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Phenotypic Variation in IgG Receptors by Nonclassical FCGR2C Alleles

Joris van der Heijden,* Willemijn B. Breunis,*+ Judy Geissler,* Martin de Boer,* Timo K. van den Berg,* and Taco W. Kuijpers*†

The balance between activating and inhibitory signals from the different FcγRs for IgG ensures homeostasis of many inflammatory responses. FCGR2C is the product of an unequal crossover of the FCGR2A and FCGR2B genes encoding the activating FcγRIIA (CD32a) and inhibitory FcγRIIB (CD32b), respectively. A single nucleotide polymorphism (SNP) in exon 3 of FCGR2C results in either expression of the activating FcγRIIC (CD32c) (FCGR2C-open reading frame [ORF]) or its absence because of a stop codon (FCGR2C-Stop). Two additional variations in FcγRIIb/c expression on leukocytes have now been identified. In case of “nonclassical” FCGR2C-ORF alleles, FcγRIIC expression was unexpectedly absent, because of novel splice site mutations near exon 7 leading to another stop codon. In some individuals with FCGR2C-Stop alleles FcγRIIB was detected on NK cells, which normally are devoid of this protein. Individuals with these nonclassical FCGR2C-Stop alleles carried a deletion of FCGR2C-FCGR3B that extends into the promoter region of the adjacent FCGR2B gene and probably deletes a negative regulatory element in the FCGR2B promoter in NK cells. FcγRIIB expression on NK cells effectively inhibited killing mediated by FcγRIIa (CD16a) in Ab-dependent cytotoxicity tests. Our findings demonstrate a more extensive and previously unnoticed variation in FcγR expression with relevance to immunity and inflammation. The Journal of Immunology, 2012, 188: 1318–1324.

FcγRs are receptors for the Fc region of IgG and are expressed on various cell types, including macrophages, dendritic cells, neutrophils, NK cells, B lymphocytes, and platelets. The binding of IgG-opsonized pathogens to FcγRs triggers a variety of cellular responses and contributes to inflammation. FcγRs are also involved in Ab-mediated tumor cell elimination. The balance between activating and inhibitory signals from the different receptor subtypes ensures control over immune complexity-driven inflammatory responses (1). Depending on their affinity for IgG, the receptors can be divided into low-affinity FcγRs, which can be subdivided into FcγRIII (CD16a, CD16b), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIIC (CD32c), FcγRIIIA (CD16a), and FcγRIIIB (CD16b), and the high-affinity FcγRI (CD64). In humans, FcγRIIB is the only inhibitory FcγR, because it contains an ITIM in its cytoplasmic tail. All other FcγRs signal through an ITAM, either encoded within their cytoplasmic tail or within associated adapter proteins such as the FcRγ.

The genes encoding the FcγRs are located on chromosome 1, of which the genes FCGR2A, FCGR2B, and FCGR2C are located in one gene cluster with the FCGR3A and FCGR3B genes.

Because of the very high homology between the genes, it is a difficult gene cluster to study. For instance, FCGR2A and FCGR2B have ~95% homology in their exons coding for the extracellular domains of FcγRIIA and FcγRIIB, respectively (2). The activating FcγRIIIa is expressed by neutrophils, monocytes, macrophages, dendritic cells, and platelets, in which it mediates various functions such as phagocytosis and Ab-dependent cellular cytotoxicity (ADCC). The inhibitory FcγRIIB exists in two splice variants, namely, FcγRIIB1 (which is exclusively expressed by B cells) and FcγRIIB2 (expressed by B cells, macrophages, and dendritic cells). FcγRIIB inhibits signaling of ITAM-associated receptors by recruitment of phosphotyrosine phosphatases that dephosphorylate the tyrosine residues in several ITAM-induced activation pathway effectors (3).

Single nucleotide polymorphisms (SNPs) have been described in both FCGR2A and FCGR2B. A functionally relevant SNP in FCGR2A leads to either a histidine or an arginine in the ligand-binding domain of FcγRIIa (H/R131) (4). This results in differences in binding affinity for human IgG2 and murine IgG1 (4, 5). In FCGR2B, one SNP leads to either an isoleucine or a threonine in the transmembrane region of FcγRIIb (I/T232). The T232 allele has been suggested to result in functional impairment through exclusion from lipid rafts (6, 7). Additional SNPs in the promoter region influence transcriptional activity of FCGR2B (and FCGR2C) (8, 9). FCGR2C, encoding FcγRIIC, appears to be the product of an unequal crossover event between FCGR2A and FCGR2B (10). FCGR2C contains eight exons, which are highly homologous to exons 1–4 from FCGR2B and exons 5–8 from FCGR2A. An SNP in exon 3 of FCGR2C determines the functional expression of this gene. This SNP results in either an open reading frame (FCGR2C-ORF) or the more common stop codon (FCGR2C-Stop), in which case FCGR2C represents a pseudogene (11). FcγRIIC is expressed on NK cells from FCGR2C-ORF donors, acting as an activating IgG receptor capable of inducing ADCC and a rise in Ca2+ concentration after receptor cross-linking (12, 13).
Differences in receptor expression levels on the cell surface can alter the balance between activating and inhibitory signals and therefore change the cellular response toward IgG. Apart from SNPs, we and others have shown that several FCGR genes are subject to copy number variation (CNV). CNV in FCGR2C, FCGR3A, and FCGR3B has been shown to correlate with protein expression levels. For instance, deletion of one FCGR3A allele results in reduced target cell killing in an FcγRIIIa-specific ADCC (14). These SNPs and CNVs in FCGR genes have been linked with (chronic) inflammatory and autoimmune diseases (12, 15). It is therefore essential to further investigate genetic variation in this gene cluster, because that may be highly relevant for the discovery of disease risk factors, therapeutic efficacy of biologicals, and the development of new treatment strategies. In this study, we identify two additional genetic variations in the FCGR gene cluster that modulate the expression of FcγRIIb and FcγRIIc.

Materials and Methods

Methods

Healthy adult white volunteers (n = 146) 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 Ethics Committee of the Academic Medical Center and Sanquin (Amsterdam, The Netherlands) and was performed in accordance with the Declaration of Helsinki.

Abs and reagents

The following mAbs were used in flow cytometry and for immunoprecipitation (IP): anti-CD3 clone SK7, anti-CD14 clone MS-4, and anti-CD56 clone B159 (BD Pharmingen, San Diego, CA), anti-FcγRII clone AT10 (AbD Serotec, Oxford, U.K.), and anti-FcγRIIIb/c clone 2B6 (Macrogenics, Rockville, MD). Western blots were stained with polyclonal IgG anti-FcγRIIc (rabbit antiserum M5E2; a gift from Dr. A. Verhoeven, Department of Medical Biochemistry, Academic Medical Centre, University of Amsterdam (16)) or FcγRIIb (Epitomics, Burlingame, CA). Abs used in cytotoxicity assays were anti-FcγRIIe clone AT10 (azide-free; AbD Serotec) and anti-FcγRIII clone 3G8 (low-endotoxin azide-free; BioLegend).

IP lysis buffer was PBS containing 10% glycerol (w/v), 1% Nonidet P-40 (w/v), and 1× Complete, EDTA-free Protease Inhibitor Mixture (Roche Applied Science, Almere, The Netherlands). Laemmli sample buffer is 50 mM Tris-HCl (pH 6.8), 10% glycerol (v/v), 5 mM DTT (ns; DTT, Sigma-Aldrich), 1% 2-ME, 1% SDS (w/v), and 100 μg/ml bromophenol blue. Complete medium is IMDM (PAA, Pasching, Austria) supplemented with 10% FCS (v/v) (Bodinco, Alkmaar, The Netherlands), 1% 2-ME, 1% SDS (w/v), and 100 μg/ml penicillin, streptomycin (100 μg/ml; PAA), and 2 mM l-glutamine (PAA).

Genotyping

Genomic DNA was isolated from whole blood with the Gentra Puregene kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions.

CNV and SNPs in the low-affinity FCGR genes FCGR2A, FCGR2B, FCGR3A, and FCGR3B were determined with an FCGR-specific multiplex ligation-dependent probe amplification (MLPA) assay. The MLPA assay was performed essentially as described previously (12, 17). The FCGR MLPA includes gene-specific probes to determine the CNV of the genes. It also includes probes to detect the following SNPs: FCGR2A 270 > W (rs9427397 and rs427398), FCGR2A 131H > R (rs1801274), FCGR2B 2321 > T (rs1050501), FCGR2C/FCGR3C > 386C > G (rs3219018), –120A > G, and FCGR3A 158V > G (rs369991), and FCGR3B (HNA1a/HNA1b/HNA1c). The assay also contains a probe specific for the open reading frame in exon 3 of the FCGR2C gene and a nonspecific FCGR2BC probe to detect the stop codon in exon 3. For selected donors, we designed and used probes to determine the extent of the FCGR2/FCGR3 deletion. Probe sequences are 5′-GGTTCCCTAAGGGTGGAGAGAGATTTTCTTATCTAAACA-GTAGTTAAATAA-3′ and 5′-AAGTGAACATTTTCTTATGAGAAAGCAAGGATGTGTCATTTCT-3′ for the region between FCGR2A and FCGR3B.

Cytotoxicity assays

Cytotoxicity of NK cells was determined by Ab-dependent cellular cytotoxicity (ADCC) and redirected ADCC (rADCC). NK cells were isolated from PBMCs by MACS with CD56 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer’s instructions. NK cell purity was verified by flow cytometry.

Quantitative PCR analysis and sequencing of FcγRIIc transcripts, as well as the gene-specific, long-range PCR analysis and sequencing of FCGR2C, were performed as described previously (12).

NK cell purification

PBMCs were isolated from whole blood by Percoll density gradient centrifugation. NK cells were isolated from PBMCs by MACS with CD56 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer’s instructions. NK cell purity was verified by flow cytometry.

mRNA isolation and reverse transcription

mRNA was isolated from 1 × 10⁶ purified NK cells by using the QiaAmp RNA blood mini kit (Qiagen), according to the manufacturer’s instructions. Subsequently, first-strand cDNA was synthesized with the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Breda, The Netherlands). In short, RNA was primed with 2.5 μM oligo(dT) for 5 min at 65˚C. Reverse transcription was performed with 10 U/μl SuperScript III in the presence of 5 mM MgCl₂, 20 mM Tris-HCl, 50 mM KCl (pH 8.4), reverse transcriptase buffer), 0.5 mM 2′-deoxynucleoside 5′-triphosphate, and 2 U/μl RNaseOUT [without DTT, for reasons described by Lekanne Deprez et al. (18)] for 50 min at 37˚C. Thereafter, Superscript III was inactivated by incubation for 5 min at 85˚C, followed by chilling on ice. Immediately thereafter, 2 U RNase H were added, and the mixture was further incubated at 37˚C for 20 min. Subsequently, cDNA was stored at 20˚C until further use.

Detection and relative quantification of FcγRII isoform-specific mRNA

The detection and relative quantification of FcγRII mRNA have been described in detail elsewhere (19). In short, intron-spanning primers were designed to specifically amplify cDNA and exclude amplification of genomic DNA, yielding products of 100 bp for β-glucuronidase (GUS), 243 bp for FcγRIIB2, and 162 bp for FcγRIIb as the most abundant productive FcγRII transcript in immune cells (12). The following primers were used: β-glucuronidase (GUS), 5′-GAAAATATGTTGGTGGAGAGCTGTT-3′ (forward primer) and 5′-CCAGTAGTAAGACCTCTTCTTCTTA-3′ (reverse primer); FcγRIIb2, 5′-GGAAAAAGCCCATATTGGGACCAATC-3′ (forward primer) and 5′-GGAAAAACATGATCTCCCTTCTGTTG-3′ (reverse primer) and FcγRIIc, 5′-TATCTGTGCTGTGCATCTGG-3′ (forward primer) and 5′-TTCCTGATGGCACAATCTTGGAGC-3′ (reverse primer). Quantification of cDNA was performed with the Lightcycler Instrument (Roche Applied Science). Serial 10-fold dilutions of the cDNA were made and quantified with the method described in Technical Note No. LC 13/2001 (Roche Applied Science).

IP and Western blotting of FcγRII

IP was performed at 4˚C. Cells were pretreated with 10 mM diisopropyl fluorophosphate for 10 min and lysed with IP lysis buffer for 1 h. Lysates were centrifuged at 21,000 × g for 15 min. Supernatants were incubated with pan-FcγRII Abs (10 μg/ml) overnight. Subsequently, they were incubated with 5% protein G-Sepharose 4 Fast Flow (w/v) (GE Healthcare Life Sciences) for 1 h. Protein G-Sepharose was washed three times with IP lysis buffer by centrifugation at 21,000 × g for 15 s and incubated in Laemmli sample buffer for 30 min at 95˚C.

Samples were run on a 12.5% Bis-Tris gel (w/v) and subsequently blotted on a nitrocellulose membrane. FcγRIIb and –c were detected by polyclonal Abs against their cytoplasmic tails. Bands were visualized by ECL substrate (Pierce, Rockford, IL).

Next generation sequencing

For the region between FCGR2C and FCGR3B, the total volume per well was 100 μl. Cells were centrifuged at 300 × g for 30 s and subsequently incubated at 37˚C and 5% CO₂ for 4 h. Cells were lysed with the detergent RIPA, and FcγRIIc transcripts, as well as the gene-specific, long-range PCR analysis and sequencing of FCGR2C, were performed as described previously (12).

NK cell purification

PBMCs were isolated from whole blood by Percoll density gradient centrifugation. NK cells were isolated from PBMCs by MACS with CD56 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer’s instructions. NK cell purity was verified by flow cytometry.
were centrifuged again at 300 × g for 30 s, and supernatants were collected and counted in a gamma counter.

Specific killing was determined by the formula: percentage specific killing = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Statistics

Flow cytometry data were analyzed with Student t test; p < 0.05 was considered statistically significant. Data are expressed as mean ± SEM.

Results

Mismatches between FCGR2C genotype and phenotype

We have analyzed the FcγR expression by genotyping CNV and SNPs within the FCGR2A, FCGR2B, FCGR2C, FCGR3A, and FCGR3B genes by MLPA in 146 healthy adult white individuals (Tables I, II; data not shown) and phenotyping a selection of 47 of these individuals with different mAbs against FcγRII (pan-CD32), FcγRIIb/c (CD32b/c), and FcγRIIIa/b (CD16).

There is no mAb available that can differentiate between surface-expressed FcγRIIb and FcγRIIc because of the completely identical extracellular domains. NK cells express FcγRIIc but not FcγRIIb (12), and these cells can therefore be used to distinguish FCGR2C-Stop from FCGR2C-ORF donors at the level of surface expression with a FcγRIIb/c-specific mAb.

When we related the genotype for FCGR2C with the FcγRIIc expression on NK cells as measured by flow cytometry (Fig. 1), we observed a strong and positive correlation between genotype and average protein surface expression levels. However, we also detected two exclusive sets of individuals that showed different genotype–phenotype mismatches. First, we identified individuals who repeatedly showed no FcγRIIb/c surface expression on NK cells in the presence of a classical FcγRIIc-specific mAb (ORF versus Stop) mutation in exon 3 of the FCGR2C gene. In normal FCGR2C-ORF donors, these noncoding transcripts were not detected (data not shown).

Nonclassical FCGR2C-ORF by novel splice site mutations (group A)

Expression of FcγRIIc is known to be determined by the p.Q13X (ORF versus Stop) mutation in exon 3 of the FCGR2C gene. Interestingly, NK cells of some of the FCGR2C-ORF donors did not express FcγRIIc. The lack of expression was not limited to NK cells but was also apparent on myeloid cells, as indicated for monocytes and neutrophils (Fig. 2A). To investigate whether RNA transcripts for FcγRIIc were detected in the PBMC fraction of nonclassical FCGR2C-ORF donors, PCR analysis was performed on the RNA from these three donors. Sequence analysis of the PCR products revealed various FcγRIIc transcripts. Most abundantly, we found transcripts from which exon 7 was spliced out. In two of these donors, the transcripts contained in addition a 62-bp insertion, which was found to be part of intron 7 (Fig. 2B). Both unusual transcripts result in a frameshift and premature stop codon. Exon 6 was spliced out from all transcripts but that is normal for myeloid cells from all FCGR2C-ORF donors. The only isoform of FcγRII in which exon 6 is not spliced out is the B cell-specific FcγRIIb1. Besides these most commonly found transcripts in the three nonclassical FCGR2C-ORF donors, additional noncoding transcripts were detected as well. Premature stop codons can result in various nonsense mRNAs. It is likely that these less frequently found transcripts fall in this category. In normal FCGR2C-ORF donors, these noncoding transcripts were not detected (data not shown).

A gene-specific, long-range PCR was performed to amplify FCGR2C from genomic DNA. Sequence analysis of the PCR product revealed that all three nonclassical FCGR2C-ORF donors studied thus far carried a G→A point mutation at the donor splice site of intron 7, which explains a splicing defect that results in the loss of exon 7. In addition to this mutation, two donors also had a G→C point mutation at the acceptor splice site of intron 7. In these donors, a cryptic acceptor splice site 62 bp upstream of the normal exon–intron boundary was used, which explains the insertion of part of intron 7 in these two donors (Fig. 2C).

Nonclassical FCGR2C-Stop because of gene deletions in the FCGR cluster (group B)

Because FCGR2C-Stop donors are expected to be unable to express any isoform of FcγRII on NK cells, we were surprised to find that the NK cells from some of these healthy individuals did bind mAb 2B6, which is specific for FcγRIIb and FcγRIIc. Although genotyping errors in FCGR2C were considered, there was an important phenotypic difference with the binding pattern with the same mAb on immune cells from individuals carrying classical FCGR2C-ORF (Fig. 3A). Neutrophils and monocytes from these four individuals from group B showed a similar lack of surface

Table I. Copy numbers of FCGR2B, FCGR2C, and FCGR3B

<table>
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<th>CNV (No. of Donors)</th>
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<th>FCGR2C</th>
<th>FCGR3B</th>
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<tr>
<td>3</td>
<td>0</td>
<td>20</td>
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</table>

Table II. FCGR2C allele combinations in 146 healthy white individuals, determined by MLPA

<table>
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<th>FCGR2C Exon 3</th>
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</tr>
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</tr>
<tr>
<td>ORF</td>
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</tr>
<tr>
<td>Stop/Stop</td>
<td>94</td>
</tr>
<tr>
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</tr>
<tr>
<td>ORF/ORF</td>
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</tr>
<tr>
<td>Stop/Stop/Stop</td>
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</tr>
<tr>
<td>Stop/Stop/ORF</td>
<td>3</td>
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<tr>
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<td>2</td>
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<td>ORF/ORF/ORF</td>
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</table>
binding of mAb 2B6 as did normal FCGR2C-Stop donors in contrast to the usual myeloid expression observed in classical FCGR2C-ORF donors. This difference excluded genotyping errors as a possible explanation of our findings.

Further studies of these donors in which the NK cells unexpectedly showed CD32b/c surface expression were performed at the genomic level. MLPA analysis of DNA from these so-called nonclassical FCGR2C-Stop donors showed that these individuals all carried a large deletion in the FCGR locus, resulting in the combined loss of both FCGR2C and FCGR3B on one of their alleles (14). MLPA probes were designed to analyze the regions located around these genes to investigate the extent of this deletion. The deletion of FCGR2C and FCGR3B was found to include part of the sequence between the FCGR3B gene and the presumed promoter region of the FCGR2B gene (Fig. 3B).

We hypothesized that a regulatory element in this region could be involved in repression of FcγRIIb expression and that deletion of this element results in FcγRIIb expression on NK cells. Therefore, RNA was isolated from NK cells from individuals genotyped and phenotyped as FCGR2C-ORF, FCGR2C-Stop, and nonclassical FCGR2C-Stop (group B) donors. FcγRIIb1 mRNA was not detectable in NK cells, but some FcγRIIb2 mRNA was found to be present in NK cells of all individuals. In subsequent quantitative PCR experiments, the relative amounts of FcγRIIb2 and the major FcγRIIc (FcγRIIc1) mRNA were determined, compared with a household gene. Upon comparison of FCGR2C-ORF and -Stop donors, the nonclassical FCGR2C-Stop donors showed strongly increased transcriptional levels of FcγRIIb2 mRNA. FcγRIIc1 mRNA was comparable between classical and nonclassical FCGR2C-Stop and was—as expected—strongly elevated in the FCGR2C-ORF donors (Fig. 3C).

This led to the hypothesis that FcγRIIb and not FcγRIIc is expressed on the NK cells of these nonclassical FCGR2C-Stop donors. The assumption was further verified at the protein level. IPs of FcγRII with pan-FcγRII mAb AT10 were performed from lysates of highly purified NK cell fractions obtained from individuals with the FCGR2C-ORF,-Stop, and nonclassical Stop signature after combined geno-phenotyping. These immunoprecipitates were used for Western blotting and stained with Abs specific for the intracellular tail of either FcγRIIa/FcγRIIc (data not shown) or FcγRIIb (Fig. 3D). The experiments confirmed that NK cells from nonclassical FCGR2C-Stop donors indeed express FcγRIIb as the single FcγRII.

To test whether expression of FcγRIIb on NK cells had functional consequences, cytotoxicity was tested in rADCC assays (Fig. 4). Killing of target cells in the presence of mAbs for FcγRII was comparable between NK cells from all donor types. As we had also previously published (11, 12), NK cells from FCGR2C-ORF donors were able to kill target cells in the presence of mAbs for FcγRII, whereas NK cells from FCGR2C-Stop donors, not able to bind these mAbs, were unable to kill under these rADCC conditions (Fig. 4A). As expected, NK cells from nonclassical FCGR2C-Stop donors, expressing the inhibitory FcγRIIb, were not able to kill target cells in the presence of mAbs for FcγRII (Fig. 4B). Rare individuals carrying both an FCGR2C-ORF allele and a nonclassical FCGR2C-Stop allele will express both FcγRIIb and FcγRIIc on NK cells. We had identified a healthy donor with this genotype. The NK cells from this individual were unable to kill the target cells in the rADCC, despite expression of FcγRIIc. Thus, FcγRIIb on NK cells is unable to mediate cytotoxicity, but effectively inhibits killing mediated through FcγRIIc when coexpressed and ligated in the rADCC test system (Fig. 4B).

Because NK cells also express FcγRIIIa, the effect of FcγRIIb and FcγRIIc expression on cytotoxicity was tested in a classical ADCC test in which the clinically applied chimeric CD20 mAb rituximab was used to opsonize the Raji B cell lymphoma cell line as target cell. In these experiments, we observed that the expression of FcγRIIb on the NK cells of nonclassical FCGR2C-Stop donors resulted in decreased NK cell-mediated killing of the Raji target cells when preincubated with the CD20 mAb (Fig. 4C).

**Discussion**

Expression of FcγR on NK cells is normally limited to the activating FcγRIIIa (CD16a). We and others (12, 13) have previously shown that NK cells from approximately 20% of healthy individuals express
a second activating FcγR (i.e., FcγRIIc). Individuals expressing FcγRIIc may have an increased response toward IgG-mediated signals on several cell types of the immune system. We have previously shown that surface expression of FcγRIIc in such individuals is not limited to NK cells but is also present on monocytes (W.B. Breunis, J. van der Heijden, J. Geissler, M. de Boer, N. Laddach, M. Tanck, D. Burgner, I.M. Kuipers, D. Roos, and T.W. Kuijpers, submitted for publication) and, as shown in this study, on neutrophils as well.

Classically, an SNP in exon 3 of the gene encoding for FcγRIIc determines whether a stop codon in exon 3 or an ORF is present. However, in a number of healthy individuals, genotyping of FCGR2C by the SNP in exon 3 alone is insufficient. Additional mutations at the splice sites of intron 7 in their FCGR2C-ORF alleles were shown to exist, resulting in another stop codon. Although our previous study on FCGR2C-ORF in ITP (12) has become even more significant because of the lower frequency of nonclassical FCGR2C-ORF patients with ITP compared with the healthy control population (data not shown), nonclassical FCGR2C-ORF alleles may lead to the misinterpretation of cohort studies when only analyzed for the typical FCGR2C-ORF exon 3 alleles. Future experiments should include analysis of the intron 7 splice sites to determine the number of classical FCGR2C-ORF donors. We have found that ∼20% of the FCGR2C-ORF donors from our cohort of healthy white adults carry nonclassical FCGR2C-ORF alleles and are not expressing any FcγRIIc.

![Image of Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Nonclassical FCGR2C-Stop donors express FcγRIIb on NK cells. A, Surface binding of FcγRIIib/c mAb 2B6 to NK cells (CD3+CD56+), monocytes (CD14+), neutrophils, and B cells (CD19+) as determined by flow cytometry, compared among classical FCGR2C-Stop (n = 29), classical FCGR2C-ORF (n = 9), and nonclassical FCGR2C-Stop (n = 4) donors. The differences in 2B6 binding between classical FCGR2C-Stop and -ORF donors in NK cells, monocytes, and neutrophils are statistically significant (p < 0.0001), in contrast to binding to B cells (p = 0.39). The differences in 2B6 binding between classical and nonclassical FCGR2C-Stop donors are statistically significant in NK cells (p < 0.0001) but not in monocytes, neutrophils, and B cells (p = 0.97, 0.18, and 0.82, respectively). Binding of 2B6 to classical FCGR2C-ORF and nonclassical FCGR2C-Stop donors is statistically different in monocytes and neutrophils (p = 0.01 and 0.003, respectively) but not in NK cells and B cells (p = 0.17 and 0.40, respectively). Data are expressed as mean ± SEM. All values are corrected by subtracting values measured with isotype mAb controls. B, Representation of the FCGR gene locus. Arrows indicate the direction of transcription. The distance between the FCGR genes is mentioned between the arrows. The dashed bar shows the extent of the deletion found in nonclassical FCGR2C-Stop donors. C, Relative amounts of FcγRIIb and FcγRIIc transcripts in NK cells from classical FCGR2C-Stop, classical FCGR2C-ORF, and nonclassical FCGR2C-Stop donors. Values are relative to mRNA levels for the household gene GUS. Values were normalized, with the relative amounts of transcripts found in FCGR2C-Stop donors set to 1. D, Western blot analysis of FcγRIIb expression. Lanes 1 and 2, Whole-cell lysates of PMN and the human B cell line Daudi. Lanes 3, 4, and 5, NK cells from different donor types subjected to IP with a mAb binding all isoforms of FcγRII. Nonclassical FCGR2C-Stop donors are abbreviated as n.c.FCGR2C-Stop.
the phenotypic point of view, nonclassical FCGR2C-ORF donors should be included in the FCGR2C-Stop group because FcγRIIc is not expressed in these donors. We have also shown in this study that some individuals express FcγRIIb on all circulating NK cells, which normally do not show any FcγRIIb expression. Although indications for this phenomenon have been reported before, this was not further explored (13, 20). We have now identified that such donors have a deletion at the FCGR locus that includes FCGR2C, FCGR3B, and part of the region between FCGR3B and FCGR2B. The prevalence of such deletions among healthy white individuals is ~3%, but another racial background may result in a different prevalence.

FIGURE 4. Cytotoxicity of NK cells from different types of donors. A and B. Killing of P815 mouse mastocytoma cells through FcγRII and FcγRIII measured in an rADCC assay, comparing NK cells from FCGR2C-ORF (n = 4) and FCGR2C-Stop (n = 3) donors (A), as well as NK cells from nonclassical FCGR2C-Stop donors (n = 3) and cells from a rare donor carrying an FCGR2C-ORF in combination with a nonclassical FCGR2C-Stop allele (B). C. Killing of Raji cell lines measured in classical ADCC using clinical-grade chimeric CD20 mAb rituximab, comparing the same types of donor as in A and B (n = 3 for all donor types). Values are corrected for killing in the absence of mAbs. Data are expressed as mean ± SEM. Nonclassical FCGR2C-Stop is abbreviated as n.c.FCGR2C-Stop. *p < 0.05.
Our data suggest that deletion of one or more inhibitory elements in the 30-kb DNA sequence between the FCGR3B and the FCGR2B genes results in the unusual expression of FcγRIib on NK cells. In these individuals, the B cell expression of FcγRIib is unaltered, and the circulating neutrophils and monocytes do not show the FcγRIib expression, as we demonstrated by flow cytometry and IP (Fig. 3). The fact that these donors do not show (increased) expression of FcγRIib on other tested immune cells may indicate that this regulatory element normally acts to selectively suppress the FcγRIib expression in NK cells.

Because of the inhibitory nature of FcγRIib (13, 21), the NK cell expression of FcγRIib is presumed to result in a reduction in Ab-dependent cytotoxicity by NK cells. Cross-linking FcγRIib alone in an rADCC assay did not induce killing of target cells. However, in case of NK cells expressing both FcγRIib and FcγRIIc, FcγRIib completely inhibited killing induced by FcγRIIc cross-linking. In a classical ADCC, all FcγR on the NK cell can bind mAbs on target cells. FcγRIIa is the main FcγR on NK cells, expressed by all donors and at much higher levels than FcγRIib or FcγRIIc. The series of experiments performed in classical ADCC was too small to identify any effect of additional FcγRIIc expression on the NK cells of FCGR2C-ORF donors when compared with FCGR2C-Stop donors. Nonetheless, expression of FcγRIib on NK cells resulted in decreased killing of Raji cells in the presence of therapeutic mAbs, indicating that the expression of the inhibitory FcγRIib in nonclassical FCGR2C-Stop donors is functionally relevant in Ab-dependent cytotoxicity.

Taken together, we have identified several novel genetic variations in the FCGRRII/III locus, thereby demonstrating that the degree of genetic variation within this locus is considerably larger than determined previously. Apart from its natural cytotoxic activity toward tumors and viral infections, evidence for a regulatory role of NK cells in inflammation and infectious disease has accumulated during the past decade (22–27). It will be of interest to see whether the presence of FcγRIib is linked to the susceptibility for cancer and inflammatory diseases.

Clearly, a more accurate FcγR genotyping, including the currently described variants, will be useful for improving our understanding of the role of FcγR in disease.

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Disclosures

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References


