Eikelspecht
Melanerpes formicivorus
Acorn woodpecker

Eikelspechten leven in groepsverband en werken veel samen. Ze leggen gemeenschappelijke voorraden eikels aan in boomstammen en de vrouwtjes leggen de eieren in één nest, waarna ze samen gaan broeden. Als een van de vrouwtjes echter eerder dan de rest begint met eieren leggen, worden deze eieren door de andere vrouwtjes het nest uit gewerkt.
Haplotypes of FcγRIIa and FcγRIIIb polymorphic variants influence IgG-mediated responses in neutrophils

Joris van der Heijden, Sietse Q. Nagelkerke, Xiwen Zhao, Judy Geissler, Theo Rispens, Timo K. van den Berg, Taco W. Kuijpers

ABSTRACT

Human blood neutrophils normally express two Fc gamma receptors (FcγRIIa and FcγRIIIb) that, upon multivalently binding of IgG in immune complexes or on opsonized targets, mediate responses such as phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and respiratory burst. Allelic variants have been described for both FcγRIIa (131H/R) and FcγRIIIb (NA1/NA2/SH), with different binding affinity for IgG subclasses. Because neither of these variants acts alone, we have set out to systematically analyze in a large cohort of healthy FCGR2/3-genotyped volunteers how the different haplotypes of neutrophil FcγRs functionally interact. Maximal IgG-induced H$_2$O$_2$ production by neutrophils from individuals with different (homozygous) haplotypes was observed in the following order: 131HH-NA2NA2 > 131RR-NA1NA1 > 131HH-NA1NA1 > 131RR-NA2NA2. Although FcγRIIa 131H is known to bind IgG1 and IgG2 more avidly, no such differences in affinity are known for FcγRIIIb variants. Nonetheless, a remarkable impact of the FcγRIIIb variants on IgG-mediated neutrophil activity was thus demonstrated, which was not explained by differences in FcγR surface expression. The FcγR expression profile changed by overnight G-CSF/IFNγ activation of the neutrophils and eliminated any haplotypic impact on the respiratory burst. To our knowledge, our results are the first to provide an integrated functional analysis of neutrophil FcγR haplotypes and suggest that particularly the early phase of IgG-mediated neutrophil reactivity is influenced by FCGR2/3 genotypic variation.
INTRODUCTION

The Fc-gamma receptors (FcγR) are receptors for immunoglobulin G (IgG), and they are expressed on virtually all immune cells. These receptors mediate various cellular responses to IgG, including phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and respiratory burst. In humans, there are three types of FcγR. Based on their affinity for monomeric IgG, they can be divided into the high-affinity FcγRI and the low-affinity FcγRII and FcγRIII. FcγRII exists in three isoforms: FcγRIIa, FcγRIIb and FcγRIIc. FcγRIII exists as a transmembrane FcγRIIIa and as a GPI-linked FcγRIIIb. Signaling by FcγR is mediated by Immunoreceptor Tyrosine-based Activating (ITAM) or Inhibitory (ITIM) Motifs. ITAMs are found either in adaptor proteins such as the Fc-receptor common gamma chain (FcRγ), which associates with FcγRI and FcγRIIIa, or within the cytoplasmic tail of the FcγR itself, in case of FcγRIIa and FcγRIIc. The only inhibitory FcγR is FcγRIIb, as it has an ITIM in its cytoplasmic tail. The genes encoding for the low-affinity FcγR are found in one cluster located on chromosome 1q23 and are highly polymorphic. Functionally relevant polymorphic variants have been described for all low-affinity receptors, and gene copy number variation (CNV) is found in the genes encoding FcγRIIc, FcγRIIIa and FcγRIIIb, but not FcγRIIa and FcγRIIb.

Neutrophils constitute the first and major line of human defense against bacterial and fungal pathogens. These cells constitutively express two main FcγRs, that is, FcγRIIa and FcγRIIIb, whereas FcγRI is only expressed on activated cells. FcγRIIb is not expressed on circulating neutrophils. In contrast, FcγRIIc can be detected in 15-20% of healthy individuals, because they carry an FCGR2C open reading frame instead of the more common pseudogene with a stop codon in exon. Apart from this basic make-up of IgG receptors of human neutrophils, functionally important single nucleotide polymorphisms (SNPs) have been described for the major FcγRs on neutrophils. For instance, a SNP in FCGR2A results in either a histidine or an arginine at position 131 (131H/R), located in the ligand-binding domain of FcγRIIa. FcγRIIa-H131 has a higher binding affinity for IgG1 and more in particular for IgG2, when compared with FcγRIIa-R131, whereas binding of IgG3 and IgG4 are similar. Functionally, phagocytes from individuals homozygous for H131 (FcγRIIa-131HH) have an increased capacity to respond with phagocytosis, degranulation, or cytokine release in response to IgG-opsonized bacteria or erythrocytes, when compared with cells from FcγRIIa-131RR individuals. Moreover, the neutrophil-specific FcγRIIIb has three polymorphic variants, NA1, NA2 and SH, also known as HNA1a, -1b and -1c, respectively. FcγRIIIb-NA1 and -NA2 nucleotide sequences differ at five positions, resulting in four amino acid differences. As a consequence, the NA2 variant has two additional N-linked glycosylation sites compared with NA1. The SH variant differs from NA2 at only one additional position, resulting in an A→D amino acid change that may affect the tertiary structure of the protein. Although binding affinities for IgG1 and IgG3 are similar for all three variants, functionally, neutrophils from FcγRIIIb-NA1NA1 individuals bind and phagocytize IgG-opsonized bacteria.
and IgG-sensitized erythrocytes more efficiently than those from NA1NA2 and NA2NA2 individuals\cite{12,13}. Apart from the differences in binding and function, both FCGR2A and FCGR3B polymorphisms have been associated with numerous autoimmune and infectious diseases\cite{14}.

Because combinations of FcγR isoforms rather than a single IgG receptor type are expressed on neutrophils, we studied the response of neutrophils to IgG in a large cohort of healthy FCGR2/3-genotyped volunteers expressing different FcγRIIa/FcγRIIIb haplotypes. As demonstrated for the respiratory burst in neutrophils, the cellular activation by IgG was not determined by a single SNP but instead by the combination of SNPs in a given haplotype.

**MATERIALS AND METHODS**

**Subjects**

Healthy adult white volunteers (n=171) volunteered to donate whole blood. 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 in Amsterdam, and was performed in accordance with the Declaration of Helsinki.

**Antibodies and reagents**

The following mAbs were used in flow cytometry: anti-FcγRI clone 10.1 (mouse IgG1; BD Pharmingen, San Diego, CA, USA), anti-FcγRII clone AT10 (mouse IgG1; AbD Serotec, Oxford, U.K.), anti-FcγRIIb/c clone 2B6 (mouse IgG1; a generous gift of Macrogenics, Rockville, MD)\cite{15}, anti-FcγRIII clone 3G8 (mouse IgG1; BD Pharmingen). Blocking Abs used in NADPH oxidase assays were anti-FcγRI (mouse IgG1, low-endotoxin azide-free, clone 10.1; Biolegend, San Diego, CA), anti-FcγRII clone AT10 (mouse IgG1, azide-free; AbD Serotec) and anti-FcγRIII clone 3G8 (mouse IgG1, low-endotoxin azide-free; Biolegend). F(ab')\textsubscript{2} fragments of AT10 were prepared from the intact antibody mentioned above by pepsin cleavage at pH 3.5 for 20 hr; F(ab')\textsubscript{2} fragments of anti-FcγRIII were from Ancell (anti-human CD16 F(ab')\textsubscript{2}; Ancell, Bayport, MN). The purity of the F(ab')\textsubscript{2} fragments was confirmed by SDS-PAGE gel electrophoresis and Coomassie staining.

Annexin V (BD Pharmingen) was used to determine neutrophil apoptosis status.

HEPES+ buffer contained 132 mM NaCl, 6 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgSO\textsubscript{4}, 1.2 mM potassium phosphate, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) human serum albumin (pH 7.4).

Complete medium used for overnight culturing was IMDM (PAA, Pasching, Austria) supplemented with 10% (v/v) FCS (Bodinco, Alkmaar, The Netherlands), penicillin (100 U/ml), streptomycin (100 μg/m; PAA Laboratories) and 2 mM L-glutamine (PAA Laboratories).
High-performance size exclusion chromatography

Ig products were analyzed for IgG monomer, IgG dimer, and IgG polymer content using high-performance size exclusion chromatography. Samples were diluted to 5 mg/ml in PBS and immediately thereafter applied to a Superdex 200 HR 10/300 column (Amersham Biosciences, Uppsala, Sweden) connected to an HPLC system (ÄKTAexplorer, Amersham Biosciences) at room temperature with a flow rate of 0.5 ml/min and PBS as running buffer. A computer program (Unicorn version 5.11) was used to integrate the areas under the curve of the peaks shown in the chromatograms. For estimation of protein size, the column was calibrated with a high molecular weight calibration kit (GE Healthcare).

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, 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. The FCGR MLPA includes gene-specific probes to determine the CNV of the genes. It also includes probes to detect the following SNPs: FCGR2A 27Q/W (rs9427397 plus rs9427398), FCGR2A 131H/R (rs1801274), FCGR2B 232I/T (rs1050501), FCGR2B/C -386C/G (rs3219018) and -120A/T (rs34701572), FCGR3A 158V/F (rs396991) and FCGR3B (HNA1a/HNA1b/HNA1c). The assay also contains a probe specific for the stop codon in exon3 of the FCGR2C gene and a nonspecific FCGR2B/C probe to detect the open reading frame in exon3.

Isolation and activation of neutrophils

Granulocytes were isolated from whole blood by Percoll density gradient centrifugation. As determined by flow cytometry, neutrophil purity was always >95% and FcγRI expression was always absent, indicating that the cells had not been preactivated in vivo (for instance by an intercurrent infection or cold). For activation, neutrophils were cultured for 16 h at a concentration of 5x10⁶ cells/ml in complete medium, supplemented with 10 ng/ml clinical grade G-CSF (Neupogen; Amgen, Breda, The Netherlands) and 50 ng/ml recombinant human Interferon-γ (PeproTech, Rocky Hill, NJ).

NADPH oxidase activity assay

NADPH oxidase activity was compared between neutrophils from donors with different FcγRIIa/FcγRIIIb combinations by measuring reactive oxygen species (ROS) as hydrogen peroxide (H₂O₂) production for 60 minutes in an Amplex Red assay (Invitrogen, Carlsbad, CA), essentially as described before. Cells were compared unstimulated or stimulated with either 100 ng/ml PMA or 17.5 mg/ml IgG. Neutrophils stimulated with IgG were also tested in the presence of blocking Abs against FcγRI, FcγRII and FcγRIII, which were added at 10 μg/ml final
concentration each. Where indicated the same mAbs were tested as F(\(ab^\prime\)2 fragments to avoid any potential influence of the Fc domain (referred to as the so-called Kurlander effect\(^{18}\)) and to confirm the genuinely blocking capacity of the mAbs used. F(\(ab^\prime\)2 fragments were used at an optimal dose of 7 \(\mu\)g/ml final concentration.

For each experiment, the maximum slope of \(H_2O_2\) production was determined by calculating the maximum value of the derivative of the \(H_2O_2\) concentration over time. Measurements were performed every 30 s. The maximum slope was based on five sequential time points.

**Statistical analysis**

Flow cytometry and NADPH oxidase activity data were analyzed with Student \(t\) test; \(p < 0.05\) was considered statistically significant. Data are expressed as mean ± SEM.

**RESULTS**

*Haplotypes of FcγRIIa and FcγRIIib polymorphisms determine neutrophil response to IgG*

We used clinical grade IgG preparations for use in patients to activate human neutrophils in vitro isolated from healthy volunteers. The protein fraction of these IgG preparations consisted of >90% IgG (54.0% IgG1, 39.6% IgG2, 2.7% IgG3 and 3.8% IgG4), with minor contaminations of IgA (2.4% of total protein content) and traces of other plasma proteins. The IgG preparations contained mainly monomeric IgG (68%), but also some dimers (28.4%) and a small amount of polymers (3%), as was determined by high-performance size exclusion chromatography (Fig. S1). For reasons of simplicity, we have used in our studies preparations for subcutaneous use only, which gave a slightly higher neutrophil response when compared with the IVIG preparations of the same plasma source and with similar Ig composition (data not shown).

Using our MLPA for *FCGR2* and *FCGR3* genes, we have genotyped CNV and SNPs within the genes coding for the different low-affinity FcγRIIa and FcγRIIib in 171 healthy adult white individuals. Subsequently, we divided these individuals into groups, representing different combinations, or haplotypes, of polymorphic variants of FcγRIIa (131H/R) and FcγRIIib (NA1/NA2), the two FcγRs expressed on resting neutrophils. To prevent creating too many groups and groups with insufficient numbers of individuals, we selected the 117 individuals that had two copies of any gene at the *FCGR2/3* locus (in particular for *FCGR3B* encoding neutrophil-specific FcγRIIib), and were also genotyped as *FCGR2C-Stop* (hence, without FcγRIIC expression on their neutrophils\(^9\)). The distribution of the resulting nine haplotypes in this selected group of healthy individuals is shown in Table 1. The compound heterozygous FcγRIIa-131HR/FcγRIIib-NA1NA2 haplotype was most prevalent. Within the selected 31 homozygous FcγRIIa-131RR individuals, only 2 were FcγRIIib-NA1NA1 while 15 were NA2NA2 (Table 1). The genotype frequencies of both FcγRIIa and FcγRIIib in this selection of individuals are similar to what has been found in other studies concerning white individuals\(^{19}\).
Upon stimulation of neutrophils with IgG we found clear differences in NADPH oxidase activity between the various haplotypes (Fig.1). The amount of H₂O₂ produced by neutrophils from individuals that were genotyped as homozygous FcγRIIIb-NA2NA2 was found to depend on the functional polymorphism in FcγRIIa; that is, the neutrophils of those that were typed as FcγRIIa-131HH produced significantly more H₂O₂ compared to those that were FcγRIIa-131RR, with FcγRIIa-131HR individuals showing an intermediate response. In contrast, in FcγRIIIb-NA1NA1 individuals this order of NADPH oxidase activity was completely reversed, being FcγRIIa-131RR > -131HR > -131HH. Although proper statistical analysis could not be performed due to a lack of sufficient healthy FcγRIIa-RR individuals to be tested in our cohort, a clear trend was observed. Individuals typed heterozygous for FcγRIIib (NA1NA2) all had equal responses when compared among each other, regardless of their FcγRIIa genotype.

To exclude the possibility that the differences in H₂O₂ production are the result of differences in FcγR expression levels, neutrophils were analyzed by flow cytometry (Fig.2). We did not detect any differences in expression of FcγRIIa between the different haplotypes. FcγRIIib ex-

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**Table 1. Distribution of FcγRIIa/FcγRIIib haplotypes in healthy white individuals**

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<tr>
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<th>NA1NA1</th>
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<td>RR</td>
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Only individuals with two copies of each gene are represented in this table (n=117 in total). H = FcγRIIa-H131; R = FcγRIIa-R131; NA1 = FcγRIIib-HNA1a; NA2 = FcγRIIib-HNA1b.

**Figure 1. H₂O₂ production by neutrophils expressing different FcγR haplotypes in response to IgG**

Data are depicted as a ratio of the maximum slopes of H₂O₂ production in response to IgG or PMA. H₂O₂ production in FcγRIIib-NA2NA2 individuals was significantly different between FcγRIIa-HH individuals and those that are FcγRIIa-HR (p = 0.0425) and FcγRIIa-RR (p = 0.0078). *p < 0.05, **p < 0.01.
pression was higher in FcγRIIIb-NA2NA2 individuals than in those homozygous for NA1, and heterozygotes had an intermediate expression. Because the mAb we used to detect expression levels does not differ in binding between FcγRIIIb-NA1 and FcγRIIIb-NA2, we know that this is truly a difference in surface expression levels. However, within the groups of individuals with the same FcγRIIIb genotype, there were no differences in expression between carriers of the different FcγRIIa variants. Although some differences in FcγR expression levels were observed, these findings cannot explain the differences in IgG-mediated NADPH oxidase activity as observed in Fig.1. As expected, FcγRI was absent on these circulating neutrophils (Figure 4).

To determine the individual contribution of FcγRIIa and FcγRIIIb in the response to IgG, we performed similar experiments in the presence of blocking mAbs or F(ab)2 fragments for both FcγRs on circulating neutrophils from healthy volunteers (Fig.3). Blocking FcγRII by either intact or F(ab’)2 CD32 mAb completely inhibited H2O2 production, showing that FcγRIIa is absolutely required for ROS production in this setting. Blocking FcγRIII by intact CD16 mAb inhibited this response much less efficiently, and blocking FcγRIII with F(ab’)2 of CD16 mAb did not inhibit the response at all, suggesting that the inhibition of the response by intact CD16 mAb was a result of the Kurlander effect18, in which one mAb can both bind its targeted receptor with its epitope-specific Fab domains and another Fc receptor by the Fc-tail. In this case this
resulted in partial blocking the IgG-binding epitope of FcγRIIA by the Fc-tails of CD16 mAbs upon binding to the surface-expressed FcγRIIB molecules on neutrophils. The F(ab′)2 fragments of CD16 mAb 3G8 have previously been described to block IgG binding to FcγRIIIb22. Moreover, the F(ab′)2 fragments of the CD16 mAb still bound to the same extent to surface-expressed FcγRIIIb on human neutrophils as indicated by flow cytometry (data not shown). The results obtained with blocking Abs were similar for the different haplotypes tested (Fig.S2).

The above-mentioned experiments indicate the relevance of the genetic haplotypes on the IgG-binding capacity of the neutrophil, which seem to depend totally on the function of FcγRIIA for the subsequent signaling capacity toward NADPH oxidase activity.

**Preactivation of neutrophils results in a loss of differences between haplotypes**

To investigate the impact of preactivation on FcγR expression and IgG-mediated neutrophil activation, we also cultured the neutrophils from these genotyped individuals overnight with a combination of G-CSF and IFN-γ, which had previously been shown to most effectively induce FcγRI20,23,24. The number of apoptotic neutrophils was <20% under these conditions (data not shown), whereas FcγRI expression was maximally upregulated, as determined by flow cytometry. Preactivation also changed the FcγRIIA and FcγRIIB expression patterns (Fig.4), as reported before20,21,25. Total FcγRII (CD32) expression was increased only slightly, whereas FcγRIIB (CD16) expression was reduced by ~50%, probably due to shedding25. Although expression of the only inhibitory IgG receptor FcγRIIB was undetectable on resting cells, low levels of expression were reproducibly detected after preactivation (Fig.4). When expression levels were compared between individuals having different haplotypes, FcγR expression follow-
ing preactivation was not identical among these individuals. FcγRI expression was found to be significantly higher in FcγRIIa-131RR individuals than in those that were 131HH (Fig.4). Even though the total FcγRII (CD32) expression was very similar among all donors, the expression of FcγRIIb (CD32b) was highest in individuals typed FcγRIIIb-NA1NA1 and almost absent in those typed FcγRIIIb-NA2NA2. In contrast, even after partial shedding by the overnight

Figure 4. Changes in neutrophil FcγR expression after overnight preactivation with G-CSF and IFN-γ
At the left side of each of the four panels, the data are shown as mean fluorescence intensity (MFI) of fluorescently labeled mAbs against FcγRI (CD64), FcγRII (pan-CD32), FcγRIIb (CD32b) or FcγRIIIb (CD16). Day 0: freshly isolated, nonactivated neutrophils (open bars); day 1: neutrophils cultured overnight in the presence of G-CSF and IFN-γ (black bars). At the right side of each panel the MFI of fluorescently labeled mAbs against the different FcγR is indicated for all genotypes separately on the neutrophils cultured overnight in the presence of G-CSF and IFN-γ (dark grey bars). 131HH, 131HR, and 131RR and NA1NA1, NA1NA2, and NA2NA2 indicate the polymorphic variants of FcγRIIa (131H/R) and FcγRIIIb (NA1/NA2), respectively. Significant differences in FcγR expression are indicated in the figure. All values were corrected by subtracting the background values measured with isotype mAb controls. Data are expressed as means ± SEM of 32 independent experiments with freshly isolated (day 0) neutrophils, and eight to nine independent experiments per polymorphic variant with overnight cultured neutrophils (day 1, total of 26).
preactivation, the FcγRIIIb expression was most pronounced in FcγRIIIb-NA2NA2 compared to NA1NA1 and NA1NA2 individuals – similar to the differences in expression levels observed in freshly isolated neutrophils from these individuals. Without preactivation with cytokines, the number of apoptotic neutrophils after overnight culture was > 70-80%, thus precluding functional studies (data not shown).

To investigate whether the altered FcγR expression pattern on preactivated neutrophils influenced the response to IgG, these cells were again tested for NADPH oxidase activity. Compared to freshly isolated neutrophils, H₂O₂ production was increased 2-fold upon stimulation with IgG but not with PMA (Fig. 5 and data not shown). Remarkably, the observed differences between haplotypes in freshly isolated neutrophils were not found anymore with the preactivated cells. Blocking mAbs against FcγRIIa – either used as alone or combined with mAbs against FcγRI and/or FcγRIIIb resulted in ~50% reduction of H₂O₂ production.

In contrast to the situation in freshly isolated neutrophils, the response to IgG could not be significantly inhibited by intact FcγRIIIb mAbs. The combination of FcγRII mAbs with FcγRI and/or FcγRIIIb mAbs was not more effective than blocking FcγRII alone, irrespective of the haplotype (Fig. S3), suggesting that, similarly to what we observed in freshly isolated neutrophils, FcγRIIIa was the dominant activating FcγR in the preactivated neutrophils as well.

**DISCUSSION**

Our studies indicate an unpredicted yet relevant interaction between common FcγR haplotypes in IgG-mediated neutrophil activation. Although FcγRIIa-131H binds more avidly to IgG1 and IgG2, we found a significant impact of FcγRIIIb variants on the respiratory burst activity, with maximal IgG-induced H₂O₂ production by neutrophils from individuals with (homozygous)
haplotype 131HH-NA2NA2 > 131RR-NA1NA1 > 131HH-NA1NA1 > 131RR-NA2NA2. These haplotypic differences in respiratory burst activity disappeared upon preactivation of the neutrophils.

Many polymorphic variants of low-affinity FcγR have been described to be functionally different from one another. Changes in binding affinity\textsuperscript{10}, receptor expression\textsuperscript{5}, promoter activity\textsuperscript{26,27}, and localization in the plasma membrane\textsuperscript{28} are all among the consequences of such polymorphisms. By far the most studies reporting on these issues analyze one receptor at a time, although most immune cells express multiple FcγRs with different affinities for IgG and different downstream signaling pathways, some activating and some inhibitory\textsuperscript{29}.

When we compared the respiratory burst after stimulation with IgG, a response both FcγRIIa and FcγRIIIb have been reported to induce\textsuperscript{30}, we found that the neutrophil response is ultimately determined by the combination of polymorphisms. For instance, FcγRIIIb-NA2NA2 individuals that are also FcγRIIa-131HH had an almost 2-fold higher respiratory burst than those that were FcγRIIa-131RR, whereas in FcγRIIIb-NA1NA1 donor cells these differences were reversed. Minor differences in FcγR expression were detected, but these could not explain the differences we observed in the respiratory burst. Blocking FcγRII, either by intact mAbs or F(ab’)\textsubscript{2} fragments, almost completely inhibited the response. In contrast, blocking FcγRIIIb by intact mAbs only partially inhibited the response and F(ab’)\textsubscript{2} fragments did not inhibit at all. This suggests that the blocking effect observed with intact FcγRIIIb mAbs is due to a so-called Kurlander effect, that is, the Fc tails of bound FcγRIIIb mAbs also bind and consequently block FcγRIIa. The fact that the response was not increased in experiments with intact mAbs compared to F(ab’)\textsubscript{2} fragments shows that possible induction of the response by Kurlander effect by crosslinking receptors on the surface was also not the case in this experimental setting. The blocking experiments suggest a predominant role of FcγRIIa in the production of ROS by neutrophils in response to IgG.

Although the experiments with blocking FcγRIIIb F(ab’)\textsubscript{2} fragments at first glance may suggest that FcγRIIIb is not involved in the neutrophil response of \textit{H}_2\textit{O}_2 production, the influence of the genetic polymorphisms in this receptor clearly shows that FcγRIIIb modulates this response. Therefore, when taken together the blocking experiments do not fully explain the differences in IgG responses between haplotypes. Possibly, FcγRIIIb plays a dual role in this response. On one hand, because expression of FcγRIIIb is much higher than FcγRIIa, it will bind many IgG molecules that are consequently not available for binding by FcγRIIa. As FcγRIIIb is not able to signal directly, this may negatively influence the level of the response. On the other hand, FcγRIIIb could increase the binding of neutrophils to immune complexes, which may increase the response. In general, these mechanisms could be in balance, explaining why no difference in response is observed after blocking. Nevertheless, there is a possibility that that this balance is slightly different between the haplotypes. Alternatively, pre-existing interactions between FcγRIIa and FcγRIIIb and other receptors in plasma-membrane micro-domains may contribute to the observed effects. Differences in the functional capacity of the FcγR complexes containing the NA1 and NA2 FcγRIIIb haplotypes to transduce outside-in signals may explain the magni-
tude of the downstream response. For example, neutrophil FcγR and the integrin CR3 (CD11b/CD18) are known to coexist in such signaling complexes and influence each other in cellular responses. These interactions have been proposed to be due to lectin-saccharide-like binding between the receptors. Because two of the five nucleotides in FcγRIIIb-NA2 that differ from FcγRIIIb-NA1 result in additional N-glycosylation sites, it might well be that glycosylation differences affect the interaction with CR3. It will clearly be of interest to study the composition and signaling capacity of the various FcγRIIa and FcγRIIIb haplotype complexes.

Large changes in receptor expression were induced by preactivating the neutrophils with a combination of IFN-γ and G-CSF. Induced expression of FcγRI and ~50% reduction in FcγRIIIb surface expression profoundly altered the FcγR expression profile and IgG-induced response. Although changes in FcγR expression were variable among haplotypes, no significant differences between the haplotypes in H₂O₂ production were detected anymore, irrespective the FcγR expression levels.

Overall, the response of preactivated neutrophils to IgG was about twice as high compared with nonactivated neutrophils. When used as intact murine Abs, the results showed that the anti-CD32 mAb was still the most potently blocking mAb. In our experimental setting we found that blocking FcγRI by intact anti-CD64 mAb alone had no effect on the response at all. Reasoning from the genetic haplotypes, it is mainly the reduction in FcγRIIIb in relationship to the relatively unchanged FcγRIIa expression that has a large influence on how these receptors interact with and respond to IgG. The role of FcγRI is unclear in this scenario, and difficult to address experimentally by blocking antibodies, as there are no anti-CD64 Abs that completely block IgG binding with their Fab alone. The 10.1 clone against FcγRI did block IgG-coated erythrocyte-binding to FcγRI-transfected cell lines using intact mAb, but not when the F(ab′)₂ fragments of this mAb were applied, demonstrating a Kurlander effect to be involved in the blocking effects of intact anti-CD64 mAb as well (data not shown). This may explain that blocking of FcγRI IgG binding by mAb 10.1 is partially dependent on IgG concentration. It might well be that the high concentration of IgG that is present in our assays was such that binding of mAb 10.1 did not significantly reduce the IgG-mediated respiratory burst in preactivated neutrophils, explaining why the IgG-response in preactivated neutrophils could not be completely blocked even in the presence of mAbs against all three FcγRs.

With respect to the genotypic impact and the surface expression of the various FcγRs on neutrophils, we observed some remarkable correlations in the induction of FcγRI and FcγRIIb. FcγRI induction was linked to the FcγRIIa-131H/R phenotype, whereas FcγRIIb induction was linked to the FcγRIIIb-NA1/NA2 phenotype. Thus, the FcγRIIa-131RR FcγRIIIb-NA2NA2 haplotype seemed to result in increased FcγRI and lower FcγRIIb induction upon activation, hence changing the weaker IgG-induced H₂O₂ production of this haplotype under resting conditions to a similar level of respiratory burst activity compared with the other haplotypes upon activation of neutrophils. Although very reproducible, with heterozygous genotypes showing an intermediary effect compared to both homozygous genotypes, an explanation for such linkage

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at the genomic level in FcγR protein expression regulation remains unclear to date. In case of FcγRIIb and FcγRIIIb, some intergenic polymorphisms in or near the promoter sites of these adjacent, head-to-head arranged genes may be linked to the NA2 allele. On the other hand, such regulatory mechanisms at the genomic level seems less likely to be involved in case of the FcγRIIa-FcγRI interaction in FcγRI upregulation because of the distance of > 11 megabases between both loci.

Numerous reports have been published about the association of FcγR polymorphic variants with disease susceptibility and severity (reviewed by Bournazos et al.\(^36\)). As is the case with studies on receptor function, nearly all of these studies have focused on variations in a single receptor type. As our study indicates, specific FcγR haplotypes can lead to a response that is clearly different from the expectations raised by some of the excellent studies investigating IgG binding by single receptor variants\(^10\).

Our studies have been performed on healthy white volunteers only. It is known that that genotype and haplotype frequencies of FcγRIIa and FcγRIIIb variants can be different in other populations\(^19\). However, we do not expect that the differences in response we have observed between haplotypes will be different in populations in which a different genetic distribution of these haplotypes is found, especially since our blocking experiments suggest that the IgG-induced respiratory burst in our assay is dependent on FcγRs only.

In conclusion, our systematic integrated functional analysis of FcγRIIa and FcγRIIIb genetic variants in an extensive cohort of healthy subjects demonstrates, to our knowledge for the first time, a novel level of functional interaction between haplotypes of allelic FcγRIIa and FcγRIIIb variants on human neutrophils. These findings provide a clear incentive for a more complete and integrated analysis of FcγR haplotypes in clinical and genetic association studies. The MLPA assay that we have developed and described before\(^7,16\), and that we have also employed in the present study, provides an important and powerful tool to perform such integrated analyses.

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DISCLOSURES

The authors have no financial conflicts of interest.
REFERENCES


SUPPLEMENTARY FIGURES

Figure S1. Mono-, di- and polymeric content of the IgG preparation
The relative amount of mono- di- and polymeric IgG in the IgG product for subcutaneous use was determined by high-pressure size-exclusion chromatography (HP-SEC), as described in Materials and Methods.

Figure S2. H₂O₂ production by freshly isolated neutrophils and the blocking effect of anti-FcγR mAbs
Data are depicted as a ratio of the maximum slopes of H₂O₂ production in response to IgG or PMA. H₂O₂ production was measured in the presence of blocking mAbs against FcγRII (CD32) and/or FcγRIII (CD16), indicated by αCD32 and αCD16, respectively. Blocking FcγRII, alone or in combination with FcγRI, FcγRIIIb or both, resulted in a significant reduction of H₂O₂ production (p<0.0001 for all). Polymorphic variants of FcγRIIa (131H/R) are indicated by 131HH, 131HR and 131RR. Data are expressed as mean ± SEM of 3-5 independent experiments per haplotype (32 in total).
All donors

Figure S3. H₂O₂ production by preactivated neutrophils and the blocking effect of anti-FcγR mAbs
Data are depicted as a ratio of the maximum slopes of H₂O₂ production in response to IgG or PMA. Neutrophils were preactivated by culturing overnight in the presence of G-CSF and IFN-γ. H₂O₂ production was measured in the presence of blocking mAbs against FcγRI (CD64), FcγRII (CD32) and/or FcγRIII (CD16), indicated by αCD64, αCD32 and αCD16, respectively. Blocking FcγRII, alone or in combination with FcγRI, FcγRIIIb or both, resulted in a significant reduction of H₂O₂ production (p<0.0001 for all). Data are expressed as mean ± SEM of 26 independent experiments.