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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*†


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 complex-driven inflammatory responses (1). Depending on their affinity for IgG, the receptors can be divided into low-affinity FcγRs, which can be subdivided into FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIc (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 adaptor 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 FcγRIIA 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 ADC (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

Subjects

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 Medical 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 MS2E and anti-CD56 clone B159 (BD Pharmingen, San Diego, CA), anti-FcγRIII clone AT10 (AbD Serotec, Oxford, U.K.), and anti-FcγRIIab/c clone 2B6 (Macrogenics, Rockville, MD). Western blots were stained with polyclonal IgG anti-FcγRIII (rabbit) and anti-FcγRIIA (goat), respectively (as 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-FcγRIII clone AT10 (azide-free; AbD Serotec) and anti-FcγRIII clone 3G8 (low-endotoxin azide-free; BioLegend).

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

Genotyping

Genomic DNA was isolated from whole blood with the Gentra Puregene genomic DNA isolation kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. CNV and SNPs in the low-affinity FCGR genes FCGR2A, FCGR2B, FCGR2C, 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, –5-CCGAGTGAAGATCCCCTTTTTA-3′ (for-fluorophore primer) and 5′-GGAAAAATACGAGATCTTCCCTCTCTG-3′ (reverse primer); FCGR2B, 5′-GGAAAAAAGCGCAATTGGAGGCACTATT-3′ (for primer) and 5′-GAAATATGGTGTTGAGGACTATT-3′ (reverse primer); FCGR2C, 5′-ATCATTGCGGCCTGTCAGCTG-3′ (for primer) and 5′-CTTCTGTGAGCGACAACTTTGACG-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 FcγRIII

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-FcγRIII 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.

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 CD36 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 use of 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 mM 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 50˚C. Thereafter, Superscript III was inactivated by incubation for 5 min at 85˚C, followed by chilling on ice. Immediately thereafter, 2 U RNase H1 were added, and the mixture was 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 FcγRIIB, and 162 bp for FcγRIIb and was the most abundant productive FcγRII transcript in immune cells (12). The following primers were used: β-glucuronidase (GUS), 5′-GAAAATATGGTGTTGAGGACTATT-3′ (forward primer) and 5′-CCGAGTGAAGATCCCCTTTTTA-3′ (reverse primer); FcγRIIB, 5′-GGAAAAAAGCGCAATTGGAGGCACTATT-3′ (forward primer) and 5′-GAAATATGGTGTTGAGGACTATT-3′ (reverse primer); and FcγRIIC, 5′-ATCATTGCGGCCTGTCAGCTG-3′ (forward primer) and 5′-CTTCTGTGAGCGACAACTTTGACG-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).

PCR and sequence analysis

Quantitative PCR analysis and sequencing of FcγRIIC transcripts, as well as the gene-specific, long-range PCR analysis and sequencing of FcγRIIB, 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 CD36 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer’s instructions. NK cell purity was verified by flow cytometry.

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were centrifuged again at 300 x 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 FcγRIIa/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 definite genotype–phenotype mismatches. First, we identified individuals who repeatedly showed no FcγRIIc expression on NK cells but was also apparent on myeloid cells, as indicated for FcγRIIa/b-Stop donors.

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 three donors. Sequence analysis of the PCR products 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/c 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 binding of FcγRIIb/c-specific mAb 2B6 to NK cells from healthy individuals genotyped as FCGR2C-Stop. 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.

Copying of FCGR2 genes

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

<table>
<thead>
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<th>Copies</th>
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<th>FCGR2C</th>
<th>FCGR3B</th>
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<tr>
<td>3</td>
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<td>20</td>
<td>16</td>
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</table>

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

<table>
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<tr>
<th>FCGR2C Exon 3</th>
<th>No. of Donors</th>
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</tr>
<tr>
<td>ORF</td>
<td>2</td>
</tr>
<tr>
<td>Stop/Stop</td>
<td>94</td>
</tr>
<tr>
<td>Stop/ORF</td>
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</tr>
<tr>
<td>ORF/ORF</td>
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</tr>
<tr>
<td>Stop/Stop/Stop</td>
<td>15</td>
</tr>
<tr>
<td>Stop/Stop/ORF</td>
<td>3</td>
</tr>
<tr>
<td>Stop/ORF/ORF</td>
<td>2</td>
</tr>
<tr>
<td>ORF/ORF/ORF</td>
<td>0</td>
</tr>
</tbody>
</table>

FIGURE 1. Binding of FcγRIIb/c-specific mAb 2B6 to NK cells from healthy individuals genotyped as FCGR2C-Stop or FCGR2C-ORF donors. Boxes A and B indicate subgroups with an unexpected mAb binding pattern when considering the individuals’ genotypes. The difference in 2B6 binding between donor groups was statistically significant (***p = 0.0005).
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 unexposed to mAb 2B6 to NK cells (CD3⁺ CD56⁺), monocytes (CD14⁺), neutrophils, and B cells (CD19⁺) compared between classical (n = 9) and nonclassical (n = 3) FCGR2C-ORF donors. The differences in 2B6 binding between donor groups in NK cells, monocytes, and neutrophils were statistically significant (p = 0.004, 0.009, and < 0.001, respectively), in contrast to B cells, where this difference was not statistically significant (p = 0.27). Data are expressed as mean ± SEM. B, Representation of FcγRIIc transcripts found in PBMCs from classical and nonclassical FCGR2C-ORF donors. White boxes, Exons that are spliced out. *, The location of stop codons; +62, the insertion of a 62-bp sequence from intron 7 (see C). C, Splice site mutations in FCGR2C intron 7 in nonclassical FCGR2C-ORF donors. The intronic splice sites are underlined. Mutated splice sites are in italics, with the altered nucleotides in bold. Gray boxes represent exons that are retained, and white boxes represent exons that are being spliced out as a consequence of these mutations. Cx, cytoplasmic domain; ECx, extracellular domain, MFI, mean fluorescence intensity; Sx, signal sequence; TM, transmembrane domain.

FIGURE 2. Nonclassical ORF donors do not express FcγRIIc. A, Binding of FcγRIIb/c mAb 2B6 to NK cells (CD3⁺ CD56⁺), monocytes (CD14⁺), neutrophils, and B cells (CD19⁺) compared between classical (n = 9) and nonclassical (n = 3) FCGR2C-ORF donors. The differences in 2B6 binding between donor groups in NK cells, monocytes, and neutrophils were statistically significant (p = 0.004, 0.009, and < 0.001, respectively), in contrast to B cells, where this difference was not statistically significant (p = 0.27). Data are expressed as mean ± SEM. B, Representation of FcγRIIc transcripts found in PBMCs from classical and nonclassical FCGR2C-ORF donors. White boxes, Exons that are spliced out. *, The location of stop codons; +62, the insertion of a 62-bp sequence from intron 7 (see C). C, Splice site mutations in FCGR2C intron 7 in nonclassical FCGR2C-ORF donors. The intronic splice sites are underlined. Mutated splice sites are in italics, with the altered nucleotides in bold. Gray boxes represent exons that are retained, and white boxes represent exons that are being spliced out as a consequence of these mutations. Cx, cytoplasmic domain; ECx, extracellular domain, MFI, mean fluorescence intensity; Sx, signal sequence; TM, transmembrane domain.

The deletion of FGCR2C and FGCR3B was found to include part of the sequence between the FGCR3B gene and the presumed promoter region of the FGCR2B 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 FcγRIIB/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 FcγRIIB 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 ~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. From
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.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.1323)
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 FcγRIII 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
The authors have no financial conflicts of interest.

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