Genetic variation in human Fc gamma receptors: Functional consequences of polymorphisms and copy number variation

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CHAPTER 2

A novel splice-variant of FeγRIIA:
a risk factor for anaphylaxis in hypogammaglobulinemia

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Abstract

Background

Our index case was a patient with Common Variable Immunodeficiency (CVID). She suffered from anaphylactoid reactions upon administration of intravenous immunoglobulins (IVIg), associated with the presence of IgG antibodies against IgA.

Objective

To determine the role of Fc gamma receptor IIa (FcγRIIa) in IVIg-induced anaphylactoid reactions

Methods

Neutrophils and PBMCs were isolated from healthy individuals and IVIg-treated patients. FcγRIIa mRNA and DNA were analyzed by RT-PCR and sequencing. IgG-mediated elastase release and intracellular Ca2+ mobilization were determined in neutrophils and transfected cell lines, respectively.

Results

A novel splice variant of FcγRIIa, containing an expressed cryptic exon 6* (FcγRIIa\textsuperscript{exon6*}) was identified in our index case. This exon is normally spliced out of all FcγRII isoforms except the inhibitory FcγRIIb1. Compared to healthy controls, the heterozygous FCGR2A\textsuperscript{c.742+871A>G} mutation was more frequent in CVID patients (n=53; p<0.013). Expression in CVID was associated with anaphylaxis upon IVIg infusion (p=0.002). Upon screening of additional IVIg-treated patient cohorts, we identified 6 FCGR2A\textsuperscript{c.742+871A>G} allele-positive patients with Kawasaki disease (KD; n=208) and 1 patient with idiopathic thrombocytopenia (ITP; n=93). None had suffered from adverse reactions to IVIg. Moreover, FcγRIIa\textsuperscript{exon6*} was also demonstrated in asymptomatic family members.

Functional studies in primary cells and transfected murine cells demonstrated enhanced cellular activation by FcγRIIa\textsuperscript{exon6*} compared to its native form, as illustrated by increased elastase release and intracellular calcium mobilization.

Conclusion

A novel splice variant FcγRIIa\textsuperscript{exon6*} was characterized as a low-frequency allele, coding for a gain-of-function receptor of IgG. In the presence of immune complexes, FcγRIIa\textsuperscript{exon6*} may contribute to anaphylaxis in CVID.
Introduction

Human Fcγ receptors are cell surface glycoproteins that bind the Fc region of IgG. Fcγ receptors are encoded on chromosome 1q23 (1,2). Three types of FcγRs, type I, II and III, are discriminated, based on their affinity for monomeric IgG. Type I is a high-affinity receptor, whereas type II and type III are low-affinity receptors (3). Of these receptors, FcγRII is the most widely distributed, as it is expressed on most types of blood cell (4). Deficiencies of FcγRII have never been observed, and it is assumed that the lack of FcγRII will be lethal. FcγRII is the only Fcγ receptor that contains its own signaling motif, whereas FcγRI and FcγRIII are dependent on the association with another molecule for signal transduction.

Several isoforms of FcγRII exist, which are highly homologous in their extracellular and transmembrane regions but differ in their intracellular domains (5,6). In this domain, FcγRII contains, depending on the isoform, either an immunoreceptor tyrosine-based activation motif (ITAM) or an immunoreceptor tyrosine-based inhibitory motif (ITIM). FcγRIIa and FcγRIIc contain an ITAM motif and therefore function as activating receptors, whereas FcγRIIb contains an ITIM motif and acts as an inhibitory receptor (7-9). The isoform FcγRIIb has two functional splice variants, FcγRIIb1 and FcγRIIb2. Apart from differential expression patterns, these splice-variants differ in the presence or absence of exon 6, respectively (10). Similar to FcγRIIb2, FcγRIIa and FcγRIIc lack this exon (although it is not recognized as a true exon in these isoforms), whereas FcγRIIb1 does contain exon 6. To date, splice variants of FcγRIIa have not been reported.

Since rodents lack an FcγRIIa homologue, transgenic animals have been generated to study (human) FcγRIIa in vivo (11). In comparison to their wild-type littermates, induction of thrombocytopenia by antibodies was more severe in hFcγRIIa transgenic mice (11), suggesting an important pro-inflammatory role for FcγRIIa in disease. In the same mouse model, FcγRIIa has recently been shown to be sufficient to trigger both anaphylaxis and airway inflammation in response to immune complexes(12). Other experimental animal models and in vitro studies suggest that the ratio between activating and inhibitory FcγRs determines the responsiveness of immune cells to immune complexes (13,14).

We have developed a highly sensitive RT-PCR for the quantification of mRNA of FcγRII isoforms (15) to test whether mRNA levels of FcγRII in neutrophils of healthy volunteers and various patient cohorts correlate with the cellular responsiveness to IgG and immune complexes. For this reason we investigated the FcγRII mRNA levels in patients whose primary treatment is intravenous immunoglobulins (IVIg) as substitution or immunomodulation therapy, i.e. hypogammaglobulinemia, idiopathic thrombocytopenia (ITP) and Kawasaki disease (KD). ITP is a common disease entity. In contrast, KD is a rare febrile childhood vasculitis of medium-sized arteries of unknown etiology. This last disease is diagnosed according to a set of clinical criteria (16). In 20-25% of KD cases coronary artery aneurysms (CAA) develop, which renders KD the leading cause of acquired heart disease under the age of 5 years. The standard treatment for KD consists of a single, high dose of IVIg (2 g/kg) in combination with oral aspirin to reduce the risk and severity of CAA (17).

We identified aberrant FcγRIIa in some of these patients. The index case suffered from an acquired form of hypogammaglobulinemia, common variable immunodeficiency (CVID), and needed antibody supplementation therapy to combat recurrent infections. She had a severe adverse reaction to her first dose of IVIg. The presence of aberrant FcγRIIa was further studied in a larger cohort of CVID cases with similar clinical findings of anaphylaxis. Although patients with aberrant FcγRIIa were also
recognized among ITP and KD patients, clinical anaphylaxis did not occur in these patients upon IVIg treatment. Here we report a novel gain-of-function Fc\(\gamma\)RII splice variant containing a cryptic exon 6\(^*\), Fc\(\gamma\)RII\(_{\text{exon6}*}\), which was expressed in granulocytes and monocytes and may be present in the healthy population as a low-frequency mutation, associated with CVID and anaphylactoid reactions toward IVIg infusion.
Methods

Patient description

Index case: A 28-year old Caucasian female was diagnosed at the age of 26 years with CVID when, after a period of recurrent upper airway infections and chronic *Giardia lamblia* related diarrhea, examination revealed wide-spread gastric and intestinal nodular lymphoid hyperplasia in combination with low serum immunoglobulins. There was no history of infectious or auto-immune diseases among her family members.

After the start of the first IVIg infusion, the patient complained of abdominal pain and nausea while developing a generalized rash, a respiration rate of 36 /min, and tachycardia (136 /min) with a fall in blood pressure, from 137/95 to 106/74 mm Hg. Half an hour later, she experienced chills followed by fever (39.2°C). IVIg infusion was stopped and treatment was started with intravenous clemastin fumarate (2 mg), corticosteroids (DAF, 25 mg) and NaCl 0.9% solution (500 ml). The condition of the patient improved within the next few hours, and she was discharged 24 hours later. Blood cultures and cultures from the immunoglobulin batch remained sterile. Concentrations of serum tryptase and complement activation products during the anaphylactoid reaction were not increased, in contrast to neutrophil-derived serum elastase, as was monitored in serial blood samples during this episode (Table E1 in the Online Repository & data not shown). Re-analysis of pre-infusion serum samples showed the presence of anti-IgA antibodies of the IgG1 subclass. Two weeks after the incident and under appropriate monitoring, the patient received immunoglobulin subcutaneously (IMIg 16% solution) in gradually increasing dosages during 3 subsequent days (18). Weekly administration of IMIg continued until she received IVIg without problems 2 months later, when anti-IgA had completely disappeared, probably by formation of IgA-anti-IgA complexes (19,20)

Measurement of elastase, tryptase and complement activation products

Detection of elastase, tryptase and activation of C4 and C3, and C1q-C4 complexes was performed as described previously in detail (21-24).

Patient cohorts and blood sampling for DNA extraction

EDTA-anticoagulated blood samples were obtained from 52 additional adult patients diagnosed with hypogammaglobulinemia for DNA extraction. Patients were selected according to the international criteria for Common Variable Immunodeficiency (CVID)(25). In 33 patients, the presence of antibodies against IgA had been assessed in serum taken prior to the initiation of IVIg infusions. In case of Kawasaki Disease (KD), blood was obtained from over 200 pediatric patients, all treated with IVIg (2 g/kg, single dose) and oral aspirin within 10 days following the onset of fever. KD was defined by the presence of fever for ≥3 days, together with at least four of the five classical diagnostic criteria (16,17). Additional blood samples were available for DNA extraction from 93 patients <18 years with ITP that had been treated with IVIg. The diagnosis of acute ITP was made according to the guidelines of the American Society of Hematology and the United Kingdom practice for management of acute childhood ITP (26,27).
Informed consent was obtained from all parents of patients younger than 12 years and from all patients older than 12 years. Ethnicity was determined by self or parental ethnic identification. Healthy controls were unrelated adult Caucasian blood donors residing in the same geographical area. These studies were approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam and were performed in accordance with the Declaration of Helsinki.

**Monoclonal antibodies and reagents**

The following monoclonal antibodies (mAb) against human Fcγ receptors (FcγR) were used: CD16 (anti-FcγRIII, clone 3G8 prepared as F(ab’)2 fragments) and CD32 (anti-FcγRII, clone IV.3) (generous gifts from Dr. Masja de Haas, Sanquin Research, Amsterdam, The Netherlands); CD16-PE (anti-FcγRIII, IgG2a isotype, clone CLB-FcR-gran/1, 5D2; Sanquin, Amsterdam, The Netherlands); CD32-FITC (anti-FcγRII, IgG1 isotype, clone KB61; DakoCytomation, Glostrup, Denmark) and anti-CD64-FITC (anti-FcγRI, IgG1 isotype, clone 10.1; InstruChemie, Delfzijl, the Netherlands). Relevant isotype controls were obtained from Sanquin. Isotype control IgG2a-PE (clone 713) and IgG1-FITC (clone 603). Streptavidin-polyHRP (Sanquin) was used for the detection of hIgGs. Rabbit polyclonal anti-FcγRIIIa was a generous gift from Dr. Arthur Verhoeven (28).

**Isolation of neutrophils and PBMCs**

Heparinized venous blood was collected from healthy donors and selected patients after obtaining informed consent, and was separated over a Percoll gradient (Amersham Biosciences) into PBMC as interphase and granulocytes with erythrocytes in the pellet. Neutrophils were purified by lysis of the erythrocytes with ice-cold isotonic NH₄Cl containing NaHCO₃, washed and suspended in HEPES buffer (25 mM HEPES, 123 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 2.5% (v/v) human-serum albumin at pH 7.4) Hepsene buffer, essentially as described before (15). Purity and viability of both cell fractions were >95%, as determined by flowcytometry and trypan blue exclusion, respectively. The neutrophil suspensions were negative for FcγRI (CD64).

**Detection and relative quantitation of FcγRII isoform-specific mRNA**

The detection and relative quantification of FcγRII mRNA have been described in detail elsewhere (15). In short, FcγRII isoform-specific primers (FcγRIIa: forward primer, 5’-ATCATTGTGGCTGTCATTGC-3’ and reverse primer, 5’-TCAGGTAGATGTCTTTATCATCG-3’; FcγRIIB2: forward primer, 5’-GGAAAAGCGCATTTGAGCCAATC-3’ and reverse primer, 5’-GGAAATACGAGATCTTCCCTCCTCTG-3’) were developed and applied to cDNA generated from mRNA. mRNA was isolated from 10⁶ purified neutrophils by use of QiaAmp RNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Quantitation of cDNA was performed with the Lightcycler Instrument (Roche Applied Science, Almere, The Netherlands). Serial 10-fold dilutions of cDNA were made and quantified with the method described in Technical Note No. LC 13/2001 (Roche Applied Science).
Sequence analysis of the introns surrounding exon 6* on genomic DNA

Specific primers were designed to investigate the intron-exon boundaries of exon 6* of FcγRIIa. Forward primer 5’-CCT CTG GAC TAG CCC TTT TCC ACG T-3’ anneals upstream of exon 6*, reverse primer 5’-TAG GCC CAG AAA TTA GAC TCA GTG T-3’ anneals downstream of exon 6*. Nomenclature of the intronic mutation is based on NCBI reference sequence NM_001136219.1

SDS-PAGE and Western blot of FcγRIIa exon 6*

Neutrophils were suspended in HEPES buffer (25 mM HEPES, 123 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 2.5% (v/v) human-serum albumin at pH 7.4) at a concentration of 5 x 10⁶ cells/ml. Subsequently, diisopropyl fluorophosphat e (DFP) was added 1:1000 to a final concentration of 5 mM, and the cell suspension was incubated for 10 minutes at 4°C. Thereafter, the cells were spun down and resuspended in 50 µl of protease inhibitor mixture (Roche) supplemented with 5 mM EDTA, and the suspension was heated to 95°C. SDS sample buffer was added and samples were incubated for 30 minutes at 95°C. Samples were stored at -80°C until further use. Samples were thawed and subsequently run on a 10% (w/v) Bis-Tris gel. Thereafter, the gel was blotted on PVDF membrane. FcγRIIa was detected on blot by means of a polyclonal antibody against the cytoplasmic tail of FcγRIIa. Bands were visualized by Enhanced Chemiluminescence substrate (ECL).

Neutrophil activation by IgG dimers

Immunoglobulin I.M. (IMIg, Lot. No. 01B26H403A, Sanquin, CLB, Amsterdam, The Netherlands) contains 160 g protein per liter, of which at least 90% is IgG. Dimeric IgG was isolated from IMIg by gelfiltration on Hiload 16/60 Superdex 200 (Amersham Biosciences, Uppsala, Sweden) and subsequent pooling of the peaks that contained mainly dimeric IgG. Dimeric IgG, 50 µg/ml (4.5 µg in 90 µl of Iscove’s Modified Dulbecco’s Medium [IMDM; Biowhittaker Europe, Verviers, Belgium]) were added to each well of a 96-round-bottom-well plate, followed by addition of neutrophils (2 x 10⁴ per well). The mixtures were incubated for 2 hours at 37 °C in humidified air containing 5% (v/v) CO₂. Elastase released from the cells was measured in the cell-free supernatants with a sandwich ELISA as described (22).

Cell lines and transfection

IIA1.6 cells were transfected with pcDNA3 containing either human FcγRIIa-R131 or FcγRIIa-R131 containing exon 6*. For each transfection 10 µg of vector per 10⁷ cells was used. Electroporation was performed with a Gene pulser II (Biorad), at 250 V, 975 µF. Two days after transfection the IIA1.6 transfectants were cultured in IMDM supplemented with 10% (v/v) fetal calf serum (FCS; Bodinco BV, Alkmaar, The Netherlands), 100 U/ml penicillin (Gibco, Paisley, UK) and 100 µg/ml streptomycin (Gibco) under selection of 0.8 mg/ml geneticin (G-418-sulfate, Life Technologies, Gibco).
Measurement of intracellular calcium in IIA1.6 cells

IIA1.6 cells were suspended at a concentration of 5×10^6 cells/ml in HEPES buffer and loaded with 1 µM Fura-2/AM (Molecular Probes Europe, Leiden, The Netherlands) by incubation at 37°C for 45 minutes. The cells were then washed with HEPES buffer and resuspended at 5×10^6/ml. Subsequently, the cells were incubated for 15 minutes at RT with 2 µg/ml of anti-FcγRII (clone AT10, Serotec, Oxford, UK) and washed again with HEPES buffer. Loaded IIA1.6 cells were incubated at 37°C for 5 minutes and transferred to a cuvette in a fluorometer (Luminescence Spectrometer LS55, Perkin Elmer, Fremont, CA, USA). Baseline concentration of free intracellular Ca^{2+} ions ([Ca^{2+}]_i) was measured for 100 seconds, after which AT10 was cross-linked with 10 µg/mL goat-anti-mouse IgG (Southern Biotech, Birmingham, AL, USA). After levels had stabilized again, thapsigargin was added at 1 µM. The increase in [Ca^{2+}]_i was measured, and the data were analyzed with FLWinlab software (Perkin Elmer).

Determination of isotype and subclass of anti-IgA antibodies

Most CVID patients sampled before IVIg administration were tested for the presence of anti-IgA antibodies as described before (29). In short, high-density polystyrene beads were coated with purified IgA molecules and then incubated with serum samples. Positive reactions were demonstrated by agglutination of the beads.

In the index case with the clinical anaphylaxis episode, additional tests were performed. A serum pool from 33 healthy volunteers and serum of the patient who had been sampled before IVIg administration were absorbed over IgA-coated sepharose by overnight incubation. The absorbed Ig’s were eluted with glycine-HCl pH 2.5 / 0.5 M NaCl and were subsequently dialyzed against 5000-fold excess of PBS. The obtained Ig’s and the original plasma pool were spotted onto nitrocellulose followed by overnight incubation with PBS/4% (v/w) milk powder. The nitrocellulose was cut into four pieces, each containing spots of the obtained Ig’s described above and these were incubated with mAbs to IgG subclasses. After extensive washing with PBS/0.01% (w/v) Tween the nitrocellulose was incubated with biotinylated rat anti-mouse κ-light chain. Subsequently, the blots were incubated with streptavidin-polyHRP and washed. The spots were then visualized with Enhanced Chemiluminescence substrate (ECL) (Amersham Biosciences, Uppsala, Sweden).

Statistical analysis

Results are depicted as means ± SD. When applicable, Student’s t test and ANOVA were used. A two-sided p-value <0.05 was considered to indicate a significant difference. Fisher exact test (2-sided) was used to analyze the allele frequency between the cohorts, a p-value <0.05 was considered to indicate a significant difference.
Results

Detection and sequencing of a novel splice variant of FcγRIIa

We have developed and validated primers that are specific for the mRNA of FcγRIIa. The forward PCR primer specific for FcγRIIa binds in exon 5, whereas the reverse primer binds in exon 8. Using these primers, we had previously generated a sensitive RT-PCR test for the quantification of FcγRIIa at the mRNA level (15). Via the melting curve generated at the end of a run, product specificity is routinely evaluated. Upon screening a patient cohort with IVIg treatment for RNA levels of FcγRIIa we found an exceptional case. The patient suffered from CVID with an anaphylactic shock upon administration of the first dose of IVIg.

**Figure 1A.** Melting curves of FcγRII isoforms in the CVID family.
Upper panels: A shift to the right in the melting curves of the patient (red lines) and her brother (blue lines) is observed for the FcγRIIa PCR product, but not for the FcγRIIb2 PCR product. Duplicates of the patient and her brother are indicated in dashed lines. Melting curves of all other tested individuals are indicated in black.
The melting curves of the FcγRIIa PCR products of the patient, as well that of her brother, were different from those of normal controls (Fig. 1A). The samples were applied to a 1.5% (w/v) agarose gel, which yielded two bands in the samples of the patient and her brother but not in the control (Fig. 1B). The sequence of all products was determined. Alignment of the cDNA product sequence with the genomic sequence of FcγRIIa revealed that the extra band was FcγRIIa containing exon 6*, which is normally absent from all transcripts of FcγRII except FcγRIIb1, where it is recognized as a true exon (exon 6).

Exon 6* was not derived from FCGR2B, but from FCGR2A, as assessed by sequence analysis. The mRNA expression of this novel splice variant of FcγRIIa (FcγRIIa<sub>exon6*</sub>) was observed in the granulocyte fraction as well as in the mononuclear fraction (Fig. 1B). A single nucleotide polymorphisms (SNP) present in the FCGR2A and absent in the FCGR2B or FCGR2C gene confirmed the exon 6* splicing defect in the gene encoding FcγRIIa (Figure E1 in the Online Repository).

**Figure 1B.** Gel electrophoresis of FcγRII isoforms in the CVID family. Analysis of the PCR products on agarose gel. The left most lanes contain markers (M), 1) healthy non-related control, 2) non-template control, 3) CVID patient, 4) mother, 5) brother.
Alternative splicing of FcγRIIa is linked to a point mutation in the splice site in intron 5

Specific primers around exon 6* of FcγRIIa were developed, based on the fact that FcγRIIa and FcγRIIb can be distinguished by several nucleotide differences between the two isoforms. Evaluation of the genomic DNA sequence showed that the patient expressing exon 6* in FcγRIIa mRNA had a heterozygous A>G point mutation at position +871 of intron 5 in her genomic DNA (Fig.2A). An overview of the genomic organization of FCGR2A shows the position of exon 6* in the cytosolic part directly following the transmembrane domain of the receptor. Thus, retention of exon 6* extends the cytoplasmic tail of FcγRIIa by 19 amino acids.

Figure 2. Site of point mutation in the genome.
A. An overview of the genomic organization of FCGR2, in which the blocks represent exons, lines indicate introns. TM indicates the transmembrane domain. Exon 6* is hatched where it is spliced out. Retention of exon 6* is linked to the +871 point mutation in intron 5 as shown by the arrow. B. Pedigree of the index case suffering from CVID, including her healthy father and brother carrying the same FCGR2A c.742+871A>G allele.
We subsequently tested the patient’s parents and identified one additional family member carrying the same heterozygous $FCGR2A^c.742+871A>G$ encoding the splice-variant of FcγRIIa: i.e. not only the patient suffering from CVID but also her brother and father carried the mutation, although both were completely healthy (Fig.2B).

**Detection of the novel splice-variant of FcγRIIa at protein level**

Further analysis of the expression of the protein was assessed by Western blotting. The molecular mass of the splice variant was estimated to be 42 kDa, as native FcγRIIa in neutrophils migrates at a molecular mass of 40 kDa [see also Van der Heijden et al. (30)] and exon 6* encodes 19 amino acids corresponding to ~2 kDa. Indeed, we detected two bands in the neutrophil lysate of the patient and in that of her father, one being 40 kD, the other being 42 kD in mass. In controls without mRNA encoding FcγRIIa^exon6* we observed only one band of 40 kD (Fig.3). Expression levels of FcγRII (CD32) on the blood cells of all affected persons in this pedigree were within the normal range (Figure E2 in the Online Repository).

**Figure 3. SDS-PAGE and Western blot of FcγRIIa and FcγRIIa^exon6*.**

Neutrophil lysates were obtained as described in Materials & Methods and run on a 10% (w/v) Bis-Tris gel. Thereafter, the proteins were blotted on a PVDF membrane, which was probed with a polyclonal antibody against the cytoplasmic tail of FcγRIIa. Lane 1, CVID patient; lane 2, father of the CVID patient; lane 3, healthy control not expressing FcγRIIa^exon6*.

**Prevalence and inheritance of the novel splice-variant of FcγRIIa**

Upon sequencing the DNA from the total cohort of CVID patients (n=53, including the index case), we found two additional cases with the same heterozygous $FCGR2A^c.742+871A>G$ allele. Most patients had never suffered from any serious side effects of IVIg in the past (n=45), whereas a selected subset of these CVID patients had severe anaphylactoid reactions during infusion of IVIg (n=8) (Table I). In this CVID cohort (n=53 in total), the presence of anti-IgA antibodies had been detected in 7 out of 33 of the patients that had been tested prior to the first IVIg infusion. Of these 7 anti-IgA-positive
patients 4 had shown a serious reaction, whereas the other 3 anti-IgA-positive patients had not suffered from any clinical anaphylactoid reaction during their first IVIg infusions. Of the anti-IgA-positive patients with clinical anaphylaxis one additional case carried an FCGR2A<sup>c.742+871A>G</sup> allele, whereas the other FCGR2A<sup>c.742+871A>G</sup> allele-positive CVID patient with anaphylaxis at the first IVIg infusion had never been tested for anti-IgA antibodies (not shown). Upon screening of two additional cohorts of IVIg-treated patients suffering either from KD (n=208) or from ITP (n=93), we found the same mutation in 6 additional patients (Table I). Side effects of the IVIg treatment had not been noticed, although 3 of these 5 KD children needed a second course of IVIg to become afebrile. The incidence of IVIg resistance varies in most centers between 10-20% of KD cases, irrespective of the ethnic background (31-33). Also remarkable is the fact that among these 5 patients some highly unusual autoimmune manifestations occurred during follow-up (i.e. diabetes mellitus in 2 cases and ITP in 1 case).

Two KD families were tested for the presence of the rare FCGR2A<sup>c.742+871A>G</sup> allele. In each of the families, apart from the patient, one of the parents was also found positive for the mutation (Figure E3 in the Online Repository), while being – once again – completely healthy. Upon subsequent screening of 287 healthy blood donors, one additional donor was identified with the FCGR2A<sup>c.742+871A>G</sup> allele, demonstrating its presence in the normal population. To date, homozygotes have not been identified.

Table I. Genotype and allele frequency of FCGR2A intron 5 +871 position in healthy individuals vs. KD, ITP, and CVID patients.

<table>
<thead>
<tr>
<th>Genotype frequency</th>
<th>Control subjects no. (%)</th>
<th>Patients with KD no. (%)</th>
<th>Patients with ITP no. (%)</th>
<th>Patients with CVID no. (%)</th>
<th>Patients with CVID/anaphylaxis no. (%)</th>
<th>Patients with CVID/no anaphylaxis no. (%)</th>
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<tr>
<td>AA</td>
<td>286 (99.7)</td>
<td>203 (97.6)</td>
<td>92 (98.9)</td>
<td>50 (94.3)</td>
<td>5 (62.5)</td>
<td>45 (100)</td>
</tr>
<tr>
<td>AG</td>
<td>1 (3.5)</td>
<td>5 (2.4)</td>
<td>1 (1.1)</td>
<td>3 (5.7)</td>
<td>3 (37.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>GG</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
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<td>p-value</td>
<td>-</td>
<td>.087*</td>
<td>.430*</td>
<td>.013*</td>
<td>.0001*</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>.002**</td>
</tr>
<tr>
<td>A</td>
<td>573 (99.8)</td>
<td>411 (98.8)</td>
<td>185 (99.5)</td>
<td>103 (97.2)</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>5 (1.2)</td>
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<td>3 (2.8)</td>
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<td></td>
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</table>

DNA from 287 healthy individuals, 208 KD patients, 93 ITP patients, and 53 CVID patients in total (including the index case) was analyzed for the A>G mutation at +871 position in intron 5 of FCGR2A, as described in Materials & Methods. AG and GG groups were combined in Fisher’s exact test to determine differences between the cohorts. Significance was calculated by Fisher-exact 2-sided tests (p<0.05), comparing patients versus controls (*) or within the CVID cohort comparing those with and without anaphylaxis (**).
Neutrophils expressing FcγRIIa*exon6* have increased sensitivity to small immune complexes

Because genetic variations in FcγR such as SNPs, CNV and splice variants are known to influence the cellular response upon receptor triggering (3,34,35) we tested whether this is also true for FcγRIIa*exon6*. Neutrophils were isolated and stimulated with 50 µg/ml dimeric IgG, a dose previously defined as optimal. Individuals expressing FcγRIIa*exon6* showed strongly increased elastase release in response to stimulation compared to individuals expressing the wild-type FcγRIIa (Fig.4), which was reminiscent of the findings made in the index case following infusion of her first IVIG dose (Table E1 in the Online Repository). Involvement of the FcγRII in this neutrophil response was demonstrated by the effect of an inhibitory antibody against FcγRII. The elastase release in response to dimeric IgG was almost completely ablated in all individuals when FcγRII was blocked. Responsiveness to stimulation with fMLP plus cytochalasin B was equal in all individuals tested (Fig.4), demonstrating that the effect was specific for FcγR stimulation and there was no generalized difference in elastase secretion capacity.

Figure 4. Neutrophils expressing FcγRIIa*exon6* respond more vigorously to dimeric IgG.

Elastase release from neutrophils of individuals expressing FcγRIIa with (n=4) or without exon 6* (n=14). Neutrophils were stimulated with dimeric IgG in the presence or absence of blocking Fab (IV.3, anti-FcγRII) fragments, or with fMLP/CytoB. The difference in the response to dimeric IgG is statistically significant in the absence (p=0.0077) and presence (p<0.0001) of blocking Fab fragments, in contrast to the response to fMLP/CytoB (p=0.7, N.S.)
To verify other responses, we tested the NADPH oxidase activity in neutrophils from donors with either FcγRIIa or FcγRIIa<sup>exon6*</sup> and studied the respiratory burst induced by aggregated IgG (with or without priming with platelet-activating factor [PAF], which itself does not induce NADPH oxidase activity). However, no differences between neutrophils from donors with either FcγRIIa or FcγRIIa<sup>exon6*</sup> were observed (n=3; data not shown).

**Increased [Ca<sup>2+</sup>]<sub>i</sub> in IIA1.6 cells transfected with FcγRIIa<sup>exon6*</sup>**

All but one FCGR2A<sup>c.742+871A>G</sup> allele-positive individuals showed FcγRIIa<sup>exon6*</sup> transcripts with the R131 SNP in exon 3 (rs1801274), as assessed by allele-specific amplification as well as by subsequent cloning strategies (data not shown). For reasons of comparison, we generated R131-positive FcγRIIa and FcγRIIa<sup>exon6*</sup> constructs and expressed these in IIA1.6 B lymphoma cells for analysis of their functionality. Both FcγRIIa and FcγRIIa<sup>exon6*</sup> IIA1.6 cells showed similar level of CD32 surface staining after selection (Figure E4 in the Online Repository). By measuring [Ca<sup>2+</sup>]<sub>i</sub> after receptor crosslinking, we found that IIA1.6 cells expressing the FcγRIIa<sup>exon 6*</sup> variant displayed consistently larger [Ca<sup>2+</sup>]<sub>i</sub> mobilization after CD32 cross-linking than cells transfected with the wild-type FcγRIIa (Fig.5). In addition, thapsigargin resulted in similar [Ca<sup>2+</sup>]<sub>i</sub> mobilization in both FcγRIIa and FcγRIIa<sup>exon 6*</sup> transfectants (Fig.5), indicating that both transfectants had similar maximal responses and equal labeling with the calcium-sensitive dye.
Figure 5. Increased $[\text{Ca}^{2+}]$ in IIA1.6 cells transfected with Fc$\gamma$RIIa$^{\text{exon6}*}$ after receptor crosslinking.

IIA1.6 cells were transfected with either Fc$\gamma$RIIa (solid line and arrows) or Fc$\gamma$RIIa$^{\text{exon6}*}$ (dashed line and arrows) and these receptors were crosslinked. A. Representative $[\text{Ca}^{2+}]$, traces from one experiment. Arrows indicate addition of goat-anti-mouse IgG or thapsigargin (after $[\text{Ca}^{2+}]$, stabilization). The experiment shown was repeated 6 times with identical results. B. Combined data from all experiments. All values are response peak levels minus baseline levels. Differences were significant in case of Fc$\gamma$R crosslinking ($p<0.03$; $n=6$), but not in case of thapsigargin ($p=0.7$, N.S.; $n=6$)
Discussion

We identified a novel gain-of-function FcγRIIa isoform containing an insertion of exon 6*. This FcγRIIa\textsuperscript{exon6*} variant IgG receptor showed a normal cell distribution and surface expression levels similar to the wild-type FcγRIIa. The FcγRIIa\textsuperscript{exon6*} isoform was first identified in IVIg-treated patients and in their related parents and/or sibs in the three separate families that consented to be tested. Subsequently, one completely unrelated healthy individual was identified upon screening a cohort of control donors.

All affected individuals expressed an identical, aberrantly spliced mRNA of FcγRIIa. Comparison with the native form of FcγRIIa showed that this novel splice variant contained exon 6* of FcγRIIa encoding an additional 19 amino acids. This exon 6* is normally spliced out from the pre-mRNA, except for FcγRIIb1 in B cells. Expression of the aberrant isoform of FcγRIIa was confirmed at the protein level by Western blot as an extra band at 42 kD. At the genomic level a heterozygous point mutation, located at the 871\textsuperscript{st} nucleotide of intron 5, was observed that was neither found in relatives without FcγRIIa\textsuperscript{exon6*} nor in 287 unrelated healthy controls. At the mRNA level, these individuals did also not express any other splice-variant of FcγRIIa. Thus, the A>G mutation in intron 5 of the FCGR2A gene (FCGR2A\textsuperscript{c.742+871A>G}) correlated with the expression of exon 6* in the FcγRIIa mRNA and corresponding FcγRIIa\textsuperscript{exon6*} protein.

The A>G mutation results in a consensus sequence for a 5' donor splice site (AG|GTGAG). Absence of this splice site could result in loss of exon 6* as the spliceosome may couple the acceptor splice site at the 3' end of intron 5 to the donor splice site at the 5' end, thereby removing intron 5, including the cryptic exon 6*. However, both FCGR2B and FCGR2C have a G at the same nucleotide position. As FcγRIIb2 and FcγRIIc do not contain exon 6*, the alternative splicing of FcγRIIa\textsuperscript{exon6*} can not be explained by the A>G mutation alone. Exon 6* in FCGR2A differs at one nucleotide from FCGR2B and FCGR2C. ESEfinder, a web-based resource that identifies Exonic Splice Enhancers (ESEs) (36), predicts that the G at nucleotide 12 in FCGR2A results in a higher score for putative serine/arginine-rich splicing factor 2 (SRSF2) binding site, when compared to the T at the same nucleotide in FCGR2B and FCGR2C. It might well be possible that the combination of the A>G splice site mutation and a SRSF2 binding site, results in the splice variant we have identified.

Because FcγRIIa\textsuperscript{exon 6*} was identified in an IVIg-treated patient that had a clinically relevant hypogammaglobulinemia, we tested a cohort of 53 CVID patients in total, including 8 individuals with CVID who had suffered severe anaphylactoid reactions upon IVIg infusion – including our index case. Three of these patients had serious anaphylaxis during the first IVIg infusion, and expressed FcγRIIa\textsuperscript{exon6*}, all carrying the same A>G mutation in intron 5, two with anti-IgA antibodies in the past and one who had never been tested. Thus, the FcγRIIa\textsuperscript{exon6*} splice variant seems to be more prevalent among CVID patients in general and particularly more in those who experienced serious side effects from IVIg infusion. However, although the cohort tested is relatively small, severe anaphylaxis towards IVIg is not always explained by this aberrant splice product of FcγRIIa (Table I).

As assessed by in-vitro elastase release, neutrophils expressing FcγRIIa\textsuperscript{exon6*} showed increased reactivity to dimeric IgG compared to neutrophils expressing wild-type FcγRIIa. A blocking mAb against FcγRII essentially abolished IgG-induced elastase release, while stimulation by cytochalasin B plus fMLP resulted in equal elastase release by the neutrophils from all tested individuals, showing that the observed difference is indeed dependent on FcγRIIa-mediated signaling. This observation was confirmed in transfected cell lines with stable expression of FcγRIIa\textsuperscript{exon6*}: compared to wild-type
FcγRIIa at equal levels of surface expression, cells expressing FcγRIIa<sup>exon6*</sup> displayed a larger increase in [Ca<sup>2+</sup>], upon CD32 receptor crosslinking. Thus, FcγRIIa<sup>exon6*</sup>-expressing neutrophils are hyperresponsive to stimulation with dimeric IgG, which may explain the severe side effects upon IVIg infusion in some of the CVID patients. On the other hand, family members expressing FcγRIIa<sup>exon6*</sup> appeared completely healthy. This lack of clinical effect can be explained by earlier observations from our group (37). Endogenous IgG at physiological concentrations occupies 95% of FcγRIIa on neutrophils and, by increasing the threshold for activation, mediates a protective effect against unwanted cell activation by immune complexes (37). Hypogammaglobulinemia patients have decreased endogenous levels of IgG. Therefore, the threshold for cell activation by immune complexes is substantially lowered (37). The index case with CVID had high levels of anti-IgA antibodies of the IgG<sub>1</sub> subclass, and the IVIg preparation used contains up to 1.5 mg/ml IgA. Therefore, small immune complexes may have formed <em>in vivo</em> that bound to FcγRIIa<sup>exon6*</sup> on neutrophils and hence caused the severe side effects observed in this patient upon infusion with IVIg. This may also explain why the KD patients did not suffer from side effects upon IVIg infusions. In addition, the disappearance of these anti-IgA IgG<sub>1</sub> molecules coincided with successful, well-tolerated intravenous infusions of IVIg after subcutaneous desensitization of the CVID patient.

Anti-IgA has been the focus of articles on anaphylactic transfusion reactions since it was first described by Vyas <em>et al.</em> in 1968 (38). Subsequent studies have indicated that most reactions are not related to anti-IgA, and testing for anti-IgA will not be helpful in establishing a requirement for IgA-deficient blood products in most cases (39-41). Thus, the practice of prescreening for anti-IgA in CVID patients has been questioned, and IVIg infusions will even be continued with IgA-containing IVIg in any patient with a known high titer of anti-IgA with a lack of symptoms. The basis of this practice is the fact that anti-IgA can be detected in as much as 25% of patients with CVID and 30% of patients with IgA deficiency, of which the majority tolerate repeated infusions of IVIg without adverse reactions (41,42).

Upon screening an additional cohorts of IVIg-exposed patients, <em>i.e.</em> 208 KD patients and 93 ITP patients of Caucasian background, we identified 5 KD patients with the FcγRIIa splice-variant, two of whom developed insulin-dependent diabetes mellitus (IDDM, type-1) in less than 6 months after their acute KD episode. A third KD patient had an episode of ITP during the following year of monitoring. In two of these KD patients, further pedigree analysis revealed the presence of a healthy parent carrying the rare allele for FcγRIIa<sup>exon6*</sup>. Within the ITP cohort, we found the mutation in one individual.

Thus, the mutation represents a low-frequency mutation in the normal Caucasian population that is associated with a ‘superactivating’ FcγRIIa, posing a risk for inflammatory disease reactions. Homozygotes for the gain-of-function <em>FCGR2A</em><sup>c.742+871A>G</sup> allele have not been identified.

The role of FcγRIIa in anaphylaxis was recently suggested (12). Why the FcγRIIa<sup>exon6*</sup> variant results in an immune cell that is hyperresponsive to IgG may be explained by the extension of its cytoplasmic tail. We explored exon 6* of <em>FCGR2A</em> for signaling motifs using the Scansite 2.0 database (http://scansite.mit.edu) but found no known motifs. Studies on CD40 have shown that the potential of the signaling motif is strongly influenced by the distance to the membrane, which determines the capability to interact with signaling molecules (43). In analogy with these cytoplasmic alterations, insertion of exon 6* into FcγRIIa may explain the hyperresponsiveness by enhanced signaling capabilities through elongation of the cytoplasmic tail. Although not further substantiated and beyond the scope of our study, an alternative explanation may consist of the fact that exon 6*
contains 4 prolines and 1 threonine possibly involved in the recruitment of additional signal transduction proteins different from those interacting with wild-type FcγRIIa.

In summary, we describe a novel splice variant of FcγRIIa containing exon 6*, which has a normal mRNA expression and a normal protein expression with a gain of function. The expression of this splice variant was found in three patients suffering from CVID with serious anaphylactoid reactions, two of whom were tested positive for anti-IgA antibodies while the third patient had never been tested. On the other hand, in six patients without any hypogammaglobulinemia but diagnosed with childhood vasculitis or ITP, clinical infusion of IVIg was unremarkable in these heterozygotes for the FCGR2A c.742+871A>G allele. As supported by pedigree analysis in three patients and the presence of one single unrelated individual upon screening of normal healthy blood donors, this allele is also present at low frequency in the healthy population.
**Table E1.** Dynamics of appearance of complement activation products, tryptase and elastase in serum during anaphylaxis upon the first IVIG infusion in the index CVID patient

<table>
<thead>
<tr>
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<th>before infusion</th>
<th>during shock</th>
<th>1 hr after shock</th>
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<tr>
<td>C3b/c (normal &lt;57 nM)</td>
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<td>21.2 nM</td>
<td>28.7 nM</td>
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<tr>
<td>C4b/c (normal &lt; 8 nM)</td>
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<td>undetectable</td>
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<tr>
<td>Tryptase (normal &lt; 10 AU)</td>
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<td>62 ng/ml</td>
<td>158 ng/ml</td>
<td>356 ng/ml</td>
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</tbody>
</table>

Figure E1: Alignment of FCGR2A, FCGR2B, FCGR2C and exon 6* of FCGR2A.
Exon 6/6* sequences of FCGR2A (wt), FCGR2B and FCGR2C and of the exon 6*-containing transcript, indicated by “exon 6*”, are depicted in the grey box. Sequences outside the grey box represent part of intron 5/6 of the three genomic sequences. Nucleotide differences are indicated by asterisks. Underlined are the nucleotides that are found in FCGR2A and the exon 6*-containing transcript but not in FCGR2B and FCGR2C. Single nucleotide polymorphisms (SNPs) are enclosed by brackets.

**Figure E2.** FcγRIIa and FcγRIIa^{exon6*} expression levels on neutrophils.
Data are shown as mean fluorescence intensity (MFI) of fluorescently labeled mAbs against FcγRII (AT10). No significant difference was found between neutrophils expressing FcγRIIa and FcγRIIa^{exon6*} (p=0.9859). Values were corrected by subtracting the background values measured with isotype mAb controls. Data are expressed as mean ± SEM of 3 experiments.
Figure E3. Pedigrees of two KD families showing inheritance of FcγRIIa containing exon 6*. Pedigrees of two KD families are indicated, showing autosomal inheritance of FcγRIIa containing exon 6*.

Figure E4. FcγRIIa and FcγRIIa<sup>exon6*</sup> expression levels on transfected IIA1.6 cells. Data are shown as mean fluorescence intensity (MFI) of fluorescently labeled mAbs against FcγRII (AT10). No significant difference was found between cells transfected with FcγRIIa and FcγRIIa<sup>exon6*</sup> (p=0.39). Values were corrected by subtracting the background values measured with isotype mAb controls. Data are expressed as mean ± SEM of 6 experiments.
Reference List


