

A novel splice variant of FcγRIIa: A risk factor for anaphylaxis in patients with hypogammaglobulinemia

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Background: Our index case was a patient with common variable immunodeficiency (CVID). She had anaphylactoid reactions on administration of intravenous immunoglobulin (IVIg) associated with the presence of IgG antibodies against IgA. **Objective:** We sought to determine the role of Fcγ receptor (FcγR) IIa in IVIg-induced anaphylactoid reactions. **Methods:** Neutrophils and PBMCs were isolated from healthy subjects and IVIg-treated patients. FcγRIIa mRNA and DNA were analyzed by using real-time 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^{exon6*}) was identified in our index patient. This exon is normally spliced out of all FcγRII isoforms, except the inhibitory FcγRIIb1. Compared with healthy control subjects, the heterozygous *FCGR2A*^{c.742+871A>G} mutation was more frequent in patients with CVID ($n = 53$, $P < .013$). Expression in patients with CVID was associated with anaphylaxis on IVIg infusion ($P = .002$). On screening of additional IVIg-treated patient cohorts, we identified 6 *FCGR2A*^{c.742+871A>G} allele-positive patients with Kawasaki disease ($n = 208$) and 1 patient with idiopathic thrombocytopenia ($n = 93$). None had adverse reactions to IVIg. Moreover, FcγRIIa^{exon6*} was also demonstrated in asymptomatic family members. Functional studies in primary cells and transfected murine cells demonstrated enhanced cellular activation by FcγRIIa^{exon6*} compared with its native form, as shown by increased elastase release and intracellular calcium mobilization.

Conclusion: A novel splice variant, FcγRIIa^{exon6*}, was characterized as a low-frequency allele, coding for a gain-of-function receptor for IgG. In the presence of immune complexes,

FcγRIIa^{exon6*} can contribute to anaphylaxis in patients with CVID. (J Allergy Clin Immunol 2013;■■■■:■■■-■■■.)

Key words: Anaphylaxis, common variable immunodeficiency, idiopathic thrombocytopenia, intravenous immunoglobulin, Fcγ receptors, Kawasaki disease, splice variant, neutrophils, elastase

Human Fcγ receptors (FcγRs) are cell-surface glycoproteins that bind the Fc region of IgG. FcγRs are encoded on chromosome 1q23.^{1,2} Three types of FcγRs, types I, II, and III, are discriminated based on their affinity for monomeric IgG. Type I is a high-affinity receptor, whereas types II and III are low-affinity receptors.³ Of these receptors, FcγRII is the most widely distributed because it is expressed on most types of blood cells.⁴ Deficiencies of FcγRII have never been observed, and it is assumed that the lack of FcγRII will be lethal. FcγRII is the only FcγR that contains its own signaling motif, whereas FcγRI and FcγRIII are dependent on 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 or an immunoreceptor tyrosine-based inhibitory motif. FcγRIIa and FcγRIIc contain an immunoreceptor tyrosine-based activation motif and therefore function as activating receptors, whereas FcγRIIb contains an immunoreceptor tyrosine-based inhibitory motif and acts as an inhibitory receptor.⁷⁻⁹ The isoform FcγRIIb has 2 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.

Because rodents lack an FcγRIIa homolog, transgenic animals have been generated to study (human) FcγRIIa *in vivo*.¹¹ In comparison with their wild-type littermates, induction of thrombocytopenia by antibodies was more severe in hFcγRIIa transgenic mice,¹¹ suggesting an important proinflammatory 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 real-time PCR for the quantification of mRNA of FcγRII isoforms¹⁵ to test whether mRNA levels of FcγRII in neutrophils of healthy volunteers and various patient cohorts correlate with the cellular

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Abbreviations used

[Ca²⁺]_i: Intracellular Ca²⁺ ions
 CVID: Common variable immunodeficiency
 FcγR: Fcγ receptor
 FITC: Fluorescein isothiocyanate
 fMLP: N-formyl-methionyl-leucyl-phenylalanine
 IMIg: Intramuscular immunoglobulin
 ITP: Idiopathic thrombocytopenia
 IVIg: Intravenous immunoglobulin
 KD: Kawasaki disease
 SNP: Single nucleotide polymorphism

responsiveness to IgG and immune complexes. For this reason, we investigated the FcγRII mRNA levels in patients whose primary treatment is intravenous immunoglobulin (IVIg) as substitution or immunomodulation therapy: patients with 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 cause. This last disease is diagnosed according to a set of clinical criteria.¹⁶ In 20% to 25% patients with KD, coronary artery aneurysms develop, which renders KD the leading cause of acquired heart disease in subjects less than 5 years of age. 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 coronary artery aneurysms.¹⁷

We identified aberrant FcγRIIa in some of these patients. The index patient had 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 patients with CVID with similar clinical findings of anaphylaxis. Although patients with aberrant FcγRIIa were also recognized among patients with ITP and those with KD, clinical anaphylaxis did not occur in these patients on IVIg treatment.

Here we report a novel gain-of-function FcγRIIa splice variant containing a cryptic exon 6*, FcγRIIa^{exon6*}, which was expressed in granulocytes and monocytes and might be present in the healthy population as a low-frequency mutation associated with CVID and anaphylactoid reactions toward IVIg infusion.

METHODS**Patient description**

A 28-year-old white woman was given a diagnosis of CVID at the age of 26 years when, after a period of recurrent upper airway infections and chronic *Giardia lamblia*-related diarrhea, examination revealed widespread gastric and intestinal nodular lymphoid hyperplasia in combination with low serum immunoglobulin levels. There was no history of infectious or autoimmune diseases among her family members.

After the start of the first IVIg infusion, the patient complained of abdominal pain and nausea while having a generalized rash, a respiration rate of 36 breaths/min, and tachycardia (136 beats/min), with a decrease 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 clemastine fumarate (2 mg), corticosteroids (Di-Adreson-F, 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. Serum tryptase and complement activation

product concentrations during the anaphylactoid reaction were not increased in contrast to neutrophil-derived serum elastase concentrations, as monitored in serial blood samples during this episode (see Table E1 in this article's Online Repository at www.jacionline.org and data not shown). Reanalysis of preinfusion 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 (intramuscular immunoglobulin [IMiG] 16% solution) in gradually increasing dosages for 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 through formation of IgA-anti-IgA complexes.^{19,20}

Measurement of elastase, tryptase, and complement activation products

Detection of elastase, tryptase, and activation of C4, 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 given a diagnosis of hypogammaglobulinemia for DNA extraction. Patients were selected according to the international criteria for CVID.²⁵ In 33 patients the presence of antibodies against IgA had been assessed in serum taken before the initiation of IVIg infusions. In case of KD, blood was obtained from more than 200 pediatric patients, all treated with IVIg (2 g/kg, single dose) and oral aspirin within 10 days after the onset of fever. KD was defined by the presence of fever for 3 or more days, together with at least 4 of the 5 classical diagnostic criteria.^{16,17} Additional blood samples were available for DNA extraction from 93 patients less than 18 years of age with ITP who 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 means of self or parental ethnic identification. Healthy control subjects were unrelated adult white blood donors residing in the same geographic 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.

mAbs and reagents

The following mAbs against human FcγRs 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-phycoerythrin (anti-FcγRIII, IgG_{2a} isotype, clone CLB-FcR-gran/1, 5D2; Sanquin); CD32-fluorescein isothiocyanate (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 IgG_{2a}-phycoerythrin (clone 713) and IgG₁-FITC (clone 203); anti-hIgG₁ (clone MH161, IgG_{2b,κ}); anti-hIgG₂ (clone MH162, IgG_{1,κ}); anti-hIgG₃ (clone MH163, IgG_{1,κ}); anti-hIgG₄ (clone MH164, IgG_{1,κ}); and biotinylated rat anti-mouse κ-light chain (clone 226). Streptavidin-polyHRP (Sanquin) was used for the detection of hIgGs. Rabbit polyclonal anti-FcγRIIa 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 separated over a Percoll gradient (Amersham Biosciences, Uppsala, Sweden) into PBMCs as interphase and granulocytes with erythrocytes in the pellet. Neutrophils were purified by means of lysis of the erythrocytes with ice-cold isotonic NH₄Cl containing NaHCO₃, washed, and suspended in HEPES buffer (25 mmol/L HEPES, 123 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L

glucose, and 2.5% [vol/vol] human serum albumin at pH 7.4), essentially as described previously.¹⁵ The purity and viability of both cell fractions were greater than 95%, as determined by using flow cytometry 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'-ATCATTGTGGCTGTGGTCATTGC-3' and reverse primer, 5'-TCAGGTAGATGTTTTTATCATCG-3'; FcγRIIb2: forward primer 5'-GGAAAAAGCGCATTTGAGCCAATC-3' and reverse primer 5'-GGAAATACGAGATCTTCCCTCTCTG-3') were developed and applied to cDNA generated from mRNA. mRNA was isolated from 10⁷ purified neutrophils by using the 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 by using 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*, and 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 National Center for Biotechnology Information reference sequence NM_001136219.1.

SDS-PAGE and Western blot of FcγRIIa^{exon6*}

Neutrophils were suspended in HEPES buffer at a concentration of 5×10^6 cells/mL. Subsequently, diisopropyl fluorophosphate was added 1:1000 to a final concentration of 5 mmol/L, 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 mmol/L 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% (wt/vol) Bis-Tris gel. Thereafter, the gel was blotted on a polyvinylidene difluoride membrane. FcγRIIa was detected on blots by means of a polyclonal antibody against the cytoplasmic tail of FcγRIIa. Bands were visualized with enhanced chemiluminescence substrate.

Neutrophil activation by IgG dimers

Immunoglobulin I.M. (IMiG, lot no. 01B26H403A, Sanquin) contains 160 g of protein per liter, of which at least 90% is IgG. Dimeric IgG was isolated from IMiG by using gel filtration on Hiload 16/60 Superdex 200 (Amersham Biosciences) and subsequent pooling of the peaks that contained mainly dimeric IgG.

Dimeric IgG, 50 μg/mL (4.5 μg in 90 μL of Iscove modified Dulbecco medium; BioWhittaker Europe, Verviers, Belgium), was added to each well of a 96-well round-bottom plate, followed by addition of neutrophils (2×10^4 per well). The mixtures were incubated for 2 hours at 37°C in humidified air containing 5% (vol/vol) CO₂. Elastase released from the cells was measured in cell-free supernatants with a sandwich ELISA, as previously 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 Genepulser II (Bio-Rad Laboratories, Hercules, Calif) at 250 V and 975 μF.

Two days after transfection, the IIA1.6 transfectants were cultured in Iscove modified Dulbecco medium supplemented with 10% (vol/vol) FCS (Bodinco BV, Alkmaar, The Netherlands), 100 U/mL penicillin (Gibco, Paisley, United Kingdom), and 100 μg/mL streptomycin (Gibco) under selection of 0.8 mg/mL Geneticin (G-418-sulfate, Life Technologies, Gibco).

Measurement of intracellular calcium levels 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 μmol/L Fura-2/AM (Molecular Probes Europe, Leiden, The Netherlands) by means of 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 room temperature with 2 μg/mL anti-FcγRII (clone AT10; Serotec, Oxford, United Kingdom) 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; PerkinElmer, Fremont, Calif). The baseline concentration of free intracellular Ca²⁺ ions ([Ca²⁺]_i) was measured for 100 seconds, after which AT10 was cross-linked with 10 μg/mL goat anti-mouse IgG₁ (Southern Biotech, Birmingham, Ala). After levels had stabilized again, thapsigargin was added at 1 μmol/L. The increase in [Ca²⁺]_i levels was measured, and the data were analyzed with FLWinlab software (PerkinElmer).

Determination of isotype and subclass of anti-IgA antibodies

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

In the index patient 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 means of overnight incubation. The absorbed immunoglobulins were eluted with glycine-HCl pH 2.5/0.5 mol/L NaCl and subsequently dialyzed against 5000-fold excess of PBS. The obtained immunoglobulins and the original plasma pool were spotted onto nitrocellulose, followed by overnight incubation with PBS/4% (wt/vol) milk powder. The nitrocellulose was cut into 4 pieces, each containing spots of the obtained immunoglobulins described above, and these were incubated with mAbs to IgG subclasses. After extensive washing with PBS/0.01% (wt/vol) 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 (Amersham Biosciences).

Statistical analysis

Results are depicted as means ± SDs. When applicable, the Student *t* test and ANOVA were used. A 2-sided *P* value of less than .05 was considered to indicate a significant difference. The Fisher exact test (2-sided) was used to analyze the allele frequency between the cohorts, and a *P* value of less than .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 real-time PCR test for the quantification of FcγRIIa at

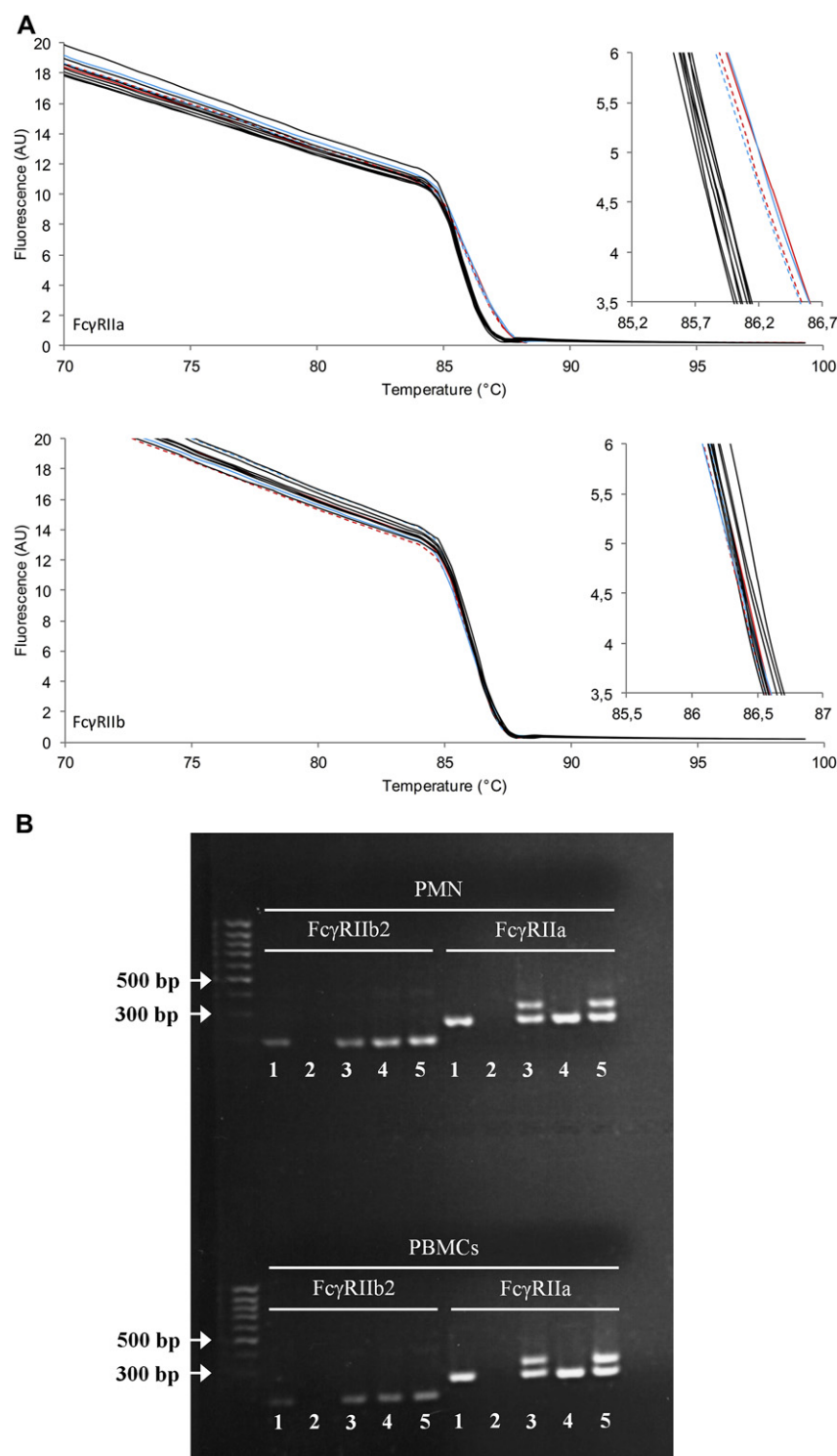


FIG 1. Melting curves and gel electrophoresis of FcγRII isoforms in the CVID family. **A, upper panels,** A shift to the right in the melting curves of the patient (*red lines*) and her brother (*blue lines*) is observed for the FcγRIIa PCR product but not for the FcγRIIb2 PCR product. Duplicates of the patient and her brother are indicated by *dashed lines*. Melting curves of all other tested subjects are indicated in *black*. **B,** Analysis of the PCR products on agarose gel. The *leftmost lanes* contain markers. *Lane 1,* A healthy nonrelated control subject; *lane 2,* a nontemplate control subject; *lane 3,* a patient with CVID; *lane 4,* mother; *lane 5,* and brother.

the mRNA level.¹⁵ Product specificity is routinely evaluated through the melting curve generated at the end of a run. On screening a patient cohort with IVIg treatment for RNA levels

of FcγRIIa, we found an exceptional case. The patient had CVID with anaphylactic shock on administration of the first dose of IVIg.

The melting curves of the FcγRIIa PCR products of the patient, as well as those of her brother, were different from those of healthy control subjects (Fig 1, A). The samples were applied to a 1.5% (wt/vol) agarose gel, which yielded 2 bands in the samples of the patient and her brother but not in the control subject (Fig 1, B). 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 using sequence analysis. The mRNA expression of this novel splice variant of FcγRIIa (FcγRIIa^{exon6*}) was observed in the granulocyte fraction, as well as in the mononuclear fraction (Fig 1, B). A single nucleotide polymorphism (SNP) present in the *FCGR2A* gene and absent in the *FCGR2B* or *FCGR2C* genes confirmed the exon 6* splicing defect in the gene encoding FcγRIIa (see Fig E1 in this article's Online Repository at www.jacionline.org).

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 2 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 2, A). An overview of the genomic organization of *FCGR2A* shows the position of exon 6* in the cytosolic part directly after the transmembrane domain of the receptor. Thus retention of exon 6* extends the cytoplasmic tail of FcγRIIa by 19 amino acids.

We subsequently tested the patient's parents and identified 1 additional family member carrying the same heterozygous *FCGR2A*^{c.742+871A>G} encoding the splice variant of FcγRIIa; that is, not only the patient with CVID but also her brother and father carried the mutation, although both were completely healthy (Fig 2, B).

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

Further analysis of protein expression was done with Western blotting. The molecular mass of the splice variant was estimated to be 42 kDa because native FcγRIIa in neutrophils migrates at a molecular mass of 40 kDa (see also Van der Heijden et al³⁰) and exon 6* encodes 19 amino acids corresponding to approximately 2 kDa. Indeed, we detected 2 bands in the neutrophil lysate of the patient and in that of her father, one being 40 kDa and the other being 42 kDa in mass. In control subjects without mRNA encoding FcγRIIa^{exon6*}, we observed only 1 band of 40 kDa (Fig 3). Expression levels of FcγRII (CD32) on the blood cells of all affected persons in this pedigree were within the normal range (see Fig E2 in this article's Online Repository at www.jacionline.org).

Prevalence and inheritance of the novel splice variant of FcγRIIa

On sequencing the DNA from the total cohort of patients with CVID (n = 53, including the index patient), we found 2 additional

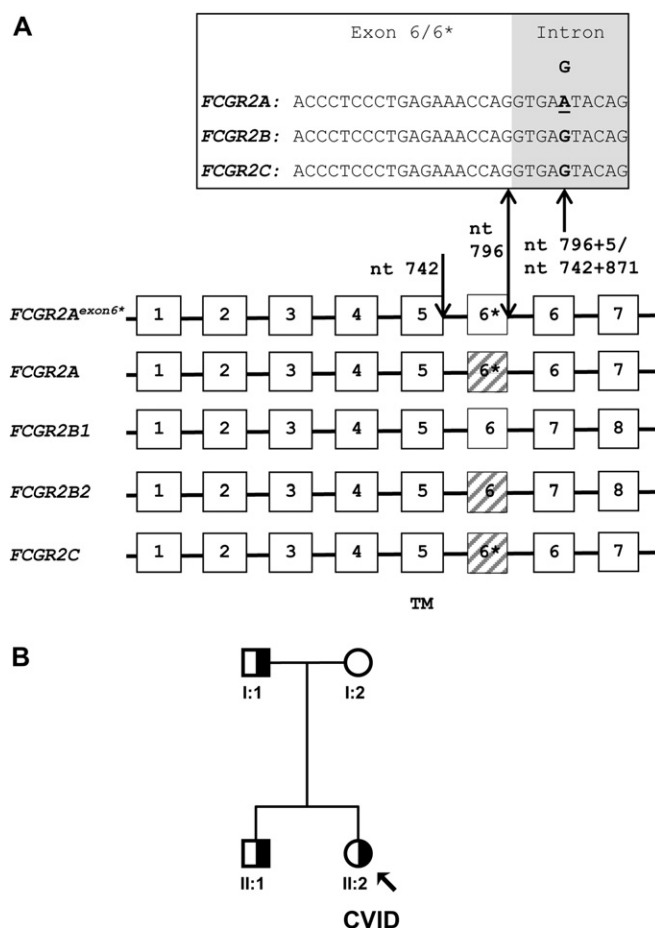


FIG 2. Site of point mutation in the genome. **A**, An overview of the genomic organization of *FCGR2*, in which blocks represent exons and lines indicate introns. TM, Transmembrane domain. Exon 6* is hatched where it is spliced out. Retention of exon 6* is linked to the +871 point mutation in intron 5, as shown by the arrow. **B**, Pedigree of the index patient with CVID, including her healthy father and brother carrying the same *FCGR2A*^{c.742+871A>G} allele.

cases with the same heterozygous *FCGR2A*^{c.742+871A>G} allele. Most patients had never experienced any serious side effects of IVIg in the past (n = 45), whereas a selected subset of these patients with CVID 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 of 33 of the patients tested before 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 did not have any clinical anaphylactoid reaction during their first IVIg infusions. Of the anti-IgA-positive patients with clinical anaphylaxis, one additional case carried an *FCGR2A*^{c.742+871A>G} allele, whereas the other *FCGR2A*^{c.742+871A>G} allele-positive patient with CVID with anaphylaxis at the first IVIg infusion had never been tested for anti-IgA antibodies (not shown).

On screening of 2 additional cohorts of IVIg-treated patients with either KD (n = 208) or 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 children with KD needed a second course of IVIg to become afebrile. The incidence of IVIg resistance varies in most centers between

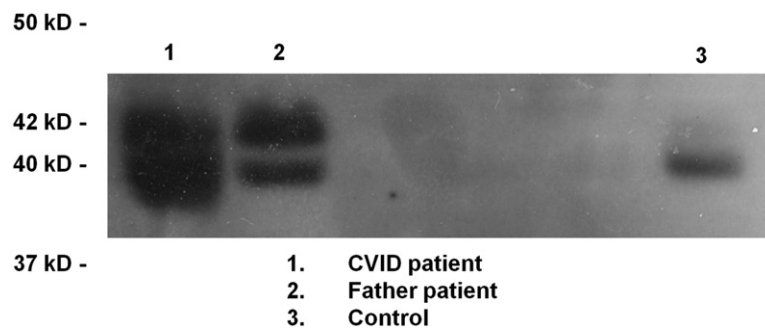


FIG 3. SDS-PAGE and Western blot of FcγRIIIa and FcγRIIIa^{exon6*}. Neutrophil lysates were obtained, as described in the Methods section, and run on a 10% (wt/vol) Bis-Tris gel. Thereafter, the proteins were blotted on a polyvinylidene difluoride membrane, which was probed with a polyclonal antibody against the cytoplasmic tail of FcγRIIIa. *Lane 1*, Patient with CVID; *lane 2*, father of the patient with CVID; *lane 3*, healthy control subject not expressing FcγRIIIa^{exon6*}.

TABLE I. Genotype and allele frequency of *FCGR2A* intron 5 +871 position in healthy subjects versus patients with KD, ITP, or CVID

<i>FCGR2A</i> ^{c.742+871A>G}	Control subjects, no. (%)	Patients with KD, no. (%)	Patients with ITP, no. (%)	Patients with CVID, no. (%)	Patients with CVID/anaphylaxis, no. (%)	Patients with CVID/no anaphylaxis, no. (%)
Genotype frequency	n = 287	n = 208	n = 93	n = 53	n = 8	n = 45
AA	286 (99.7)	203 (97.6)	92 (98.9)	50 (94.3)	5 (62.5)	45 (100)
AG	1 (3.5)	5 (2.4)	1 (1.1)	3 (5.7)	3 (37.5)	0 (0)
GG	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>P</i> value	—	.087*	.430*	.013*	.0001*	1.00
						.002†
Allele frequency						
A	573 (99.8)	411 (98.8)	185 (99.5)	103 (97.2)		
G	1 (0.2)	5 (1.2)	1 (0.5)	3 (2.8)		
<i>P</i> value	—	.088*	.430*	.013*		

DNA from 287 healthy subjects, 208 patients with KD, 93 patients with ITP, and 53 patients with CVID in total (including the index patient) was analyzed for the A>G mutation at +871 position in intron 5 of *FCGR2A*, as described in the Methods section. The AG and GG groups were combined in the Fisher exact test to determine differences between the cohorts. Significance was calculated by using Fisher exact 2-sided tests (*P* < .05) comparing patients versus control subjects (*) or within the CVID cohort comparing those with and without anaphylaxis (†).

10% and 20% of patients with KD, irrespective of ethnic background.³¹⁻³³ Also remarkable is the fact that among these 5 patients, some highly unusual autoimmune manifestations occurred during follow-up (ie, diabetes mellitus in 2 patients and ITP in 1 patient).

Two families with KD were tested for the presence of the rare *FCGR2A*^{c.742+871A>G} allele. In each of the families, apart from the patient, one of the parents was also found to be positive for the mutation (see Fig E3 in this article's Online Repository at www.jacionline.org) while remaining, once again, completely healthy. On subsequent screening of 287 healthy blood donors, 1 additional donor was identified with the *FCGR2A*^{c.742+871A>G} allele, demonstrating its presence in the healthy population. To date, homozygotes have not been identified.

Neutrophils expressing FcγRIIIa^{exon6*} have increased sensitivity to small immune complexes

Because genetic variations in FcγRs, such as SNPs, copy number variation, and splice variants, are known to influence the cellular response on receptor triggering,^{3,34,35} we tested whether this is also true for FcγRIIIa^{exon6*}. Neutrophils were isolated and stimulated with 50 μg/mL dimeric IgG, a dose previously

defined as optimal. Subjects expressing FcγRIIIa^{exon6*} showed strongly increased elastase release in response to stimulation compared with subjects expressing the wild-type FcγRIIIa (Fig 4), which was reminiscent of the findings made in the index patient after infusion of her first IVIg dose (see Table E1). Involvement of 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 subjects when FcγRII was blocked. Responsiveness to stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP) plus cytochalasin B was equal in all subjects tested (Fig 4), demonstrating that the effect was specific for FcγR stimulation and that there was no generalized difference in elastase secretion capacity.

To verify other responses, we tested the nicotinamide adenine dinucleotide phosphate oxidase activity in neutrophils from donors with either FcγRIIIa or FcγRIIIa^{exon6*} and studied the respiratory burst induced by aggregated IgG (with or without priming with platelet-activating factor, which itself does not induce nicotinamide adenine dinucleotide phosphate oxidase activity). However, no differences between neutrophils from donors with either FcγRIIIa or FcγRIIIa^{exon6*} were observed (n = 3, data not shown).

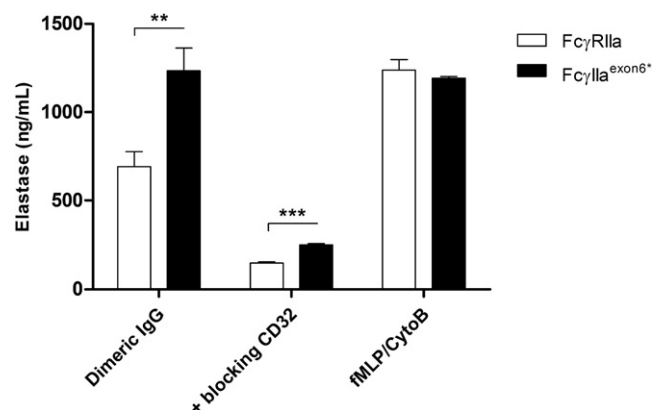


FIG 4. Neutrophils expressing FcγRIIa^{exon6*} respond more vigorously to dimeric IgG. Elastase release from neutrophils of subjects expressing FcγRIIa with (n = 4) or without (n = 14) exon 6*. Neutrophils were stimulated with dimeric IgG in the presence or absence of blocking Fab (IV.3, anti-FcγRII) fragments or with fMLP/cytochalasin B (CytoB). The difference in the response to dimeric IgG is statistically significant in the absence (P = .0077) and presence (P < .0001) of blocking Fab fragments in contrast to the response to fMLP/CytoB (P = .7, not significant). **P between .001 and .01; ***P < .001.

Increased [Ca²⁺]_i levels in IIA1.6 cells transfected with FcγRIIa^{exon6*}

All but one *FCGR2A*^{c.742+871A>G} allele-positive subjects showed FcγRIIa^{exon6*} transcripts with the R131 SNP in exon 3 (rs1801274), as assessed by means of allele-specific amplification, as well as by means of subsequent cloning strategies (data not shown). For reasons of comparison, we generated R131-positive FcγRIIa and FcγRIIa^{exon6*} constructs and expressed these in IIA1.6 B lymphoma cells for analysis of their functionality. Both FcγRIIa and FcγRIIa^{exon6*} IIA1.6 cells showed a similar level of CD32 surface staining after selection (see Fig E4 in this article's Online Repository at www.jacionline.org). By measuring [Ca²⁺]_i levels after receptor cross-linking, we found that IIA1.6 cells expressing the FcγRIIa^{exon6*} variant displayed consistently larger [Ca²⁺]_i mobilization after CD32 cross-linking than cells transfected with the wild-type FcγRIIa (Fig 5). In addition, thapsigargin resulted in similar [Ca²⁺]_i mobilization in both FcγRIIa and FcγRIIa^{exon6*} transfectants (Fig 5), indicating that both transfectants had similar maximal responses and equal labeling with the calcium-sensitive dye.

DISCUSSION

We identified a novel gain-of-function FcγRIIa isoform containing an insertion of exon 6*. This FcγRIIa^{exon6*} variant IgG receptor showed a normal cell distribution and surface expression levels similar to those of the wild-type FcγRIIa. The FcγRIIa^{exon6*} isoform was first identified in IVIg-treated patients and in their related parents, siblings, or both in the 3 separate families that consented to be tested. Subsequently, 1 completely unrelated healthy subject was identified on screening a cohort of control donors.

All affected subjects 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 means of Western blotting as an extra band at 42 kDa. At the genomic level, a heterozygous point mutation,

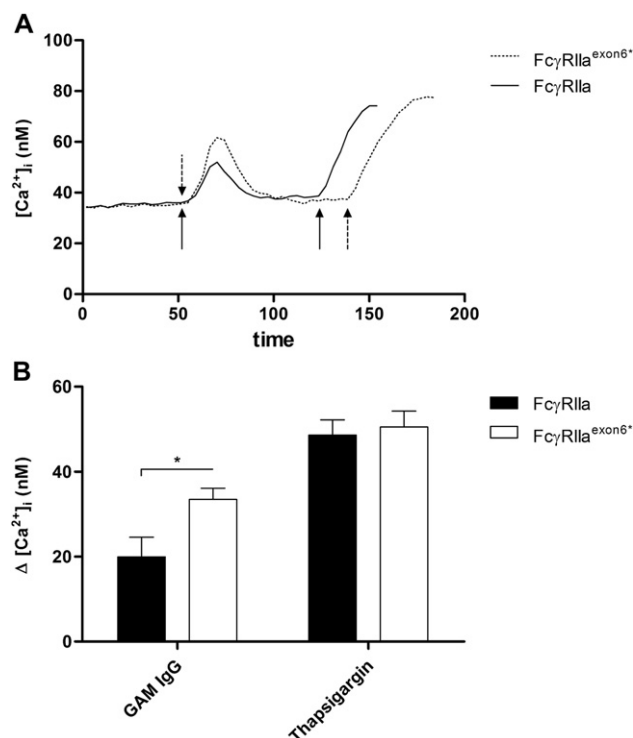


FIG 5. Increased [Ca²⁺]_i levels in IIA1.6 cells transfected with FcγRIIa^{exon6*} after receptor cross-linking. IIA1.6 cells were transfected with either FcγRIIa (solid line and arrows) or FcγRIIa^{exon6*} (dashed line and arrows), and these receptors were cross-linked. **A**, Representative [Ca²⁺]_i traces from 1 experiment. Arrows indicate addition of goat anti-mouse IgG or thapsigargin (after [Ca²⁺]_i 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 the case of FcγR cross-linking (P < .03, n = 6) but not in case of thapsigargin (P = .7 [not significant], n = 6). *P between .01 and .05.

located at the 871st nucleotide of intron 5, was observed that was neither found in relatives without FcγRIIa^{exon6*} nor in 287 unrelated healthy control subjects. At the mRNA level, these subjects did not express any other splice variant of FcγRIIa. Thus the A>G mutation in intron 5 of the *FCGR2A* gene (*FCGR2A*^{c.742+871A>G}) correlated with the expression of exon 6* in the FcγRIIa mRNA and corresponding FcγRIIa^{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* because the spliceosome might 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. Because FcγRIIb2 and FcγRIIc do not contain exon 6*, the alternative splicing of FcγRIIa^{exon6*} cannot 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,³⁶ predicts that the G at nucleotide 12 in *FCGR2A* results in a higher score for the putative serine/arginine-rich splicing factor 2 binding site when compared with 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 an serine/arginine-rich splicing factor 2 binding site results in the splice variant we have identified.

Because FcγRIIa^{exon6*} was identified in an IVIg-treated patient with clinically relevant hypogammaglobulinemia, we tested a

cohort of 53 patients with CVID, including 8 patients with CVID who had severe anaphylactoid reactions on IVIg infusion (including our index patient). Three of these patients had serious anaphylaxis during the first IVIg infusion and expressed FcγRIIa^{exon6*}, all carrying the same A>G mutation in intron 5, 2 with anti-IgA antibodies in the past, and 1 who had never been tested. Thus the FcγRIIa^{exon6*} splice variant seems to be more prevalent among patients with CVID 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 toward IVIg is not always explained by this aberrant splice product of FcγRIIa (Table I).

As assessed by using *in vitro* elastase release, neutrophils expressing FcγRIIa^{exon6*} showed increased reactivity to dimeric IgG compared with that seen in neutrophils expressing wild-type FcγRIIa. A blocking mAb against FcγRII essentially abolished IgG-induced elastase release, whereas stimulation by cytochalasin B plus fMLP resulted in equal elastase release by the neutrophils from all tested subjects, 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^{exon6*}: compared with wild-type FcγRIIa at equal levels of surface expression, cells expressing FcγRIIa^{exon6*} displayed a larger increase in [Ca²⁺]_i levels on CD32 receptor cross-linking.

Thus FcγRIIa^{exon6*}-expressing neutrophils are hyperresponsive to stimulation with dimeric IgG, which might explain the severe side effects of IVIg infusion in some of the patients with CVID. On the other hand, family members expressing FcγRIIa^{exon6*} appeared completely healthy. This lack of clinical effect can be explained by earlier observations from our group.³⁷ Endogenous IgG at physiologic 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 Patients with hypogammaglobulinemia have decreased endogenous IgG levels. Therefore the threshold for cell activation by immune complexes is substantially lowered.³⁷ The index patient with CVID had high levels of anti-IgA antibodies of the IgG₁ subclass, and the IVIg preparation used contains up to 1.5 mg/mL IgA. Therefore small immune complexes might have formed *in vivo* that bound to FcγRIIa^{exon6*} on neutrophils and hence caused the severe side effects observed in this patient on infusion with IVIg. This could also explain why the patients with KD did not have side effects on IVIg infusion. In addition, the disappearance of these anti-IgA IgG₁ molecules coincided with successful, well-tolerated intravenous infusions of IVIg after subcutaneous desensitization of the patient with CVID.

Anti-IgA has been the focus of articles on anaphylactic transfusion reactions since it was first described by Vyas et al³⁸ in 1968. 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 patients with CVID 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, the majority of whom tolerate repeated infusions of IVIg without adverse reactions.^{41,42}

On screening an additional cohort of IVIg-exposed patients (ie, 208 patients with KD and 93 patients with ITP of a white ethnic background), we identified 5 patients with KD with the FcγRIIa splice variant, 2 of whom had insulin-dependent diabetes mellitus (type 1) in less than 6 months after their acute KD episode. A third patient with KD had an episode of ITP during the following year of monitoring. In 2 of these patients with KD, further pedigree analysis revealed the presence of a healthy parent carrying the rare allele for FcγRIIa^{exon6*}. Within the ITP cohort, we found the mutation in 1 subject.

Thus the mutation represents a low-frequency mutation in the healthy white population that is associated with a “superactivating” FcγRIIa, posing a risk for inflammatory disease reactions. Homozygotes for the gain-of-function *FCGR2A*^{c.742+871A>G} allele have not been identified.

The role of FcγRIIa in anaphylaxis was recently suggested.¹² Why the FcγRIIa^{exon6*} variant results in an immune cell that is hyperresponsive to IgG might be explained by the extension of its cytoplasmic tail. We explored exon 6* of *FCGR2A* 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 might 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 might 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 normal mRNA and protein expression with a gain of function. The expression of this splice variant was found in 3 patients with CVID with serious anaphylactoid reactions, 2 of whom had positive test results for anti-IgA antibodies, whereas the third patient had never been tested. On the other hand, in 6 patients without any hypogammaglobulinemia but given a diagnosis of childhood vasculitis or ITP, clinical infusion of IVIg was unremarkable in these heterozygotes for the *FCGR2A*^{c.742+871A>G} allele. As supported based on results of pedigree analysis in 3 patients and the presence of a single unrelated subject on screening of healthy blood donors, this allele is also present at low frequency in the healthy population.

We thank Martin de Boer for helpful discussions.

Key messages

- A novel gain-of-function splice variant of FcγRIIa has been identified in a patient with an anaphylactoid reaction on IVIg transfusion.
- Cells expressing FcγRIIa^{exon6*} show increased responses toward IgG, which might contribute to inflammatory disease.

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	Intron	Exon 6/6*	Intron
		*	
FCGR2A :	TCCCTCCCAGCTCTC (T/C) CAGGAGACCCTG...	CTGAGAAACCAGGTGAATACAG	
Exon 6* :		CTCTC (T/C) CAGGAGACCCTG...	CTGAGAAACCAG
FCGR2B :	TCCCTCCCAGCTCTC (T/C) CAGGACACCCTG...	CTGAGAAACCAGGTGAGTACAG	
FCGR2C :	TCCCTCCCAGCTCTC (T/C) CAGGACACCCTG...	CTGAGAAACCAGGTGAGTACAG	

FIG E1. Alignment of *FCGR2A*, *FCGR2B*, *FCGR2C*, and exon 6* of *FCGR2A*. Exon 6/6* sequences of *FCGR2A* (wild-type), *FCGR2B*, and *FCGR2C* and of the exon 6*-containing transcript, as indicated by “exon 6*”, are depicted in the gray box. Sequences outside the gray box represent part of intron 5/6 of the 3 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*. SNPs are enclosed by parentheses.

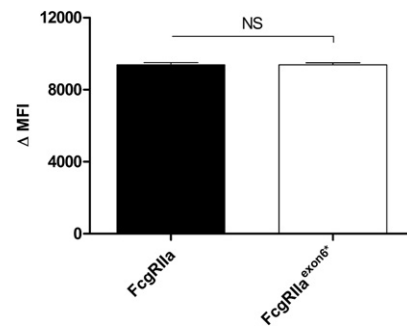


FIG E2. FcγRIIa and FcγRIIa^{exon6*} expression levels on neutrophils. Data are shown as the mean fluorescence intensity (MFI) of fluorescently labeled mAbs against FcγRII (AT10). No significant difference (NS) was found between neutrophils expressing FcγRIIa and FcγRIIa^{exon6*} ($P = .9859$). Values were corrected by subtracting the background values measured with isotype mAb controls. Data are expressed as means $\pm$ SEMs of 3 experiments.

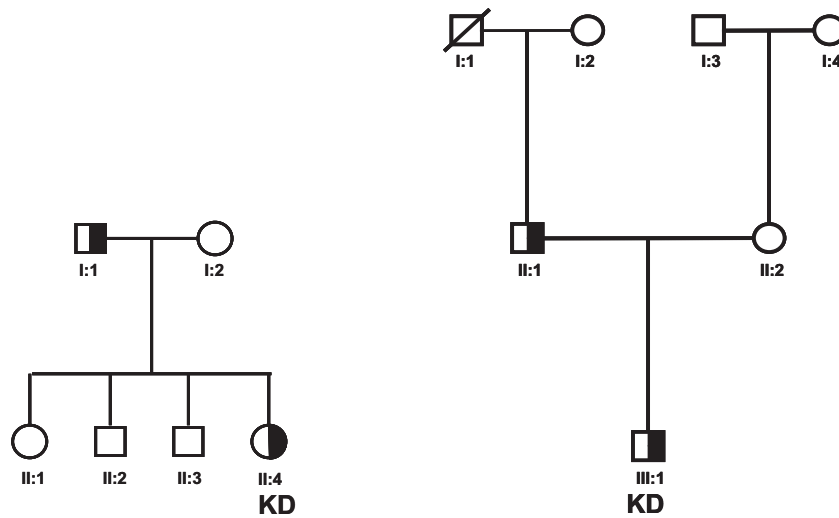


FIG E3. Pedigrees of 2 families with KD showing inheritance of FcγRIIa containing exon 6*. Pedigrees of 2 families with KD are indicated, showing autosomal inheritance of FcγRIIa containing exon 6*.

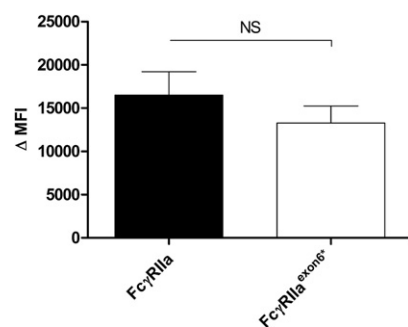


FIG E4. FcγRIIa and FcγRIIa^{exon6*} expression levels on transfected IIA1.6 cells. Data are shown as the mean fluorescence intensity (*MFI*) of fluorescently labeled mAbs against FcγRII (AT10). No significant difference (*NS*) was found between cells transfected with FcγRIIa and FcγRIIa^{exon6*} ($P = .39$). Values were corrected by subtracting the background values measured with isotype mAb controls. Data are expressed as means $\pm$ SEMs of 6 experiments.

TABLE E1. Dynamics of appearance of complement activation products, tryptase, and elastase in serum during anaphylaxis on the first IVIg infusion in the index patient with CVID

	Before infusion	During shock	1 h after shock
C3b/c (normal, <57 nmol/L)	18 nmol/L	21.2 nmol/L	28.7 nmol/L
C4b/c (normal, <8 nmol/L)	2.4 nmol/L	7.7 nmol/L	2.7 nmol/L
C1q-C4 (normal, <0.9 AU)	Undetectable	Undetectable	Undetectable
Tryptase (normal, <10 AU)	<10 AU	<10 AU	<10 AU
Elastase (normal, <100 ng/mL)	62 ng/mL	158 ng/mL	356 ng/mL