

Genetic CD21 deficiency is associated with hypogammaglobulinemia

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Background: Complement receptor 2 (CR2/CD21) is part of the B-cell coreceptor and expressed by mature B cells and follicular dendritic cells. CD21 is a receptor for C3d-opsonized immune complexes and enhances antigen-specific B-cell responses.

Objective: Genetic inactivation of the murine *CR2* locus results in impaired humoral immune responses. Here we report the first case of a genetic CD21 deficiency in human subjects.

Methods: CD21 protein expression was analyzed by means of flow cytometry and Western blotting. *CD21* transcripts were quantified by using real-time PCR. The *CD21* gene was sequenced. Wild-type and mutant *CD21* cDNA expression was studied after transfection of 293T cells. Binding of EBV-gp350 or C3d-containing immune complexes and induction of calcium flux in CD21-deficient B cells were analyzed by means of flow cytometry. Antibody responses to protein and polysaccharide vaccines were measured.

Results: A 28-year-old man presented with recurrent infections, reduced class-switched memory B cells, and hypogammaglobulinemia. CD21 receptor expression was undetectable. Binding of C3d-containing immune complexes and EBV-gp350 to B cells was severely reduced. Sequence analysis revealed a compound heterozygous deleterious

mutation in the *CD21* gene. Functional studies with anti-immunoglobulin- and C3d-containing immune complexes showed a complete loss of costimulatory activity of C3d in enhancing suboptimal B-cell receptor stimulation. Vaccination responses to protein antigens were normal, but the response to pneumococcal polysaccharide vaccination was moderately impaired.

Conclusions: Genetic CD21 deficiency adds to the molecular defects observed in human subjects with hypogammaglobulinemia. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: *CD21*, complement receptor, hypogammaglobulinemia, common variable immunodeficiency, B lymphocyte

A high degree of interdependency exists between innate and adaptive elements of the immune system in which complement factors and their receptors represent an important interface. In particular, the complement system is involved in inflammatory reactions and enhancement of B- and T-cell functions and has regulatory effects on autoreactive B cells.¹⁻³ CD21, the complement receptor (CR) type 2, is mainly expressed on mature B cells and follicular dendritic cells. The human CD21 protein consists of 15 to 16 short consensus repeats, a transmembrane domain, and a short intracytoplasmic tail. Different isoforms, including 1 transcript variant lacking an in-frame segment and resulting in a shorter protein, have been described.⁴ Interactions with the human ligands of CD21 are known to require the first 2 short consensus repeats.^{5,6} In addition to interacting with the complement component C3d, CD21 binds CD23⁷ and IFN- α .⁸ CD21 is also the receptor for the gp350/220 viral coat protein of EBV and serves as the main cellular entry receptor for EBV.⁵ CD21, together with CD19, CD81, and CD225 molecules, forms the B-cell coreceptor complex, which lowers the activation threshold of the B-cell antigen receptor.^{9,10} Genetic defects have been described both in the B-cell coreceptor complex and the complement cascade and their receptors. In particular, mutations in the human *CD19* and *CD81* genes, as well as *CD20* genes, have recently been identified as the underlying cause of a primary antibody deficiency¹¹⁻¹³ resulting in the clinical phenotype of common variable immunodeficiency (CVID), a heterogeneous group of antibody deficiency syndromes defined by hypogammaglobulinemia, poor responses to vaccination, and recurrent bacterial infections (www.esid.org). Defects within the complement cascade are known to cause immune

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Abbreviations used

α -kLC: Goat F(ab')₂ anti-human κ light chain antibodies
 BCR: B-cell receptor
 CR: Complement receptor
 CVID: Common variable immunodeficiency
 PnPS14: Pneumococcal polysaccharide type 14

dysfunction, leading to either immunodeficiency or autoimmune disease.¹⁴ Thus far, no genetic defects have been described in the human *CR1* and *CR2* genes, which are also referred to as *CD35* and *CD21*. The human *CD21* and *CD35* genes lie in close vicinity on chromosome 1q.¹⁵ In mice *CD21* and *CD35* are encoded within the *CR2* locus and generated from a single gene by means of differential splicing.¹⁶ Therefore only knockout mice deficient in both receptors are available, which might not reflect the phenotype of a human deficiency in only one of these receptors. In *CD21/CD35*-deficient mice a substantial decrease in antigen-specific IgM and IgG isotypes can be observed¹⁷; furthermore, long-term B-cell memory and effector responses are adversely affected.^{18–20} Here we report the first case of human *CD21* deficiency in a patient with hypogammaglobulinemia and a severe reduction in IgD⁺CD27⁺ memory B cells.

METHODS**Genetic analysis**

Informed written consent was obtained from each subject before participation under the internal ethics review board–approved clinical study protocol (ZERM, University Hospital Freiburg, and the ethic votum 78/2001 [#239/99]). The coding sequence of *CD21* (GenBank NM001006658.1), including flanking intronic and 5' and 3' untranslated regions, was sequenced.

CD21 protein expression

The expression of the *CD21* protein was tested by using Western blot analysis of protein lysates from EBV-immortalized B cells with a polyclonal anti-*CD21* antibody (sc-7025; Santa Cruz Biotechnology, Santa Cruz, Calif). ELISA for soluble *CD21* was performed as previously described.²¹

Real-time quantification of *CD21* gene transcripts

cDNA was reverse transcribed from total RNA isolated from EBV-immortalized B cells. Two primer pairs for real-time quantification were designed in an intron-spanning fashion. Sequences are available on request. Gene expression was quantified by using the Light Cycler 480 (Roche, Mannheim, Germany) and calculated from the real-time PCR efficiency in relation to the mean of the housekeeping gene *CDC-like kinase 2* (*CDK2*).²² Absolute *CD21* copy numbers were calculated by using serial dilutions of *CD21*-containing plasmids with known copy numbers.

Cloning pcDNA3.1 and mutagenesis

The wild-type *CD21* cDNA PCR fragment was cloned into the pcDNA 3.1 directional Topo expression vector (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. By using the Phusion Site-Directed Mutagenesis Kit (Finnzymes, Vantaa, Finland), the mutations were inserted into the *CD21* wild-type plasmid.

Serum immunoglobulin determination and lymphocyte phenotyping

Serum immunoglobulin levels and IgG subclasses were assayed by using routine laboratory tests. Lymphocyte subpopulations were measured by using

4-color flow cytometry (FACSCalibur; BD Biosciences, San Jose, Calif) with either a whole-blood method or density gradient–enriched mononuclear cells.²³

Binding of pneumococcal polysaccharide type 14/C3d-containing immune complexes to B cells

Binding of pneumococcal polysaccharide type 14 (PnPS14; 12.5 μ g/mL; LGC Promochem, Wesel, Germany) alone and of preformed PnPS14- and C3d-containing immune complexes (12.5 μ g/mL PnPS14 incubated in fresh human serum for 60 minutes at 37°C)²⁴ to bead-enriched (DynalBeads, Invitrogen) B cells was detected after an incubation for 30 minutes at 4°C by means of flow cytometry with rabbit anti-C3d (DAKO, Glostrup, Denmark) or rabbit anti-PnPS14 (Statens Serum Institute, Copenhagen, Denmark) antisera. In some experiments an inhibitory anti-*CD21* mAb (clone FE8, 1–10 μ g/mL; Upstate/Millipore) stayed present during the entire incubation with immune complex.

Synthetic immune complex formation containing anti- κ and C3d

Biotinylated goat F(ab')₂ anti-human κ light chain antibodies (α -kLC; SouthernBiotech, Birmingham, Ala) were incubated at suboptimal concentrations (0.05 μ g/mL) with 0.2 μ g/mL biotinylated C3d or mutated C3d D163A²⁵ and 1 μ g/mL R-phycoerythrin–labeled NeutrAvidin (Molecular Probes/Invitrogen) for 30 minutes at 4°C in the dark. Optimal concentrations for costimulatory experiments were determined by using titration of each reagent.

Measurement of intracellular Ca²⁺ after B-cell receptor–CD21 costimulation

PBMCs were incubated with Fluo4 AM (1 μ mol/L) and Pluronic (0.1%; Molecular Probes, Eugene, Ore) in Hank's balanced salt solution containing 1% BSA for 30 minutes at 37°C. Staining antibodies against *CD19* and the human λ light chain (BioLegend, San Diego, Calif) were added, and the cells were incubated for an additional 25 minutes at room temperature. The concentration of intracellular free Ca²⁺ was recorded as the fluorescence of Fluo-4 by using the FACSCalibur flow cytometer (BD Biosciences). Ionomycin (2 μ g/mL; Sigma-Aldrich, St Louis, Mo) served as a positive control. The data acquired were analyzed by gating on *CD19*⁺ λ ⁺ B cells and visualized as mean values with Gaussian smoothing of the curve by using the kinetics tool of the FlowJo software (TreeStar, Inc, Ashland, Ore).

Tetanus toxoid ELISpot

Antibody-secreting cells specific for tetanus toxoid were analyzed with ELISpot. Mononuclear cells were incubated in triplicate for 18 hours at 37°C in tetanus toxoid (Statens Serum Institute)–coated 96-well microplates (Nunc, Roskilde, Denmark) at cell concentrations ranging from 5×10^5 to 2×10^6 cells per well. After washing, the plates were incubated for 2 hours at 37°C with alkaline phosphatase–conjugated anti-IgG. Development of anti-tetanus toxoid spots occurred during an 18-hour incubation period with BCIP substrate (BluePhos microwell substrate kit; KPL, Gaithersburg, Md) in 0.7% agarose. Spots were counted, and mean values of triplicates were converted to IgG anti-tetanus toxoid–producing cells/10⁶ mononuclear cells.

Vaccination response to pneumococcal polysaccharides

The patient was vaccinated against pneumococcal polysaccharides (Pneumovax). Specific IgG antibodies were assayed by using a commercially available 23-valent ELISA (Binding Site, Birmingham, United Kingdom) and serotype-specific (serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F) ELISA assays performed according to World Health Organization recommendations, with reference serum 89-SF as standard.^{26,27}

***In vitro* B-cell functional analysis and protein vaccination responses**

The materials and methods used in *in vitro* B-cell functional analysis and protein vaccination responses are described in detail in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS

Clinical and immunologic presentation

The patient is a 28-year-old man with healthy parents with no known consanguinity and no family history for recurrent infections. In the immunologic workup of first-degree relatives (mother, father, and sister of the patient), normal serum immunoglobulin levels and lymphocyte subpopulations were found. The patient had recurrent infections of the upper respiratory tract in early childhood. After tonsillectomy was performed at the age of 6 years, he remained healthy for the following 20 years. He then presented with persistent myalgias, fevers, sore throat, chronic diarrhea with weight loss, and recurrent respiratory tract infections. Respiratory swabs showed large amounts of *Haemophilus influenzae* repeatedly, and during infectious episodes, the patient's white blood cell count was increased up to 12.8×10^3 leukocytes/ μ L. Infections resolved on antibiotic treatment, but because of their high frequency, the patient intermittently received immunoglobulin substitution therapy. The clinical examination revealed a splenomegaly. The immunologic workup showed hypogammaglobulinemia affecting mainly IgG (3.8 g/L; normal range, 7.0–16.0 g/L). IgG subclass analysis showed reduced IgG₁ values (3.3 g/L; normal range, 4.9–11.4 g/L) and absent IgG₄ (<0.01 g/L; normal range, 0.08–1.51 g/L), whereas IgG₂ and IgG₃ values were normal (1.8 g/L and 0.84 g/L, respectively). IgA values were slightly reduced (0.6 g/L; normal range, 0.7–4 g/L), and IgM values were within the lower normal range (0.7 g/L; normal range, 0.4–2.4 g/L). Isohemagglutinins were of low titers (1:4). Total complement activity, C3, C4, and C3d plasma levels were within the normal range. Autoantibodies (antinuclear antibodies, rheumatoid factor, antineutrophil cytoplasmic antibodies, and phospholipid antibodies) were not detectable. Antibodies against recall antigens, such as measles, mumps, and varicella zoster virus, were in the normal range. Antibodies against EBV antigens (p18, p23, p54, and p138, including p72/EBNA 1) were present, indicating a previous exposure to EBV. IgM and IgG antibodies against pneumococcal polysaccharides were detectable before vaccination. Differential blood counts and counts for T and B cells were within normal ranges. Because the patient presented with hypogammaglobulinemia, splenomegaly, and chronic diarrhea, an incipient CVID syndrome was suspected, and a flow cytometric analysis of the B-cell phenotype according to the Freiburg CVID classification²³ was performed.

The patient's B cells were completely devoid of CD21 surface protein (Fig 1, A). The absence of CD21 surface expression on the patient's B cells was confirmed with 4 different anti-CD21 mAbs directed against different epitopes distributed along the CD21 extracellular domain (Fig 1, B). No intracellular CD21 protein was detectable after permeabilization of cells (Fig 1, C) and sera of the patient showed complete absence of soluble CD21 (data not shown). In contrast, the expression levels of CD19, CD81, and CR1/CD35 were normal (Fig 1, D, and data not shown), and the patient's B cells represented a mature B-cell phenotype based on the expression profiles of IgD, IgM, CD10, CD20, CD22, CD23, CD24, CD38, and CD40 (see Fig E1 in this article's Online

Repository at www.jacionline.org and data not shown). Within the patient's family, regular but slightly decreased CD21 expression on B cells (Fig 1, A) was found. We analyzed lysates from B-cell lines of the patient and his relatives using Western blotting. Again, we were unable to show any CD21 protein expression in the patient, whereas in the other family members CD21 protein expression could be detected but seemed to be reduced compared with that of a healthy control subject (Fig 1, E).

Genetic and molecular characterization of the CD21 defect

Sequencing of the patient's genomic DNA revealed a heterozygous mutation disrupting the highly conserved "GT" splicing donor site of exon 6 (genomic DNA: NC_000001.9_v1:g.15779G>C, cDNA: NC_000001.9 (CR2_v1):c.1225+1G>C). This causes an in-frame skipping of the entire exon 6. The result is a shorter *CD21* mRNA (Fig 2, A and B) that putatively codes for a truncated CD21 protein that is predicted to lack the extracellular short consensus repeats 5 and 6.^{15,28} The semiquantitative RT-PCR suggested that the second allele is not expressed at the mRNA level because the patient only expressed the transcript variant lacking exon 6 (Fig 2, B). The second mutation was detected in exon 13 and introduced a premature stop codon at amino acid position 766 (cDNA: NM_001006658.1 (CR2):c.2297G>A, p.W766X; Fig 2, A). The patient's father and sister are carriers for the exon 6 splicing site mutation, whereas the stop codon mutation in exon 13 is carried by the patient's mother (Fig 2, A). Neither the exon 6 splice site mutation nor the exon 13 mutation was found in 100 healthy control subjects.

Based on the results of semiquantitative PCR, we speculated that the premature stop codon in exon 13 (p.W766X) results in nonsense-mediated mRNA decay of the patient's maternal *CD21* allele. This would explain why the patient only expressed the aberrant splice variant (Fig 2, B). We carried out an allele-specific quantification of *CD21* mRNA levels using 2 primer sets, of which one primer set amplifying the *CD21* allele containing exon 6 had its lower primer in exon 6, thus allowing the discrimination between mRNA copies transcribed from either allele. The exon 6–specific primer set did not prime the amplification of *CD21* in the patient (Fig 2, C), demonstrating the lack of *CD21* mRNA encoded by his maternal allele. The second primer set, which is specific for both alleles, yielded less *CD21* cDNA copies in the patient (Fig 2, C). In the mother PCR with both primer pairs confirmed a reduced copy number of *CD21* mRNA carrying the premature stop codon (p.W766X; Fig 2, C).

Expression of mutated *CD21* alleles in 293T cells

The mutations were introduced into the pcDNA3.1 expression vector carrying the wild-type *CD21* cDNA sequence by means of PCR-based mutagenesis. Transfection of 293T cells with the variant devoid of exon 6 did not lead to detectable CD21 expression on the cell surface (Fig 2, D, left panel). In addition, no CD21 expression was detectable after transduction of 293T cells with the vector containing the p.W766X mutation by using FACS and Western blot analysis (Fig 2, D, right panel, and data not shown). In summary, the absence of CD21 protein in the patient is explained by a recessive, compound heterozygous mutation in the *CD21* gene.

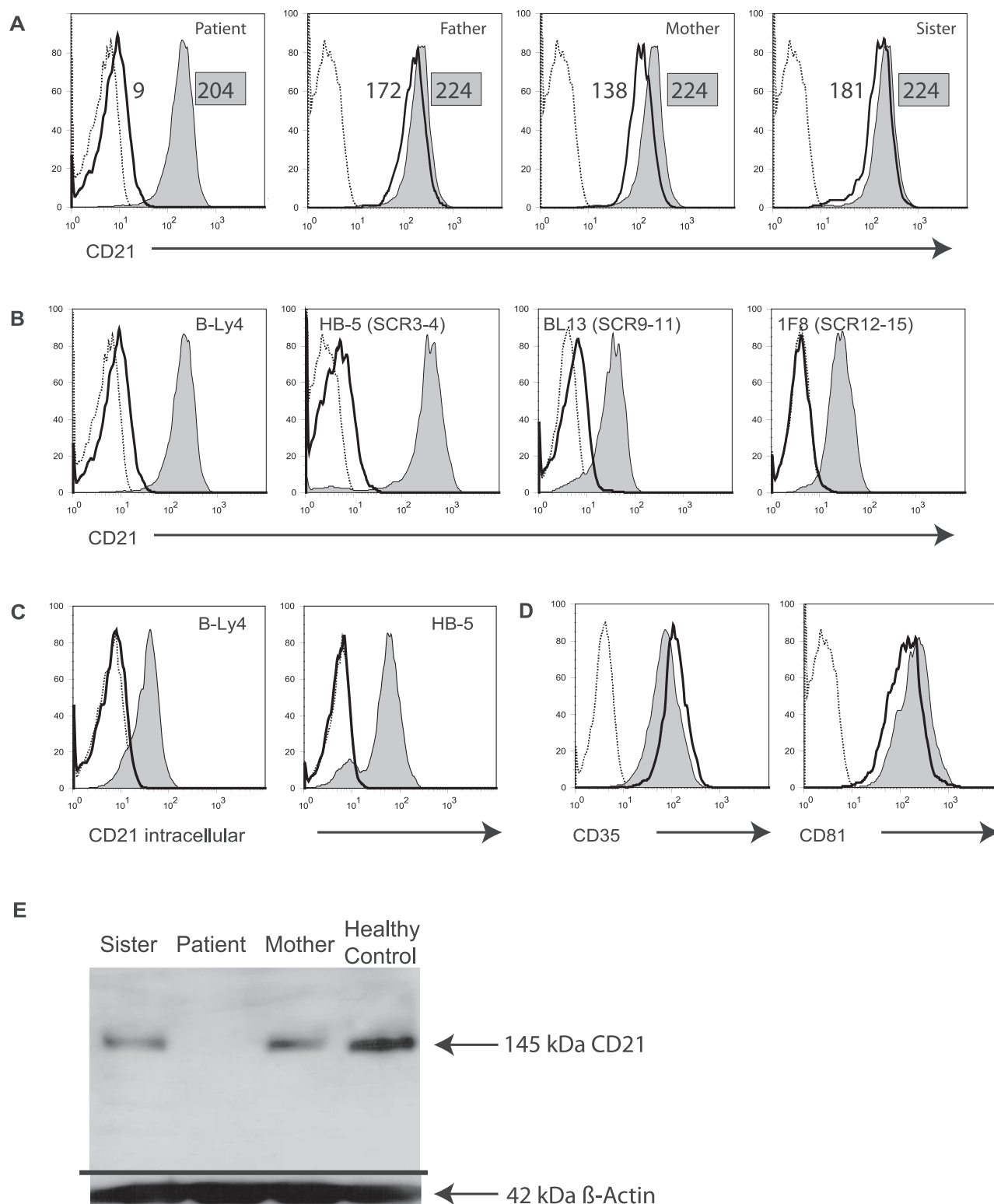


FIG 1. CD21 expression on B cells of the patient and his family members. Histograms (**A-D**) show isotype controls as dotted lines, a healthy donor as a gray-shaded area, and the patient or family members as solid lines. **Fig 1, A**, CD21 expression and mean fluorescence intensities of CD19⁺ B cells. **Fig 1, B-D**, Surface staining of the patient's B cells with mAbs recognizing different epitopes of CD21 (**Fig 1, B**), intracellular staining of CD21 (**Fig 1, C**), and staining for CD35 and CD81 (**Fig 1, D**). **E**, Western blot of CD21.

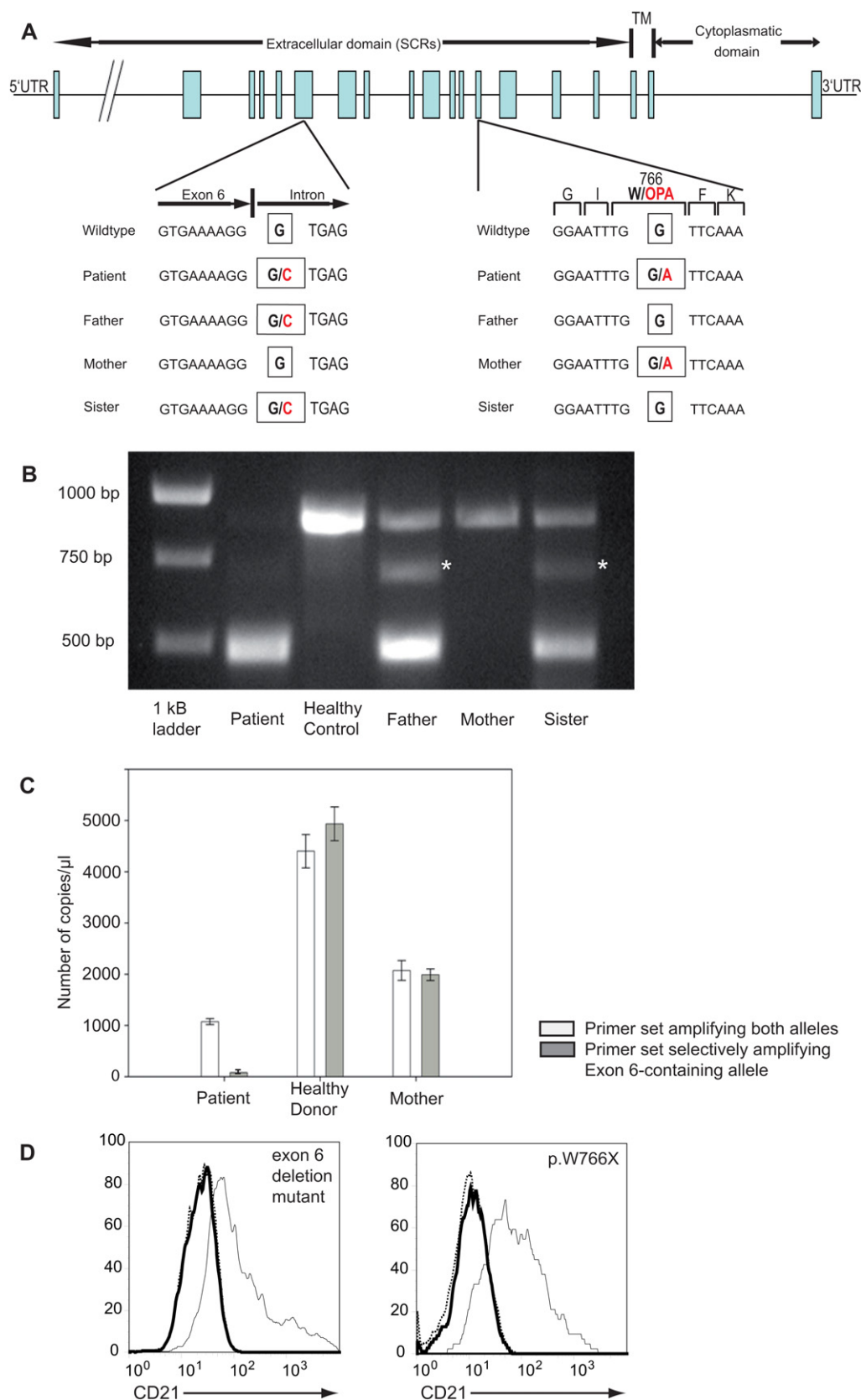


FIG 2. Mutational analysis and determination of *CD21* mRNA levels. **A**, Illustration of the *CD21* gene mutations. **B**, PCR amplification of *CD21* cDNA. Artificial PCR heteroduplexes are marked by asterisks. **C**, Allele-specific *CD21* mRNA quantification. **D**, Expression of *CD21* mutants in 293 T cells. Dotted line, Empty vector; fine solid line, wild-type *CD21*; and bold solid line, mutated *CD21* vectors. SCR, Short consensus repeat; UTR, untranslated region.

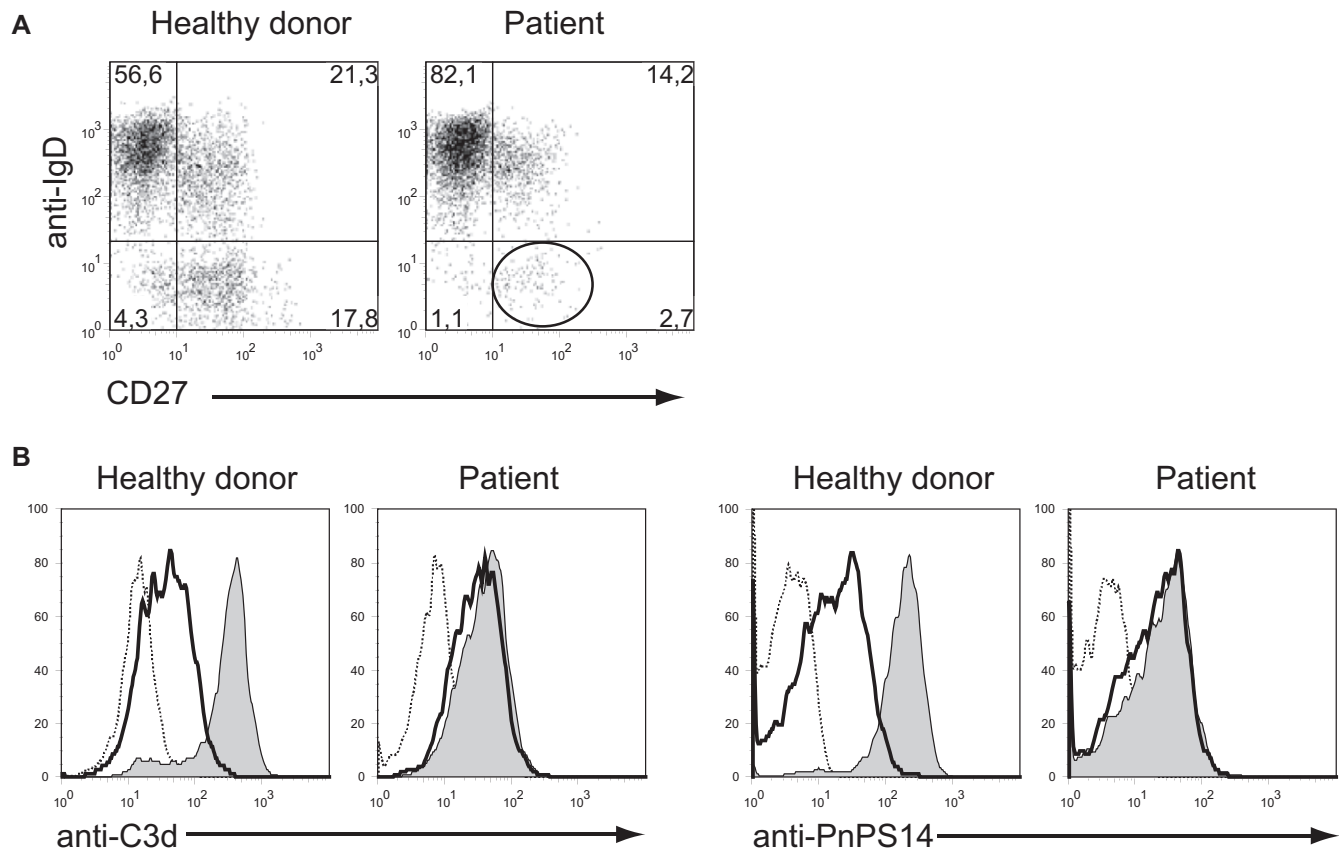


FIG 3. Reduced class-switched IgD⁺CD27⁺ memory B-cell numbers and deficient binding of CD21 ligands. **A**, CD19⁺ B cells of the control subject (*left*) and patient (*right*) were analyzed for IgD and CD27 expression. **B**, Deficient binding of C3d-containing immune complexes. PnPS14 binding alone is shown as *dotted lines*, and PnPS14 immune complex binding without (*shaded histograms*) or in the presence of (*solid lines*) the inhibitory CD21 mAb FE8 is shown. Staining with anti-C3d (*left*) or anti-PnPS14 (*right*) antisera is shown.

Functional relevance of human CD21 deficiency

In correlation to the reduced IgG serum levels, we found a strongly reduced percentage of class-switched memory B cells (2.7% of CD19⁺ B cells; normal range, 6.5% to 29.1%), which remained low throughout the observation period (Fig 3, A).

A hallmark of CD21 function is binding of C3d-containing immune complexes supporting B-cell stimulation through the B-cell receptor (BCR) and antigen trapping to B-cell follicles.^{10,29} To test immune complex binding to the patient's B cells, we constructed a C3d-containing immune complex using incubation of PnPS14 in fresh serum.²⁴ Binding of this complex to B cells was detected by antisera to C3d or PnPS14 (Fig 3, B). Applying these conditions, the patient's B cells exhibited only one tenth of the immune complex binding observed with normal B cells. Although the binding of immune complexes to B cells of a healthy donor could be strongly inhibited by the anti-CD21 mAb FE8, this had no effect on the residual immune complex binding of the patient's B cells (Fig 3, B). Similar results were obtained when we tested recombinant EBV-gp350 as another physiologic ligand of CD21 (see Fig E2 in this article's Online Repository at www.jacionline.org).

To assess the costimulatory activity of CD21 in the context of BCR triggering, we used synthetic immune complexes containing biotinylated goat F(ab')₂ anti-human κ light chain antibodies (α -kLC) and biotinylated C3d or a mutated inactive isoform

(C3d D163A).^{25,30} These reagents were cross-linked by using NeutrAvidin to elicit calcium flux through the BCR and its coreceptor, CD21. Using a suboptimal concentration of α -kLC alone (0.05 μ g/mL), we could trigger a calcium response neither in the patient nor in the control subject (Fig 4, upper panels, light grey lines).

However, when the same amount of α -kLC was combined with 0.2 μ g/mL C3d in a synthetic immune complex, a robust calcium influx could be detected in the control subject (Fig 4, upper right panel, black line) but not in the CD21-deficient patient (Fig 4, upper left panel, black line). Stimulation with the same amount of immune complexes containing a mutated C3d (D163A) did not significantly enhance the response by α -kLC stimulation alone in either the patient or the control subject (Fig 4, upper panels, dark grey lines). A titration from suboptimal to optimal α -kLC concentration alone showed almost identical dose-dependent responses in the patient's and control subject's cells (Fig 4, lower panels). Similarly, stimulation of the CD21-deficient B cells through the BCR by using anti-IgM antibodies resulted in equivalent calcium flux as observed in normal B cells (data not shown).

Thus calcium signaling triggered directly through the BCR alone is normal in the CD21-deficient patient, whereas the costimulatory activity of C3d-containing complexes through CD21 is completely abolished. We estimate that the BCR signal is amplified at least 100-fold when C3d is added in an immune complex (Fig 4, upper and lower right panels, and data not shown).

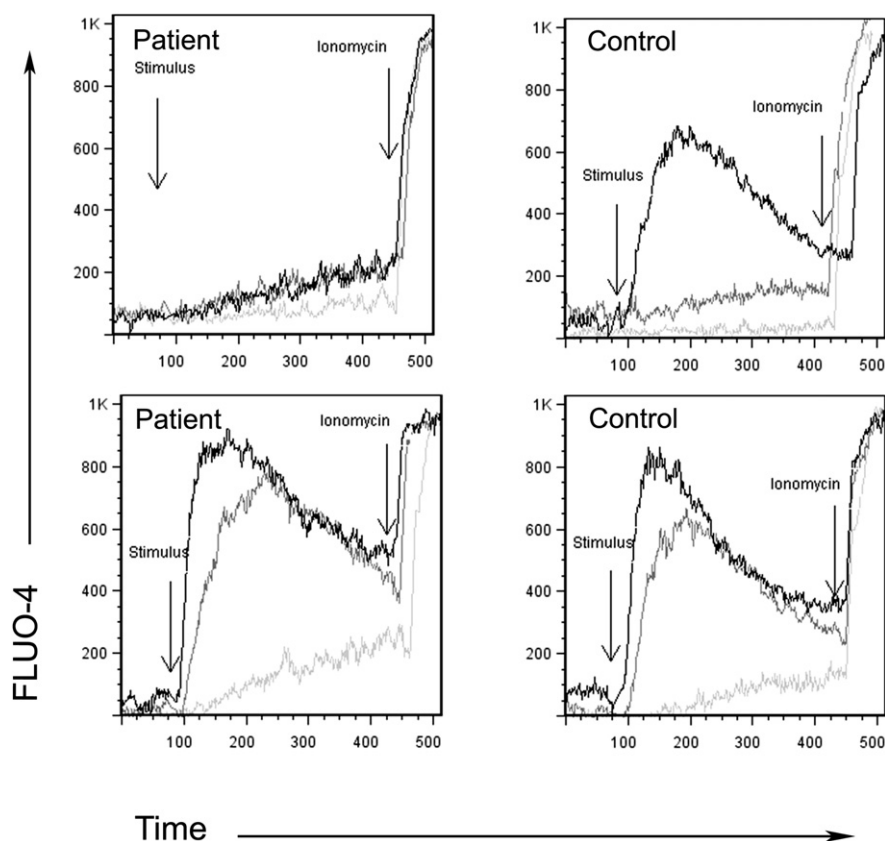


FIG 4. Abolished C3d-mediated costimulation of calcium flux. *Upper panels*, PBMCs were stimulated with α -kLC alone (light gray lines), α -kLC antibody and wild-type C3d (black lines) or C3d D163A (dark gray lines). *Lower panels*, Titration of α -kLC. PBMCs were stimulated with increasing concentrations of α -kLC (0.1 μ g/mL, light gray lines; 1 μ g/mL, dark gray lines; 5 μ g/mL, black lines).

The proliferative response and production of immunoglobulins *in vitro* on stimulation with anti-IgM and anti-CD40 was regular in the patient's B cells (see Fig E3 in this article's Online Repository at www.jacionline.org). The CD21-deficient B cells showed strong IgM but reduced IgG and IgA production, which reflects the reduction of circulating class-switched memory B cells. Finally, we also tested *in vitro* plasmablast formation and immunoglobulin class-switching after stimulation with soluble CD40 ligand and IL-21 by using sorted naive ($\text{IgM}^+\text{IgD}^+\text{CD27}^-$) B cells from the patient and a control subject.³¹ $\text{CD27}^{++}\text{CD38}^{++}$ plasmablast formation after 9 days, *in vitro* class-switching to IgG after 6 days, and IgM and IgG antibody production after 9 days (see Fig E4 in this article's Online Repository at www.jacionline.org) were all normal in the patient.

The patient was vaccinated with protein antigens (tetanus and diphtheria toxins and hepatitis A and B), protein-polysaccharide conjugate (*Haemophilus influenzae*), and pneumococcal polysaccharides to analyze vaccination responses *in vivo*. At day 7, anti-tetanus toxoid IgG-antibody secreting cells were detected by using ELISpot in the patient with a frequency between that of healthy donors and patients with CVID (Fig 5, A). Specific IgG antibody titers were determined up to 68 months after vaccinations (see Fig E5 in this article's Online Repository at www.jacionline.org). The patient mounted a normal response to all protein vaccines, and antibody titers were maintained during the whole observation period.

Regarding the pneumococcal polysaccharide vaccination response, we tested IgG antibody titers against 10 individual serotypes using ELISA (Fig 5, B) based on World Health Organization recommendation.²⁶ The patient only showed a greater than 4-fold increase for 3 serotypes and a seroconversion from titers of less than 1.3 μ g/mL for only 2 serotypes of anti-pneumococcal polysaccharide antibodies, indicating an impaired response according to published criteria.³² By using a commercial 23-valent assay, the patient had a less than 3-fold increase of IgG anti-pneumococcal polysaccharide antibodies (Fig 5, B). A lower reference value (10 μ g/mL)³³ is only available for unimmunized subjects, and normal vaccination responses are not yet established for this assay.

DISCUSSION

We report the first case of a genetic CD21 deficiency in human subjects caused by a compound heterozygous mutation in the *CD21* gene. Both mutations represent "null alleles" of *CD21* and preclude protein expression. In the heterozygous carriers we were able to detect weaker bands for CD21 protein in Western blots (Fig 1, E) and slightly lower CD21 expression on the cell surface by means of flow cytometry (Fig 1, A). However, this did not translate into manifest haploinsufficiency because the carriers have a completely normal immunologic and clinical phenotype. Together with the defects in CD19¹¹ and CD81,¹² CD21

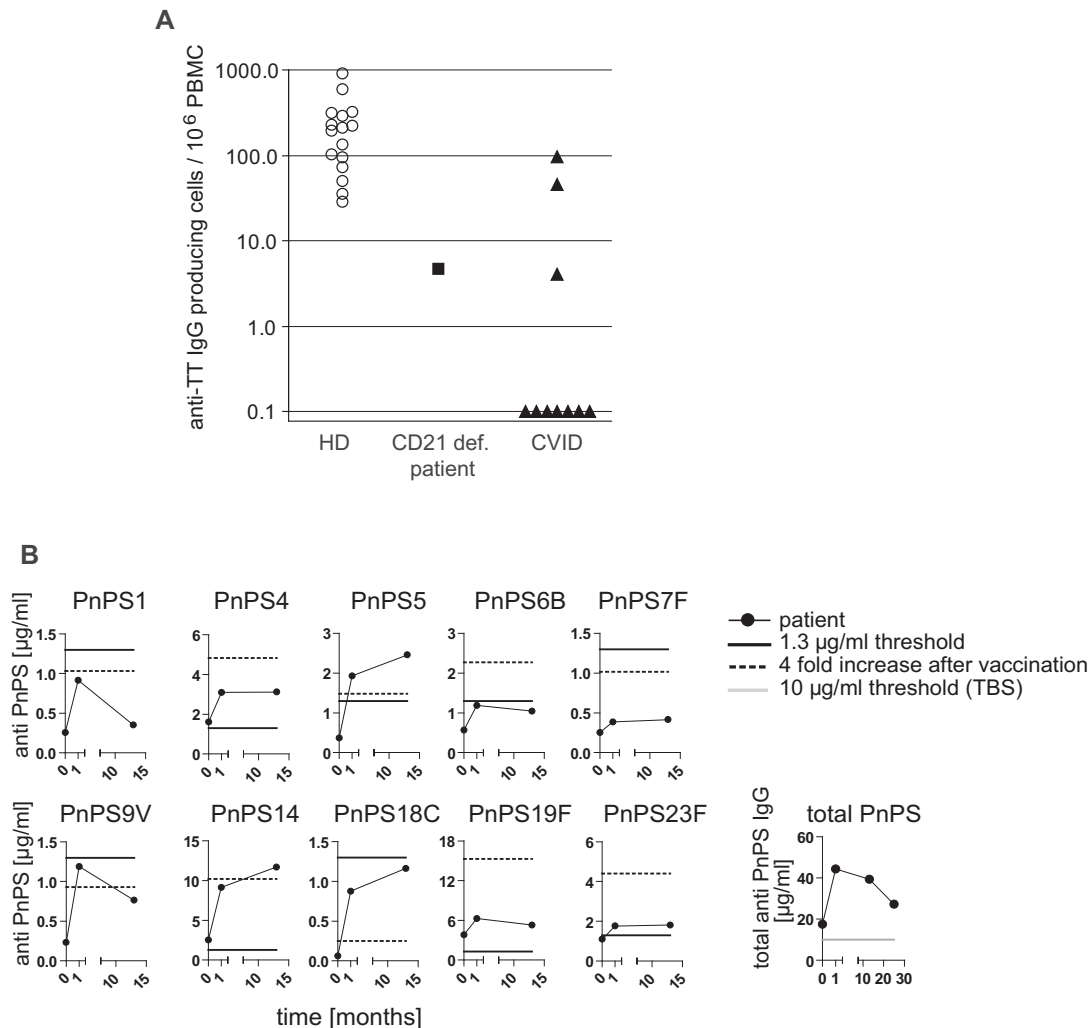


FIG 5. Vaccination responses of the patient. **A**, Tetanus toxoid-specific antibody ELISpot. Response of healthy donors (open circles; HD), patients with CVID (solid triangles), and the CD21-deficient patient (solid square) are shown. **B**, Pneumococcus-specific IgG antibody titers measured for 10 individual serotypes or 23-valent antigen by means of ELISA. Different threshold criteria are indicated by lines and explained in the results. TBS, Tris-buffered saline.

deficiency is the third genetic defect affecting the B-cell coreceptor complex in human subjects. All 3 defects, together with the recently described CD20 deficiency,¹³ phenotypically share the features of severely decreased memory B-cell numbers, hypogammaglobulinemia, and recurrent infections, with some variability in the clinical presentation. The clinical phenotype of CD21 deficiency was less severe than that reported for the CD19- and CD81-deficient patients. The CD21-deficient patient was able to mount specific antibody responses on vaccinations *in vivo*, although this was less efficient against polysaccharide antigens similarly to what is observed in patients with CD20 deficiency.¹³ However, there are additional differences that distinguish CD21 deficiency from the other 2 defects in the B-cell coreceptor. Deficiencies in CD19 and CD81 affect the expression of the other coreceptors, namely reduced expression of CD21 in patients with CD19 deficiency¹¹ and almost absent expression of CD19 in patients with CD81 deficiency.¹² This fact most probably accounts for the normal responses to BCR stimulation of the CD21-deficient B cells in contrast to CD19- or CD81-deficient B

cells because CD19 is part of the BCR complex. However, in the case of suboptimal BCR stimulation, the patient's B cells showed a strong deficiency in the response to anti- κ /C3d/NeutrAvidin complexes because of missing costimulation through CD21. The CD21-mediated costimulation is considered essential for B-cell responses to low antigen concentrations¹⁰ and also was found to be deficient in *Cr2*^{-/-} mice.³⁴

In *Cr2*^{-/-} mice, which are deficient for both CD21 and CD35, slightly reduced total serum IgG values but strongly affected primary and secondary specific IgG responses to T-dependent antigens and polysaccharide, as well as impaired persistence of specific IgG responses, have been described.^{17,18,20,35,36} Secondary specific antibody responses could be restored in *Cr2*^{-/-} mice on stable expression of a human *CR2* gene, indicating the importance of CD21 for specific antibody responses.³⁷

Although generally accepted criteria for positive or protective responses to pneumococcal polysaccharides in human subjects are still missing, we would interpret the patient's vaccination response as moderately impaired because of 3 criteria. First, 4

weeks after vaccination, the patient responded to only 2 of 10 serotypes with an at least 2-fold increase in antibody titers reaching at least 20% of a pooled serum from vaccinated subjects (World Health Organization reference serum 89-SF).^{38,39} Second, for only 3 of 10 tested serotypes, a more than 4-fold increase in anti-pneumococcal polysaccharide antibodies was monitored (Fig 5, B). Third, for only 2 of 7 serotypes, a seroconversion from titers of less than 1.3 µg/mL to values of greater than 1.3 µg/mL can be observed (Fig 5, B).³²

It should also be considered that with an age of 28 years, the patient came to clinical attention at an early stage of a possibly still developing, more severe, full-blown antibody deficiency disorder because clinical progression toward CVID from lesser antibody deficiencies (eg, selective IgA deficiency and IgG subclass deficiencies) has been described.⁴⁰ Furthermore the patient's clinical condition improved on intermittent immunoglobulin substitution therapy. On the other hand, we assume that null mutations in *CD21* are very rare in antibody-deficient patients because CD21 has been used as a B-cell surface marker to classify several hundred patients with CVID according to the Freiburg classification.^{23,41}

In summary, we present the first case of a genetic CD21 deficiency in human subjects and its extended immunologic and clinical phenotype. The human case of CD21 deficiency is also the first exclusive genetic deficiency for CD21 in mammals because the murine knockout removed both CD21 and CD35. Although the patient's B cells were unable to recognize the EBV-gp350 antigen, the patient underwent uneventful EBV seroconversion *in vivo*, and his B cells were easily immortalized by EBV *in vitro*. This supports views that the requirement for CD21 as an entry receptor for EBV is not absolute.⁴² Although CD21 deficiency is very likely a rare defect in patients with severe hypogammaglobulinemia, extending the testing of CD21 expression to patients with mild hypogammaglobulinemia or IgG subclass deficiency might uncover new cases of CD21 deficiency.

We thank the patient and his family for their participation in the study.

Key messages

- This is the first description of genetic human CD21 deficiency caused by a compound heterozygous deleterious mutation in the *CD21* gene.
- The patient's B cells do not bind and respond adequately to C3d-containing immune complexes.
- Human CD21 deficiency is clinically associated with recurrent infections of the respiratory tract, hypogammaglobulinemia, and reduced response to pneumococcal polysaccharide vaccination.

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METHODS

Binding of recombinant EBV-gp350 protein to B cells

Biotinylated gp350 (50 $\mu\text{g/mL}$)^{E1} and NeutrAvidin-phycoerythrin (40 $\mu\text{g/mL}$, Invitrogen) were mixed at equal volumes and incubated for 30 minutes at room temperature. Mononuclear cells were treated with CD19-allophycocyanin (BD Biosciences) either alone or together with the inhibitory anti-CD21 mAb FE8 (5 $\mu\text{g/mL}$) or anti-CD21 mAb HB5 (5 $\mu\text{g/mL}$, Santa Cruz Biotechnology) for 5 minutes at 4°C. Subsequently, the preformed gp350-biotin-NeutrAvidin-phycoerythrin complex was added at a final dilution of 1:5 and incubated for 30 minutes at 4°C. Cells were washed once and analyzed for gp350-biotin-NeutrAvidin-phycoerythrin binding by means of flow cytometry after gating on CD19⁺ B cells.

B-cell proliferation and immunoglobulin production

Activation-induced B-cell proliferation was measured by using tritiated thymidine incorporation (18-hour pulse) after stimulation of bead-enriched B cells with goat IgG-F(ab')₂ anti-IgM (10 $\mu\text{g/mL}$; MP Biochemicals, Santa Ana, Calif), anti-CD40 (clone 82111, 5 $\mu\text{g/mL}$; R&D Systems, Minneapolis, Minn), or both in the presence of IL-4 (20 ng/mL, BD Biosciences) for 3 days.

In vitro IgM, IgG, and IgA production of PBMCs was assayed by means of ELISA in culture supernatants after stimulation with *Staphylococcus aureus* Cowan I and IL-2 for 8 days.

In vitro plasmablast formation and class-switching

In vitro plasmablast formation was done as previously described.^{E2} Briefly, FACS-sorted naive B cells (IgM⁺CD27⁻CD19⁺) were seeded in 96-well

round-bottom plates at 30,000 cells/well and activated with IL-21 (ImmunoTools, Friesoythe, Germany) and a soluble trimeric form of CD40 ligand, as previously described. Stimulated cells were split 1:2 at day 3, and fresh medium containing CD40 ligand and IL-21 was added at days 3 and 6. At days 3, 6 and 9, culture supernatant was collected, and IgM and IgG concentrations were determined by means of sandwich ELISA with rabbit polyclonal anti-human IgM or IgG antibodies (SouthernBiotech) for coating and alkaline phosphatase-conjugated anti-human immunoglobulin (Jackson ImmunoResearch Laboratories, Bar Harbor, Me) as detection antibodies. Antibody concentrations were calculated from human IgM or IgG standards (Jackson ImmunoResearch Laboratories) processed in parallel. At days 3, 6, and 9, activated B cells were analyzed by means of FACS for the expression of cell-surface markers: CD19, CD27, CD38, IgG, and IgA.

Protein vaccination responses

The patient was vaccinated against *Haemophilus influenzae* (Act-HiB; Sanofi Pasteur, Leimen, Germany), hepatitis A and hepatitis B (Twinrix; GlaxoSmithKline, Berlin, Germany), and tetanus and diphtheria toxins (Td-Behring; CSL Behring, Marburg, Germany); booster vaccinations with Twinrix were performed 3.5 and 6 months after the primary immunization. Vaccination-induced specific serum IgG antibody titers were analyzed by using routine laboratory tests.

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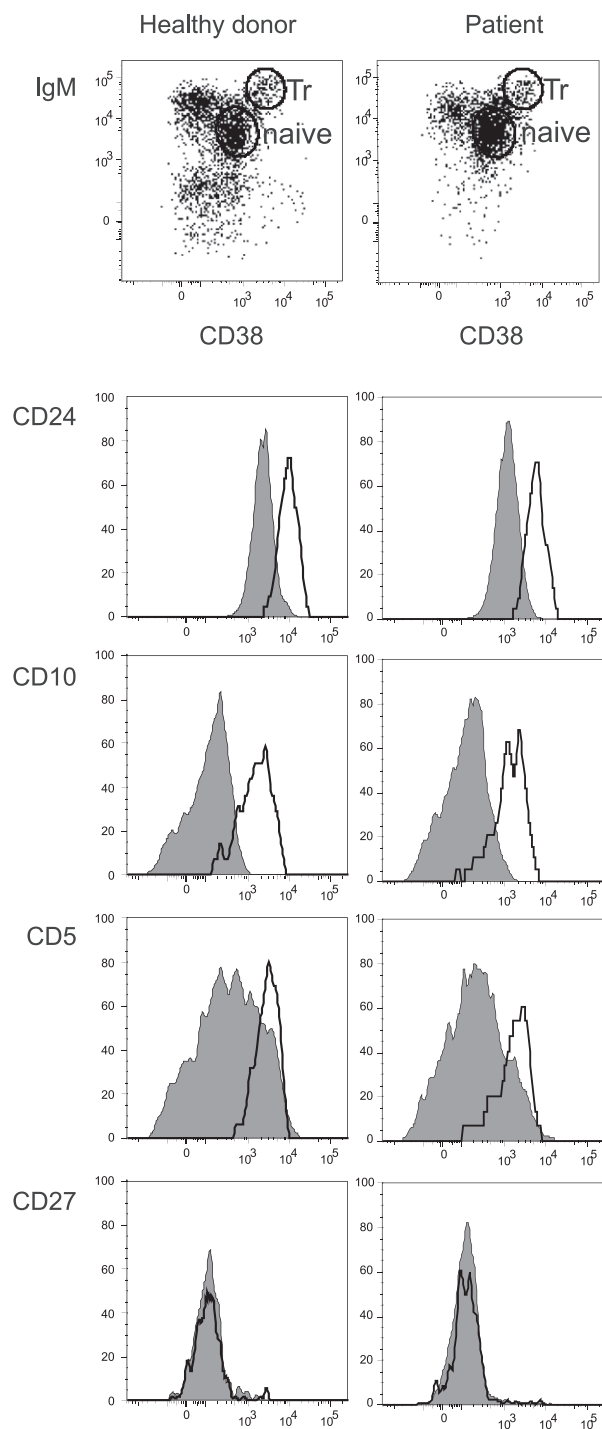


FIG E1. Phenotype of transitional and naive B cells. CD19⁺ B-cell subpopulations of a healthy donor (*left panels*) and the patient (*right panels*) were defined by means of anti-IgM and CD38 staining (*circles*). Histograms show the extended phenotype of transitional B cells (*Tr*, *bold line*) in comparison with naive B cells (*shaded area*).

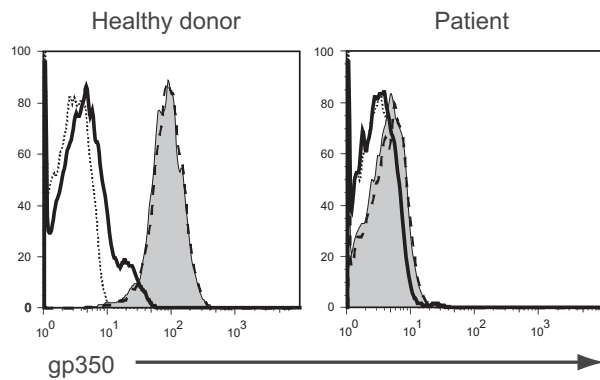


FIG E2. Deficient binding of EBV-gp350. B cells of a healthy donor and the patient were stained with EBV-gp350-biotin-NeutrAvidin-phycoerythrin in the absence (*shaded histograms*) or presence of the CD21 mAb FE8 (*solid line*) or the CD21 mAb HB5 (*dashed line*).

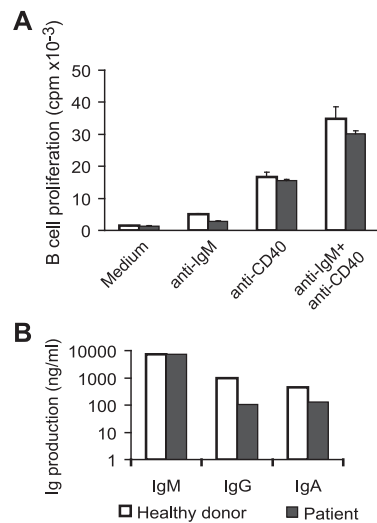
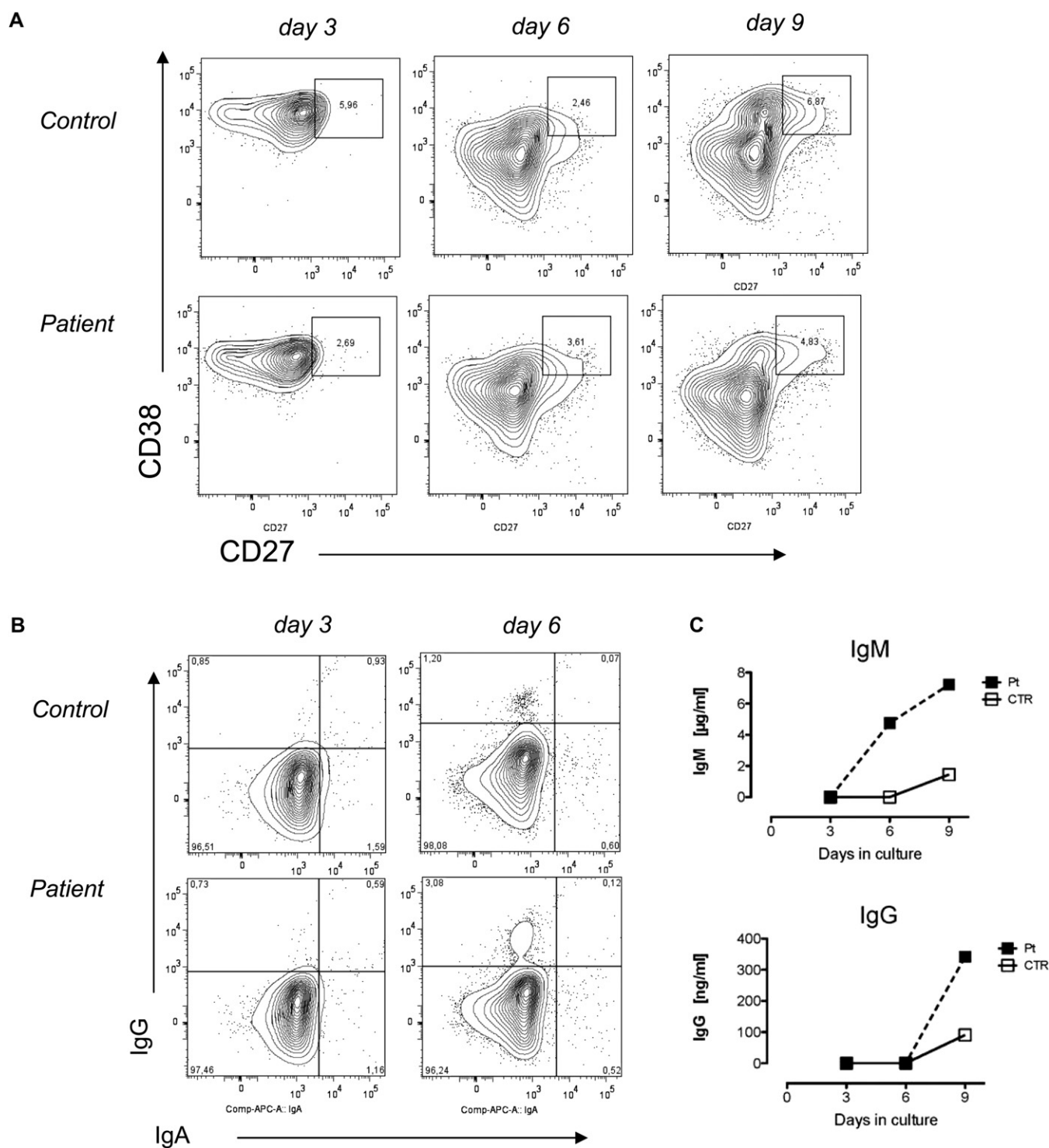


FIG E3. Proliferative response and immunoglobulin production. **A**, CD19⁺ B cells were cultured for 3 days in medium plus IL-4 only or in the presence of anti-human-IgM, anti-CD40 mAb, or anti-IgM and anti-CD40 mAb. Proliferative responses of triplicate cultures are depicted as mean counts per minute and SDs. **B**, *In vitro* IgM, IgG, and IgA production after *Staphylococcus aureus* Cowan I plus IL-2 stimulation of PBMCs from a healthy donor (white columns) and the patient (black columns).



IgG antibody vaccination response against

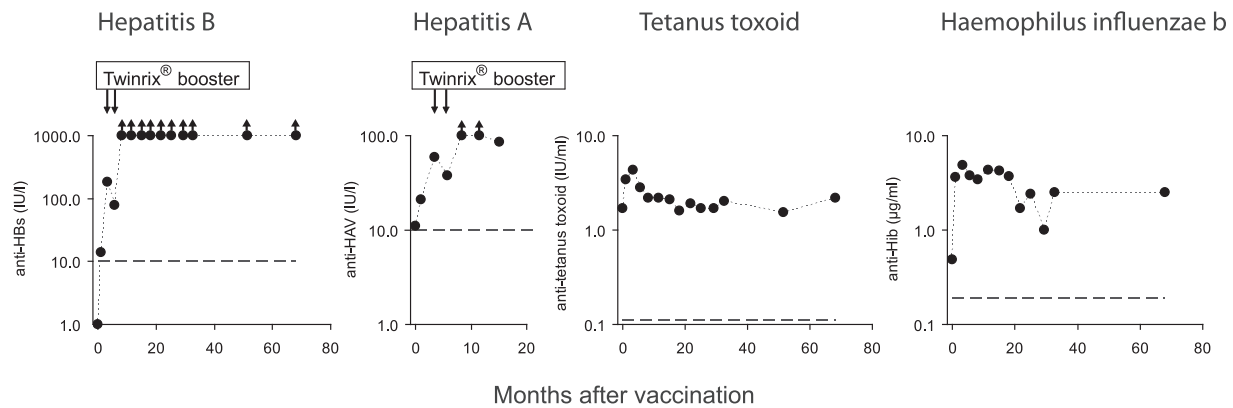


FIG E5. Regular vaccination response of the patient to protein and conjugate antigens. Specific IgG antibody titers were analyzed during 68 months after vaccinations by using routine laboratory tests. *Dashed lines* indicate the lower normal ranges, and *arrows above symbols* represent values of greater than the upper detection limits of the assay.