2C-ORF and -STOP alleles. In addition, the SNaPshot genotyping method for the simultaneous detection of both FCGR2C-STOP and -ORF alleles is a semi-quantitative method. Thus, further optimization of the described SNaPshot genotyping might provide an alternative approach to detect the relative proportion of FCGR2C-ORF and -STOP alleles and thereby provide a first insight into the copy number variation that often occurs within this genomic region.

Taken together, both methods described here amplified FCGR2C using forward primers that bind both FCGR2B and FCGR2C, while reverse primers bind FCGR2A/2C sequence. This procedure ensured amplification and unambiguous identification of existing FCGR2C-ORF alleles in a donor sample. Furthermore, each of the two described methods provides complementing information, thus, only a combination of both methods provides a comprehensive genetic status of the FCGR2C gene.

3.4 | Expression of Fcpress protein in donors carrying FCGR2C ORF allele

The genotyping methods described above identified individuals with a functional FCGR2C gene, thereby revealing the potential to express FcyRIIC. The tools to identify FcyRIIC protein expression on cell surface of FCGR2C-ORF allele containing donors by flow cytometry are restricted to the antibodies that cannot distinguish between FcyRIIb and FcyRIIC. In contrast to other immune cells which show broader expression of FcyRII protein surface expression, CD56<sup>+</sup>CD3<sup>−</sup> NK cells are known to almost exclusively express FcyRIIC receptor in presence of an FCGR2C-ORF allele. FcyRIIC expression was detected in only a minor subset of NK cells (< 1%), which would therefore not interfere to any significant extent with FcyRIIC expression analysis. To investigate whether the identified FCGR2C-ORF alleles correlate with the receptor protein expression on the cell surface, samples from 20 individuals carrying either FCGR2C-ORF or -STOP allele were analyzed by flow cytometry. FcyRIIC expression was monitored using two FcγRIIb/c-specific antibodies, SM201<sup>22</sup> and 2B6<sup>23</sup> and controlled by a “Fluorescence minus one” (FMO) method or an universal isotype control (control for all Recombinant Engineered Antibodies (REAL)) (Figure 4A).

The flow cytometry analysis displayed distinct expression staining patterns for the 7 FCGR2C-ORF donors and the 13 FCGR2C-STOP donors. Whereas no protein expression was observed in donors carrying the FCGR2C-STOP allele, in donors encoding for the FCGR2C-ORF allele a robust FcyRIIC or FcyRIIC expression was detected (Figure 4A and B). Since sequence identity within the extracellular part of the two proteins does not allow one to distinguish between FcyRIIC and FcyRIIC by flow cytometry, the detection of the expression on NK cells will be denoted throughout the report as FcyRIIC/c expression. For all 7 FCGR2C-ORF donors a considerable percentage of CD56<sup>+</sup>CD3<sup>−</sup> cells had distinguishable surface protein amounts on their surface. Interestingly, while the majority of CD56<sup>dim</sup>CD3<sup>−</sup> NK cells expressed FcyRIIC/c, the expression was much lower on CD56<sup>bright</sup>CD3<sup>−</sup> NK cells (Figure 4A and B).

Genetic analysis did not correlate with protein expression in the case of two donors, Fc_2014 and Fc_2125, both of which carried only the FCGR2C-STOP allele, yet showed expression of FcyRIIC/c protein by flow cytometry at the level comparable to the FCGR2C-ORF allele carrying donors (Figure 4B). To test whether these individuals carry a genomic deletion within the FCG2R gene cluster reported previously, which would result in juxtaposition of the FCGR2B gene in proximity of the regulatory elements directing the expression of the FCG2R gene, we have performed low coverage WGS and analyzed the copy number variation of the gene segments within the FCG2R gene cluster. Indeed, in the two samples, Fc_2014 and Fc_2125, the observed vs. expected (diploid) CNV value ratio within the FCGR2 gene cluster was lower compared to the flanking chromosome regions, indicating a deletion, thereby validating the low coverage WGS approach (Figure 4C). High resolution CNV analysis revealed the log2 of the observed vs. expected CNV values ratio to be around ~1 in the genomic interval from the FCGR2C to the FCG3B gene of samples Fc_2014 and Fc_2125.
FIGURE 3  Genotyping strategy for identification of FCGR2C-ORF/-STOP alleles and determining homo-/hetero-zygosity. A. Method overview for the identification of FCGR2C-ORF using genomic DNA. Pf: forward primer; Pr: reverse primer. B. DNA sequence alignment of regions selected for FCGR2C-specific PCR. The selected region is located upstream of exon 1 (top), within intron 2 (middle) and within intron 6 (bottom) for forward primer, nested forward primer and reverse primer selection, respectively. Identical bases are shaded grey. Forward, forward-nested and reverse primer sequences are marked in bold-italics. C. Overview of the SNaPshot method for identification of FCGR2C-ORF/-STOP alleles (rs759550223 SNP) and homo-/hetero-zygosity of the alleles. (top) Nucleotide sequence alignment of the target SNaPshot region. rs759550223 SNP is shaded red. (bottom) Representative donor chromatograms depicting the FCGR2C-STOP (left), FCGR2C-ORF (middle) and FCGR2C-ORF/STOP (right) allele detection. Identical bases are shaded grey

(Figure 4D), which corresponds to a heterozygous deletion. Interestingly, while control samples Fc_5280 and Fc_9968 displayed a neutral copy number ratio indicating a diploid state of the FCGR2 gene cluster, the ORF allele carrying Fc_6889 sample contained a deletion within the FCGR2 gene cluster, whereas sample Fc_5735 displayed a gain in sequences within the locus resulting in three copies of the FCGR2C and FCGR3B genes (Figure 4D). In conclusion, the genetic analyses described above provide a comprehensive approach for the determination of the allele composition of the FCGR2C gene, while in combination with flow cytometry, it uncovered the existence and status of the CNV.
FIGURE 4  Genetic identification of FCGR2C–ORF allele correlates with protein expression on cell surface. A. Multi-color flow cytometry analysis of FcγRIIb/c expression on NK cells. Representative dot-plots of CD56 NK-cell marker vs. FcγRIIb/c expression detected by two FcγRIIb/c-specific antibodies SM201 and 2B6. The percentage of NK cells expressing FcγRIIb/c is indicated in the upper left quadrant of each dot-plot. FMO: Fluorescence Minus One; REA: control for all recombinant engineered antibodies. B. Cumulative representation of flow cytometry analysis of FcγRIIb/c expression on NK cells from 20 donors. The graphs show percentage of FcγRIIb/c-positive cells (left) and mean fluorescence intensity of the FcγRIIb/c expression (right) within the gating region defined in panel A. Each symbol represents one donor. Two donors with genotype not matching to protein expression are indicated by red box. C. Whole-genome sequencing of non-matching donors 2014 and 2125 reveals CNV (large genomic deletion) within the FCGR2 gene cluster. D. Representation of the copy number ratio between the observed and expected values within the FCGR2 gene cluster. Each dot represents a measured genomic interval. Values around 0 indicate diploid status, values below 0 indicate deletion, while values above 0 indicate duplication of the genomic region. FCGR2C gene location: chr1: 161551129–161571010, FCGR2B gene location: chr1: 161632905–161648444 (GRCh37/hg19 genome assembly)
The antigen-recognizing IgG-immune complexes trigger the immune response by forming a stable interaction with low affinity Fcy receptors and are therefore essential part of the acquired immunity. Besides the activating receptors FcγRIa and FcγRIIa, and the inhibitory FcγRIib receptor, a third, activating receptor, FcγRIlc, was shown to be expressed on NK cells, monocytes, neutrophils and B-cells, however only in the presence of a functional FcγRIIC-ORF allele. FcγRIIC expression is shown to be associated with idiopathic thrombocytopenic purpura, an autoimmune disease of an unknown etiology mediated by immune complexes, as well as SLE, systemic sclerosis, more severe malaria phenotypes and augmented humoral immune response.

Thus, the precise determination of the functional FCGR2C gene and its expression is highly relevant when considering the development of therapies for immune complex-mediated autoimmune disorders. The high degree of homology within the FCGR2/3 gene cluster complicates development of a simple and accurate method for identification of FcγRIIC expression to facilitate patient stratification.

In this study, we describe a reliable genetic protocol for determination of the genetic status of the FCGR2C locus consisting of: (i) messenger RNA sequencing for identification of the FCGR2C-ORF allele; (ii) flow cytometry of FcγRII expression on NK cells; (iii) SNaPshot genotyping for determining zygosity; and (iv) low coverage next-generation sequencing for detection of CNV, in a two-step process. The first step determines the presence of the functional FCGR2C allele using the first two methods above, while the second step determines the zygosity and the copy number of the FCGR2C alleles in cases where SNP analysis and protein expression on NK cells are inconsistent. The data presented here show that the described genetic analysis correlates with the FcγRIIC protein expression on the cell surface. Conversely, sequencing of the whole FCGR2C cDNA provides information about all variants potentially affecting the expression or function of the receptor.
alleles and revealing the high homology of the FCGR2C-ORF allele towards FCGR2B in this region.\(^25,26\) which in turn suggested that the differences between FCGR2B and FCGR2C described in publicly available genomic databases apply exclusively to FCGR2C-STOP alleles.\(^27\) These further SNPs also raise the question of possible additional differences within intronic regions originating from the evolutionary divergence of the FCGR2B and FCGR2C genes. Hence, to detect existing FCGR2C-ORF allele(s) within a given sample, it is important to utilize methods and reagents that are not relying on sequence differences between FCGR2B and FCGR2C, which would a priori create bias towards one or the other allele.

Due to a lack of appropriate reagents, detection of FcγRIIc protein expression has been a daunting task. Specific antibodies targeting the extracellular part of the protein cannot discriminate between FcγRIIb and FcγRIIc molecules, while intracellularly there is no distinction between FcγRIIa and FcγRIIc protein. Previous studies have shown that CD56\(^{+}\)CD3\(^{-}\) NK cells typically lack FcγRIIa and FcγRIIb expression, thus only if an individual encodes for the FCGR2C-ORF allele, was FcγRII expression detected.\(^11\) Of the 20 donors that could be recalled for further analysis, the analysis of NK cells confirmed FcγRIIc protein expression in seven genetically identified FCGR2C-ORF donors. Interestingly, among CD56\(^{+}\)CD3\(^{-}\) NK cells, about 70% of CD56\(^{dim}\)CD3\(^{-}\) NK cells expressed FcγRIIc, whereas CD56\(^{bright}\)CD3\(^{-}\) NK cells appeared to be negative for FcγRIIc expression. The expression of FcγRIIc on CD56\(^{dim}\)CD3\(^{-}\), rather than CD56\(^{bright}\)CD3\(^{-}\) NK cells suggests that FcγRIIc receptor expression correlates with the developmental transition from CD56\(^{bright}\)CD3\(^{-}\) towards CD56\(^{dim}\)CD3\(^{-}\) NK cells that also express CD16/FcγRIIa.\(^28\) To our knowledge, the functional relevance of FcγRIIc expression on mature NK cells in addition to CD16/FcγRIIa has not been described. However, it could affect the effector mechanisms of these NK cells and thus potentially play a role in the pathology of autoimmune diseases or malignancies. In support of this hypothesis, FcγRIIc expression in other FcγII-expressing immune cells altered the activation threshold resulting in augmented immune responses.\(^3\)

Previously reported cases of FcγRIIb expression on NK cells were shown to result from a major deletion within the FCGR2/3 genomic region that brought FCGR2B gene under the control of FCGR2C regulatory elements.\(^4\) Indeed, our analysis demonstrated that in two FCGR2C-STOP donors, which expressed FcγRIIb/c, the expression correlated with the presence of a large deletion encompassing FCGR2C and FCGR3B genes that occurred on one of the alleles, which probably brought the FCGR2B gene under the control of cis-regulatory elements of the FCGR2C locus. Thus, by identifying the expected genomic deletions, low-coverage WGS explained the discrepancy in the genetic status vs. protein expression. The data shown here not only corroborate previous findings of genetic variability within the FCGR2 gene cluster, but also demonstrate the suitability of the methods such as targeted sequencing described recently\(^29\) or low-coverage WGS approach described here, for providing both qualitative and quantitative insight into the nature of the CNV, thus enabling the elucidation of the complete information of the genetic status of the FCGR2 gene cluster. Over the past few years Multiplex Ligation-dependent Probe Amplification (MLPA) has become a method of choice to analyze CNV, even within complex genomic regions such as the FCGR gene cluster, due to its simplicity in design and the availability of developed computational analysis solutions. However, MLPA comes with its own shortcomings such as its inability to provide information regarding the exact location of a duplicated sequence or its orientation, its lack of sensitivity for regions not directly encompassed by the probe sets used, and the possible occurrence of false-positive results attributable to polymorphism-induced allele dropouts.\(^30\) Here we describe an alternative method to MLPA that does not rely on the generation of appropriate primers, avoids potential amplification bias, and precisely maps the CNV boundaries.\(^31\) Thus, it provides accurate CNV results normalized to diploid genomic surroundings and with further minor modifications might be amenable to address additional genotyping culprits of this complex genomic region. Despite the high homology of FCGR2C and FCGR2B promoter sequences and their partly overlapping expression pattern,\(^3,4\) the two genes are regulated in distinct manner, particularly in NK cells, where only FcγRIIc is expressed.\(^4,11\) Identification of cis-regulatory elements and trans-factors required for cell type- and developmental stage-specific FCGR2C expression, might be instrumental for the modulation of effector functions in a variety of pathologies ranging from immune response to malignancies, to autoimmune disorders and organ transplant rejection.

The analysis of samples from the SLE patients revealed a trend in correlation between FCGR2C expression and disease incidence, reaching significance when leveraged on the larger sample size provided by the inclusion of additional data from a previously published study of genetic associations within the FCGR2/3 locus and Kawasaki disease.\(^17\) This suggests that if a larger sample size had been included in the present study, the conclusion regarding the correlation of the FcγRIIc prevalence and susceptibility to autoimmunity would have been further strengthened statistically. This also emphasizes the relevance and the need for precise genotyping of the FCGR2C allele as one of the predisposing factors in the context of autoimmune diseases. While incidence of SLE in males is rare,\(^24\) the prevalence of FCGR2C-ORF allele is the highest among all sample sets analyzed (25% healthy males, 23% whole sample set, 18% healthy females). Thus, it is conceivable that the male population, while not being overtly affected in terms of susceptibility to SLE, might actually be carriers of the FCGR2C-ORF allele, which would then, in combination with a plethora of female-specific parameters, e.g. female sex hormones,\(^32–34\) impact females by predisposing them to SLE.

The methods described in this study present a comprehensive approach for the identification of FcγRIIc protein expression on the cell surface of hematopoietic cells using novel technologies that can map both single nucleotide alterations, as well as large genomic modifications with an improved precision over previously reported methods, which is particularly relevant for the analysis of highly homologous genomic regions. Given the high relevance of the Fcγ receptors in the immune responses to immune complexed antigens, their involvement in immune responses by effector cells, as well as functional disequilibrium in a number of autoimmune disorders, the approach described here opens up novel opportunities for better understanding the interplay of the FcγRII receptors in immune responses. Finally, several
therapies for autoantibody-mediated diseases are in development, which exploit modulation of FcyR-mediated pathways either by blocking interaction of immune complexes with activating Fcy receptors or by stimulating inhibitory FcyRlb. In such cases, inadvertent stimulation of activating FcyRlb due to sequence homology might result in disease escalation rather than remission, thus the precise genotyping and stratification of patients is of great importance.

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CONFLICT OF INTEREST
Dorit Lehmann, Sabine Unterthurner, Verena Berg, Karin Hock, Padmapriya Ponnuswamy, Mantas Malisauskas, Brian Crowe, Birgit M. Reipert, Greg Hather and Ivan Bilic were employees of Baxalta Innovations, now part of Takeda, when the studies were done. Brian Crowe, Sabine Unterthurner, Ivan Bilic and Birgit M. Reipert, Greg Hather hold stocks and/or stock options of Takeda. All authors have read and approved the final version of the manuscript.

AUTHOR CONTRIBUTIONS
Dorit Lehmann, Sabine Unterthurner, Mantas Malisauskas, Birgit M. Reipert and Ivan Bilic formulated technical and scientific questions, conceptualized the work, and oversaw analyses, Dorit Lehmann, Sabine Unterthurner and Verena Berg organized sample accrual, performed genotyping analyses and interpreted data, Karin Hock and Padmapriya Ponnuswamy performed protein expression analysis using flow cytometry and interpreted data, Bekir Erguener and Christoph Bock performed CNV analysis using NGS, interpreted data and reviewed the manuscript, Greg Hather performed statistical analysis, Dorit Lehmann, Sabine Unterthurner, Verena Berg, Brian A. Crowe, and Ivan Bilic wrote the manuscript and interpreted the findings.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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