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A novel IKAROS haploinsufficiency kindred with unexpectedly late and variable B-cell maturation defects

Delfien J. Bogaert, MD, PhD^{a,b,c,d,e}, Hye Sun Kuehn, PhD^f, Carolien Bonroy, MPharm, PhD^{d,g}, Katherine R. Calvo, MD, PhD^h, Joke Dehoorne, MD, PhDⁱ, Arnaud V. Vanlander, MD, PhD^j, Marieke De Bruyne, MSc^{a,b,c,d}, Urszula Cytlak, PhD^{k,l}, Venetia Bigley, MD, PhD^{k,l}, Frans De Baets, MD, PhD^{b,d}, Elfride De Baere, MD, PhD^{c,d}, Sergio D. Rosenzweig, MD, PhD^f, Filomeen Haerynck, MD, PhD^{#a,b,d}, and Melissa Dullaers, PhD^{#a,d,e,m}

^aClinical Immunology Research Laboratory, Department of Pulmonary Medicine, Ghent University Hospital, Ghent, Belgium

^bDepartment of Pediatrics, Division of Pediatric Immunology and Pulmonology, Ghent University Hospital, Ghent, Belgium

^cCenter for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium

^dCenter for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, Belgium

^eLaboratory of Immunoregulation, VIB Inflammation Research Center, Ghent, Belgium

^fImmunology Service, Department of Laboratory Medicine, NIH Clinical Center, National Institutes of Health, Bethesda, Md

^gDepartment of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium

^hHematology Section, Department of Laboratory Medicine, NIH Clinical Center, National Institutes of Health, Bethesda, Md

ⁱDepartment of Pediatrics, Division of Pediatric Nephrology and Rheumatology, Ghent University Hospital, Ghent, Belgium

^jDepartment of Pediatrics, Division of Pediatric Neurology and Metabolism, Ghent University Hospital, Ghent, Belgium

^kInstitute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom

^lNorthern Centre for Bone Marrow Transplantation, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, United Kingdom

^mDepartment of Internal Medicine, Ghent University, Ghent, Belgium

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Melissa.Dullaers@ugent.be.

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These authors contributed equally to this work.

To the Editor

IKAROS, encoded by the IKAROS family zinc finger protein 1 (*IKZF1*) gene, is a hematopoietic zinc finger transcription factor essential in human hematopoiesis and B-cell development.¹ Somatic changes in *IKZF1* have been associated with B-cell leukemia.¹ Germline *IKZF1* mutations have been identified recently as a cause of common variable immunodeficiency and dysgammaglobulinemia through IKAROS haploinsufficiency.^{2,3} They are autosomal dominantly inherited or appear *de novo*.^{2–4} Eleven of 42 germline *IKZF1* mutation carriers were clinically asymptomatic at the time of publication, suggesting incomplete penetrance.^{2–4} However, since age of onset ranged from infancy to the sixth decade, younger asymptomatic subjects might still develop disease later in life.^{2,3} Symptomatic subjects mainly presented with bacterial infections, especially of the respiratory tract.^{2,3} In addition, some patients had antibody-mediated autoimmune manifestations or B-cell acute lymphoblastic leukemia.^{2,3} Affected subjects typically showed a (progressive) decrease of peripheral B-cell numbers accompanied by a (progressive) reduction in at least one major immunoglobulin isotype.^{2,3} Total T-cell numbers were not decreased, but patients often displayed quantitative changes in various T-cell subsets.^{2,3} Here we report an autosomal dominant kindred with a novel truncating mutation in *IKZF1* and describe new insights in B-cell maturation in 2 symptomatic and 1 asymptomatic family members.

The parents (subjects I:1 and I:2) were healthy and non-consanguineous and of Dutch-Italian origin (Fig 1, A). Subject II:3, currently 17 years old, was given a diagnosis of monocyclic systemic-onset juvenile idiopathic arthritis at the age of 6 years and reactive arthritis at the age of 9 years. Test results for autoantibodies were negative. Her younger sister (subject II:4, currently 12.5 years old) had multiple episodes of reactive arthritis between the ages of 3 and 5 years. In addition, both patients had recurrent bacterial sinopulmonary infections. Patient II:4 was given a recent diagnosis of juvenile myasthenia gravis confirmed by positive acetylcholine receptor autoantibody levels.

The immunologic workup of patients II:3 and II:4 is provided in Table E1 and Figure E1 in this article's Online Repository at www.jacionline.org. In summary, patient II:3, who was first examined at the age of 9 years, had absent serum IgA and IgM and severe B lymphopenia (Fig 2, A). Patient II:4, who was first evaluated at 4.5 years of age, had absent IgA but normal IgM levels. Her B-cell counts were initially in the lower normal range but decreased progressively over time (Fig 2, A). Both patients had poor polysaccharide vaccination responses. Total IgG and IgG subclass levels have thus far remained normal. There were no important abnormalities in peripheral T-cell subsets. The youngest sibling (subject II:5) is currently 8 years old and in good health.

Whole-exome sequencing revealed a heterozygous frameshift mutation in *IKZF1* (NM_006060: c.136delA, p.S46Afs*14) in the 2 affected siblings (patients II:3 and II:4) and asymptomatic mother (subject I:2; Fig 1, A). The mutation is not reported in public or in-house databases. The frameshift introduces a premature stop codon before the first zinc

finger motif in all *IKZF1* transcripts (Fig 1. *B*). Although nonsense-mediated mRNA decay was predicted *in silico*, mutant transcripts were detected at cDNA level (see Fig E2 in this article's Online Repository at www.jacionline.org), and patients had normal levels of *IKZF1* cDNA (Fig 1, *C*), suggesting mutant transcripts escape nonsense-mediated mRNA decay. Nonetheless, IKAROS protein expression levels were, on average, 40% lower in all 3 mutant subjects compared with the wild-type family members and healthy control subjects (Fig 1, *D*). The latter suggests that the mutant transcript encodes an unstable truncated protein that is rapidly degraded.⁵ Alternatively, the truncated protein might not be recognized by the antibody; however, epitope details are lacking. Either way, our findings indicate a molecular diagnosis of IKAROS haploin-sufficiency in patients II:3 and II:4 and their asymptomatic mother (subject I:2). Because the mutation was also present in the clinically asymptomatic 43-year-old subject I:2, we performed indepth immunologic testing, which revealed mildly reduced serum IgM levels, as well as marginally inadequate antibody responses to a polysaccharide pneumococcal vaccine (see Table E1).

Because germline IKAROS haploinsufficiency is a newly identified primary immunodeficiency and data on asymptomatic mutation carriers are scarce, we evaluated the peripheral and central B-cell compartments both in the affected siblings and their asymptomatic mother. In patient II:3 the few circulating B cells consisted for 50% of transitional B cells (Fig 2, *B*). Total naive B-cell levels, encompassing transitional B cells, were also increased at the expense of switched memory and marginal zone B cells (Fig 2, *B*). In patient II:4 normal numbers of peripheral B-cell subsets revealed an arrest at a later stage in development. Particularly, transitional B-cell levels were normal, whereas total naive B-cell levels were relatively increased and memory-type B-cell percentages were decreased (Fig 2, *B*). This points to a block after the naive mature B-cell stage. Analogous to previously reported asymptomatic *IKZF1* mutation carriers,^{2,3} the asymptomatic mother (subject I:2) exhibited peripheral B-cell frequencies and subsets within normal range for age, although memory-type B cells were at the lower end and naive B cells were at the upper end. This remained stable over a period of about 2 years (Fig 2, *B*).

Bone marrow aspirates from patient II:3 and the asymptomatic mother (subject I:2) revealed normal frequencies of hematopoietic stem cells. Multipotent progenitors were reduced, whereas common lymphoid progenitors were moderately expanded (Fig 2, *C*, and see Fig E3 in this article's Online Repository at www.jacionline.org), and total B-lineage cell frequencies were profoundly decreased in both patients (Fig 2, *C* and *D*, upper panel). In addition, symptomatic patient II:3 exhibited a partial block from pro- to pre-B cells (Fig 2, *D*, middle panel) and an almost complete block from immature/transitional to mature B cells (Fig 2, *D*, lower panel). In the asymptomatic mother (subject I:2), on the other hand, the reduced B-lineage cells demonstrated a normal maturation profile and were sufficient to sustain normal peripheral B-cell numbers. Patient II:4 was unavailable for bone marrow analysis.

The multilevel central B-cell developmental block observed in this *IKZF1* family appears later than observed in previously published IKAROS-haploinsufficient cases.^{2,3} First, early hematopoiesis in subjects I:2 and II:3 showed a partial arrest at the multipotent progenitor and B-lineage stages, whereas 2 patients reported by Hoshino et al³ exhibited blockages at

the earlier hematopoietic stem cell and common lymphoid progenitor stages in addition to the B-lineage block. Second, within B-lineage cells, symptomatic patient II:3 exhibited arrests at the pre-B and mature B-cell stages, whereas the Hoshino et al patients showed normal composition of the B-lineage, and 2 patients described by Kuehn et al² had a block at the earlier pro-B cell stage. Whether these differences are linked to the genotype of our patients remains to be established because all 4 previously reported patients harbored missense mutations in the second zinc finger motif of *IKZF1*, whereas our patients have a loss of IKAROS protein caused by a frameshift mutation.

To our knowledge, there have been no previous reports on central B-cell development in asymptomatic *IKZF1* mutation carriers. In subject I:2 the absence of clinical involvement was reflected in a quasinormal peripheral B-cell development. However, considering the important reduction in central B-lineage cells and the recently documented specific antibody deficiency, the B-cell compartment is clearly affected, and the subject might still become symptomatic at a later age.² Interestingly, a Kaplan-Meier curve for onset of symptoms generated based on 45 reported symptomatic and asymptomatic cases suggests that all germline *IKZF1* mutation carriers might eventually go on to develop symptoms (see Fig E4 in this article's Online Repository at www.jacionline.org). Although Kaplan-Meier estimates at the utmost ends of a time interval need to be interpreted with caution, from a clinical perspective, this warrants longitudinal follow-up of asymptomatic subjects. Peripheral B-cell numbers seem to be a suitable marker for monitoring disease progression, whereas serum IgG levels have been shown to remain normal for years after development of B-cell lymphopenia.²

IKAROS haploinsufficiency shares important genetic aspects with other recently identified primary immunodeficiencies of haploinsufficiency. Notable are nuclear factor *κB1*, cytotoxic T lymphocyte-associated antigen 4, and GATA-2 haploinsufficiencies, disorders that also affect the B-cell compartment and are characterized by incomplete penetrance, as well as variable expressivity and a highly variable age of onset in manifesting subjects.^{6,7} The molecular basis of incomplete penetrance and variable expressivity is poorly understood.⁶ Murine models have implicated an important role for IKAROS at nearly every step of B-cell development and function.^{8,9} Besides the more obvious defects in hematopoietic progenitor cell development, impaired IKAROS function during recombination of immunoglobulin gene segments and peripheral B-cell responses might provide a link with the antibody-mediated autoimmune diseases seen in patient II:4 and previously reported cases and with the specific polysaccharide antibody deficiency detected in the asymptomatic mother (subject I:2), respectively.^{2,3} Furthermore, the largely different B-cell profile in the reported mutant subjects despite similarly low IKAROS protein levels might suggest the presence of compensatory mechanisms at multiple levels capable of (partially) surmounting the IKAROS defect.^{8,9} The actions of modifier genes, epigenetic changes, and/or environmental exposures might affect these compensatory mechanisms in different ways, which could, in part, explain the phenotypic heterogeneity in IKAROS-haploinsufficient patients.^{8,9} Disease-influencing mechanisms in *IKZF1*-associated immunodeficiency await further study.

In conclusion, we report on the first truncating *IKZF1* mutation associated with IKAROS haploinsufficiency and illustrate an unexpectedly late and variable block in central and peripheral B-cell development in 2 patients and their asymptomatic mother. Given the observed immunologic abnormalities, we recommend close follow-up of asymptomatic *IKZF1* mutation carriers.

Methods

Clinical information

The first 2 pregnancies in the index family were complicated by premature labor of unknown cause at 25 (subject II:1) and 15 (subject II:2) weeks of gestational age, respectively. Neither child was viable. The 3 remaining sisters (subjects II:3, II:4, and II:5) were born around 35 weeks of gestational age and had an uneventful postnatal period.

Ethics

All reported subjects provided written informed consent for participation in the study in accordance with the 1975 Helsinki Declaration. The research protocol was approved by the ethical committee of Ghent University Hospital (2012/593).

Whole-exome sequencing

Whole-exome sequencing was performed in the 2 affected siblings (patients II:3 and II:4) and both parents (subjects I:1 and I:2). Genomic DNA was isolated from whole blood leukocytes by using the Puregene DNA Isolation Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Whole-exome enrichment was performed with the SureSelectXT Human All Exon V6 kit (Agilent Technologies, Santa Clara, Calif). Paired-end massively parallel sequencing (150 cycles) was performed on a NextSeq 500 (Illumina, San Diego, Calif). Read mapping against the human genome reference sequence (National Center for Biotechnology Information, GRCh37) and postmapping duplicate read removal, quality-based variant calling, and coverage analysis were performed with CLC Genomics Workbench v6.0.4 (Qiagen). More than 98% of enriched regions had a read depth of at least 20. Called variants with a coverage of 3 or greater were annotated with Alamut Batch (Interactive Biosoftware, Rouen, France). Only variants with population frequencies of less than 10% were considered, according to the public databases National Center for Biotechnology Information's dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>); National Heart, Lung, and Blood Institute's Exome Sequencing Project–Exome Variant Server (<http://evs.gs.washington.edu/EVS/>); Exome Aggregation Consortium Browser (<http://exac.broadinstitute.org/>); and 1000 Genomes Project Browser (<http://browser.1000genomes.org/>). Variants were prioritized further based on allele frequency, functional prediction scores, nucleotide conservation scores, and biological relevance. Both Mendelian and non-Mendelian inheritance patterns were taken into account. Afterward, variants of interest were evaluated by using Alamut Visual mutation interpretation software (v2.7, rev. 1; Interactive Biosoftware), Ingenuity Variant Analysis (Qiagen, 2015 Release Spring), CADD scores (v1.3; <http://cadd.gs.washington.edu/home>), the Genome Aggregation Database Browser (<http://gnomad.broadinstitute.org>), literature search,

segregation analysis in available family members, and frequency in an in-house database containing variants of more than 1000 exomes at the time of analysis.

Sanger sequencing of genomic DNA

The DNA template (GRCh37/hg19) of *IKZF1* (NM_006060) was obtained from the UCSC Genome Browser (<https://genome.ucsc.edu>). Primers for amplification and sequencing of exon 3 and adjacent intron-exon boundaries were designed with Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>)E2: forward primer 5' - CTCATGCCACCCTCTCAAG-3' and reverse primer 5' - GAGTGTCCATCCTCCCACAC-3'. Genomic DNA was amplified by mean of PCR using the specific primers and KAPA2G Robust Hotstart Ready Mix (KAPA Biosystems, Wilmington, Mass). PCR products were enzymatically purified with Exonuclease I and Antarctic phosphatase (both from New England BioLabs, Ipswich, Mass). Purified PCR products were sequenced with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, Calif) on a 3730xl DNA Analyzer (Applied Biosystems). Sequence reads were analyzed with SeqScape v2.5 (Thermo Fisher Scientific, Waltham, Mass).

Sanger sequencing of cDNA

Total RNA was isolated from PBMCs of all available family members (subjects I:1, I:2, II:3, II:4, and II:5) and 2 healthy control subjects by using the RNeasy Plus Mini Kit (Qiagen) and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif), according to the manufacturer's instructions. The cDNA template (GRCh37/hg19) of *IKZF1* (NM_006060) was obtained from the Ensembl Genome Browser (<http://www.ensembl.org/index.html>). Primers for amplification and sequencing of exon 3 and adjacent coding regions were designed with Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>)E2: forward primer 5' - ATGGATGCTGATGAGGGTCAAG-3' and reverse primer 5' - CGGAATGCAGCTTGATGTGCAGGAGC-3'. PCR amplification was performed by using GoTaq Hot Start Colorless Master Mix (Promega, Madison, Wis). Purified PCR products were Sanger sequenced with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on a 3130xL Genetic Analyzer (Applied Biosystems). Sequence reads were analyzed with SeqMan (DNASTAR, Madison, Wis).

IKZF1 gene expression

Total RNA was isolated from PBMCs of 2 mutant family members (II:3 and II:4) and 3 healthy control subjects by using the RNeasy Plus Mini Kit (Qiagen) and converted to cDNA by using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Two amplicons of *IKZF1* were investigated: amplicon 1 located upstream of the mutation at genomic position Chr7:50358652-50367269 and amplicon 2 located downstream of the mutation at genomic position Chr7:50469628-50469734 (GRCh37/hg19). These amplicons were chosen to target as many *IKZF1* transcripts as possible. Primers were designed by using PrimerXL and IDT PrimerQuest (available on request). *GPI* and *PSMB2* were used as reference genes. Gene expression was analyzed by using real-time quantitative PCR with LightCycler 480 SYBR Green I Master reagent and a LightCycler 96 Instrument (both from Roche), according to the

manufacturer's instructions. All reactions were performed in triplicate. The relative quantification of gene expression was calculated with the comparative cycle threshold method.

Flow cytometric analysis of peripheral blood B- and T-cell subsets

Peripheral blood B- and T-cell subsets were evaluated in the *IKZF1* mutation carriers (subjects I:2, II:3 and II:4) and age-matched healthy control subjects, as previously described.^{E3} In brief, cryopreserved PBMCs were stained with Fixable Viability Dye 506 (eBioscience, San Diego, Calif) and fluorescently labeled mAbs under saturation conditions. The following antibodies (clones) were used: CD8 (RPA-T8), CD16 (3G8), CD19 (HIB19), CD20 (2H7), CD21 (B-LY4), CD27 (M-T271), CXCR5 (RF8B2), IgD (IA6-2), and $\gamma\delta$ T-cell receptor (11F2; all from BD Biosciences, San Jose, Calif); CD3 (SK7), CD4 (SK3), CD24 (ML5), CD25 (BC96), CD45RO (UCHL1), and CCR7 (G043H7; all from BioLegend, San Diego, Calif); CD4 (RPA-T4), CD38 (HIT2), CD56 (TULY56), and forkhead box protein 3 (Foxp3; PCH101; all from eBioscience). Cells were acquired on an LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (version X; TreeStar, Ashland, Ore).

IKAROS intracellular staining

Cryopreserved PBMCs were first stained with fluorescently labeled anti-CD3 (clone UCHT1) and anti-CD19 (clone SJ25C1; both from BD Biosciences). Afterward, cells were fixed and permeabilized with the FoxP3 staining kit (eBioscience), according to the manufacturer's instructions, and incubated with fluorescently labeled anti-IKAROS (clone R32-1149; BD Biosciences).^{E4} Cells were acquired on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (version X; TreeStar).

Flow cytometric analysis of bone marrow aspirates

Bone marrow aspirates from the hipbone were obtained from subjects I:2 and II:3 with informed consent and ethical approval of the Newcastle and North Tyneside Research Ethics Committee. Human bone marrow control samples were obtained from a femoral head after total hip replacement with informed consent and ethical approval of Newcastle and North Tyneside Research Ethics Committee. The femoral head was excavated, the cavity and fragments were washed with PBS, and it was then passed through a 50- μ m filter to obtain cells from the hip arthroplasty specimen. Mononuclear cells were prepared from the resulting cell suspension by using density centrifugation and stained for flow cytometry according to standard protocols.

Analysis of hematopoietic stem and lineage progenitor cells was based on the method of Doulatov et al.^{E5} The following antibodies (clones) were used: CD3 (SK7-Leu9), CD19 (HIB19), CD20 (L27), CD56 (NCAM16.2), CD38 (HB7), CD45RA (HI100), CD90 (G20-127), and CD10 (HI10A; all from BD Biosciences) and CD34 (581; BioLegend). Cells were acquired on an LSR Fortessa X-20 flow cytometer (BD Biosciences), with dead cell exclusion by using 4'-6-diamidino-2-phenylindole dihydrochloride (Sysmex, Lincolnshire, Ill).

For analysis of B-lineage cells, cryopreserved bone marrow samples were stained, as previously described.^{E6} The following antibodies (clones) were used: CD5 (L17F12), CD10 (HI10a), CD19 (SJ25C1), CD20 (2H7), CD45 (2D1), CD34 (8G12); all from BD Biosciences). Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences).

All data were analyzed with FlowJo software (version X; TreeStar).

Statistical analysis

IKZF1 gene expression and IKAROS protein expression levels were compared by using the Mann-Whitney *U* test with GraphPad Prism software (v7; GraphPad Software, La Jolla, Calif). A 2-sided *P* value of .05 or less was considered statistically significant.

Extended Data

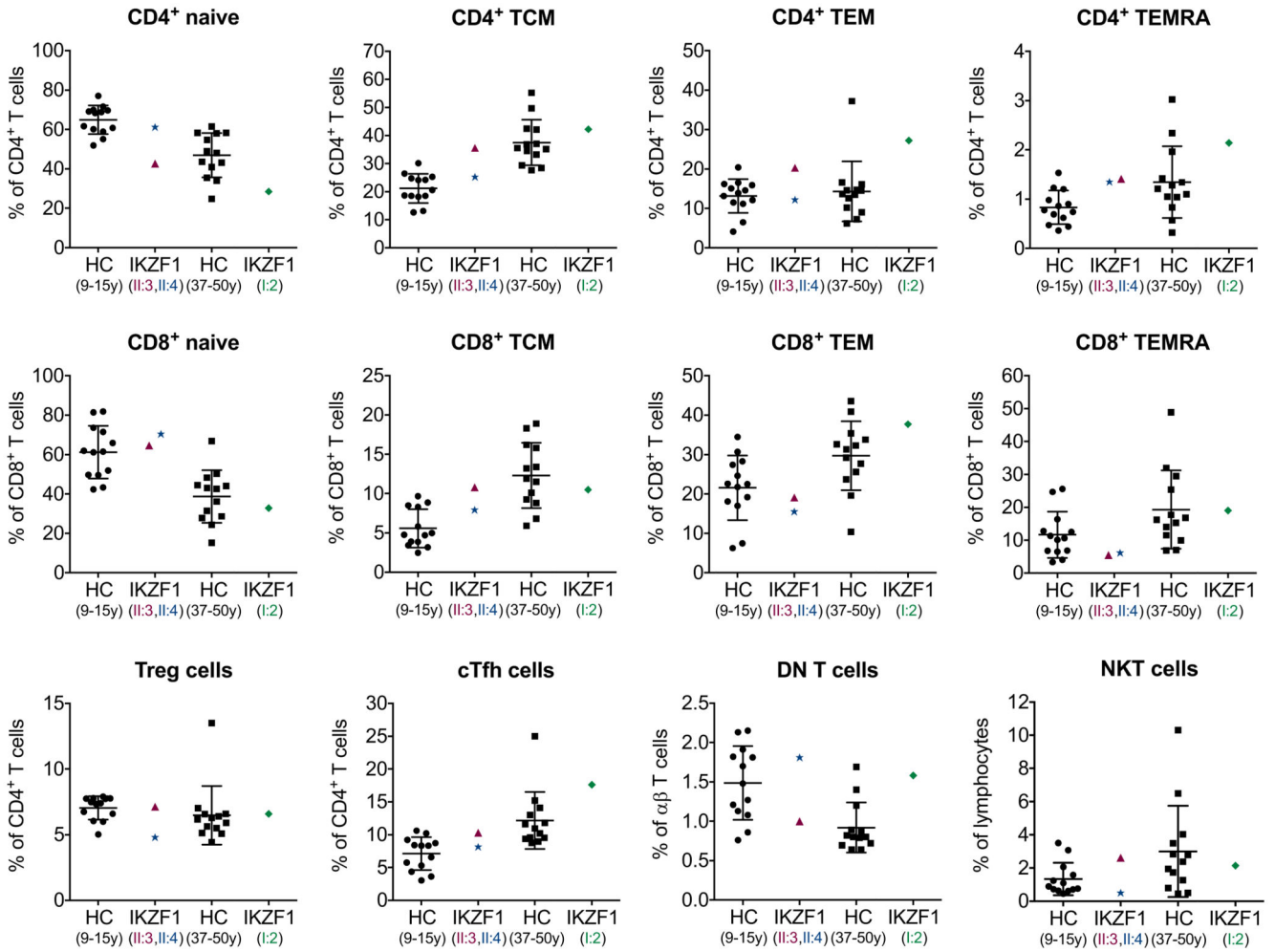


Fig E1.

Peripheral blood T-cell subsets. Flow cytometric immunophenotyping of T-cell subsets was performed on patients' PBMCs in comparison with age-matched healthy control subjects (*HC*). At the time of analysis, subjects I:2, II:3 and II:4 were 40, 14, and 9.5 years old, respectively. Patient II:3 is indicated as a *purple triangle*, and patient II:4 is indicated as a *blue star*. Total T cells were gated as $CD3^+$ in alive PBMCs. $\alpha\beta$ T cells were gated as $\gamma\delta^-$ T-cell receptor–negative in total T cells. $CD4^+$ and $CD8^+$ T cells were gated in $\alpha\beta$ T cells. In $CD4^+$ and $CD8^+$ T cells naive cells were gated as $CD45RO^-CCR7^+$, central memory cells (*TCM*) as $CD45RO^+CCR7^+$, effector memory cells (*TEM*) as $CD45RO^+CCR7^-$, and terminally differentiated cells (*TEMRA*) as $CD45RO^-CCR7^-$. Regulatory T (*Treg*) cells were gated as $CD25^+Foxp3^+$ and circulating follicular helper T (*cTfh*) cells were gated as $CXCR5^+CD45RO^+$ in $CD4^+$ T cells. Double-negative (*DN*) T cells were gated as $CD4^-CD8^-$ in $\alpha\beta$ T cells. Natural killer (*NK*) T cells were gated as $CD56^+$ in total $CD3^+$ T cells.

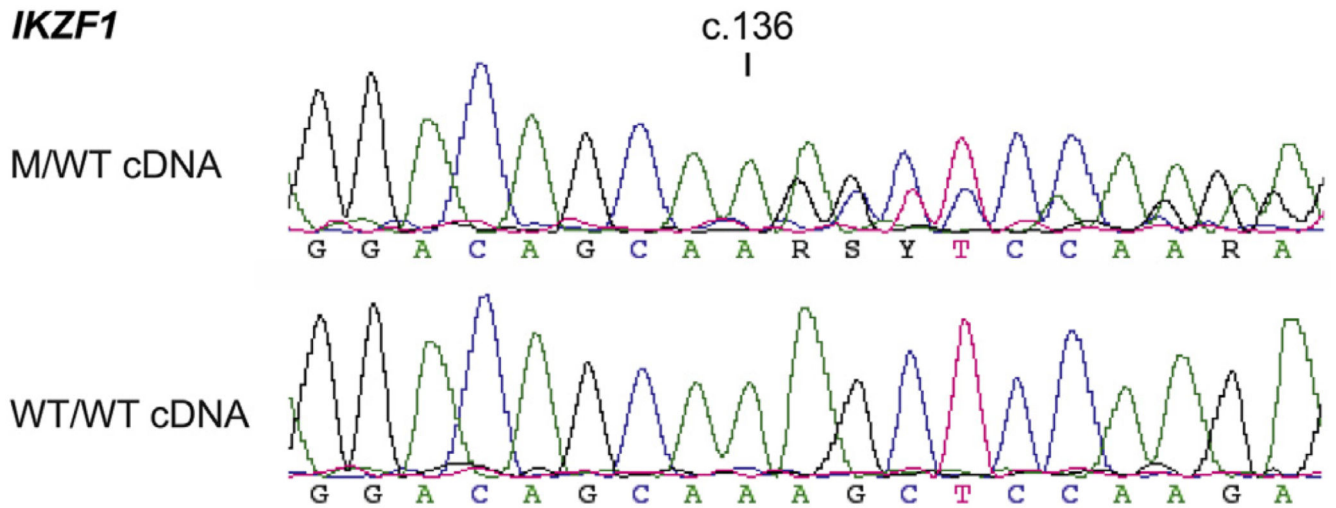


Fig E2. *IKZF1* cDNA sequencing.

Representative *IKZF1* cDNA sequences of a mutant (*M/WT*) and wild-type (*WT/WT*) family member. cDNA was derived from total PBMCs. The position of the nucleotide deletion (c.136) is shown at the *top*. The *M/WT* subject has both a mutant and a wild-type cDNA sequence, suggesting that the mutant transcripts escape nonsense-mediated mRNA decay.

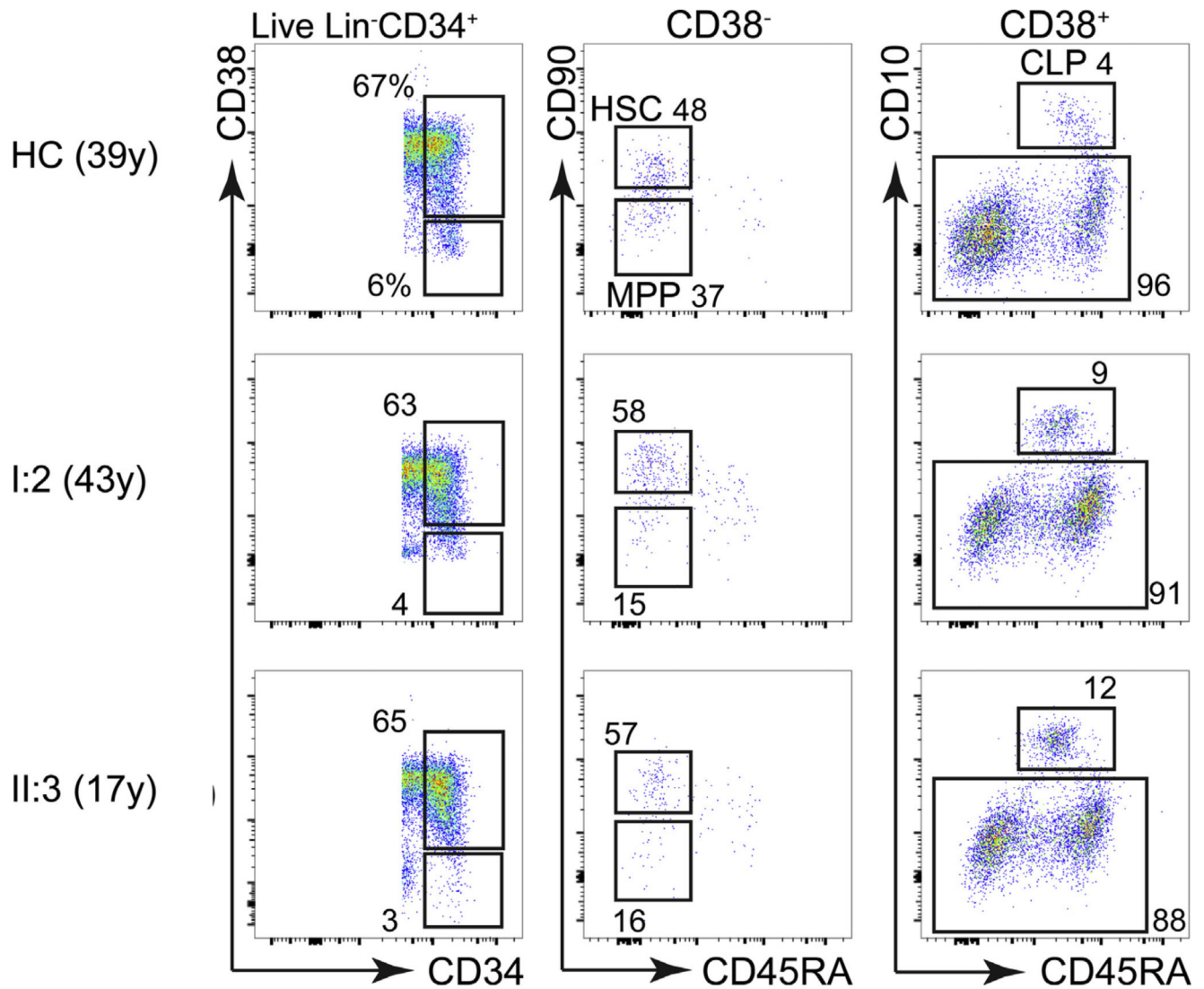
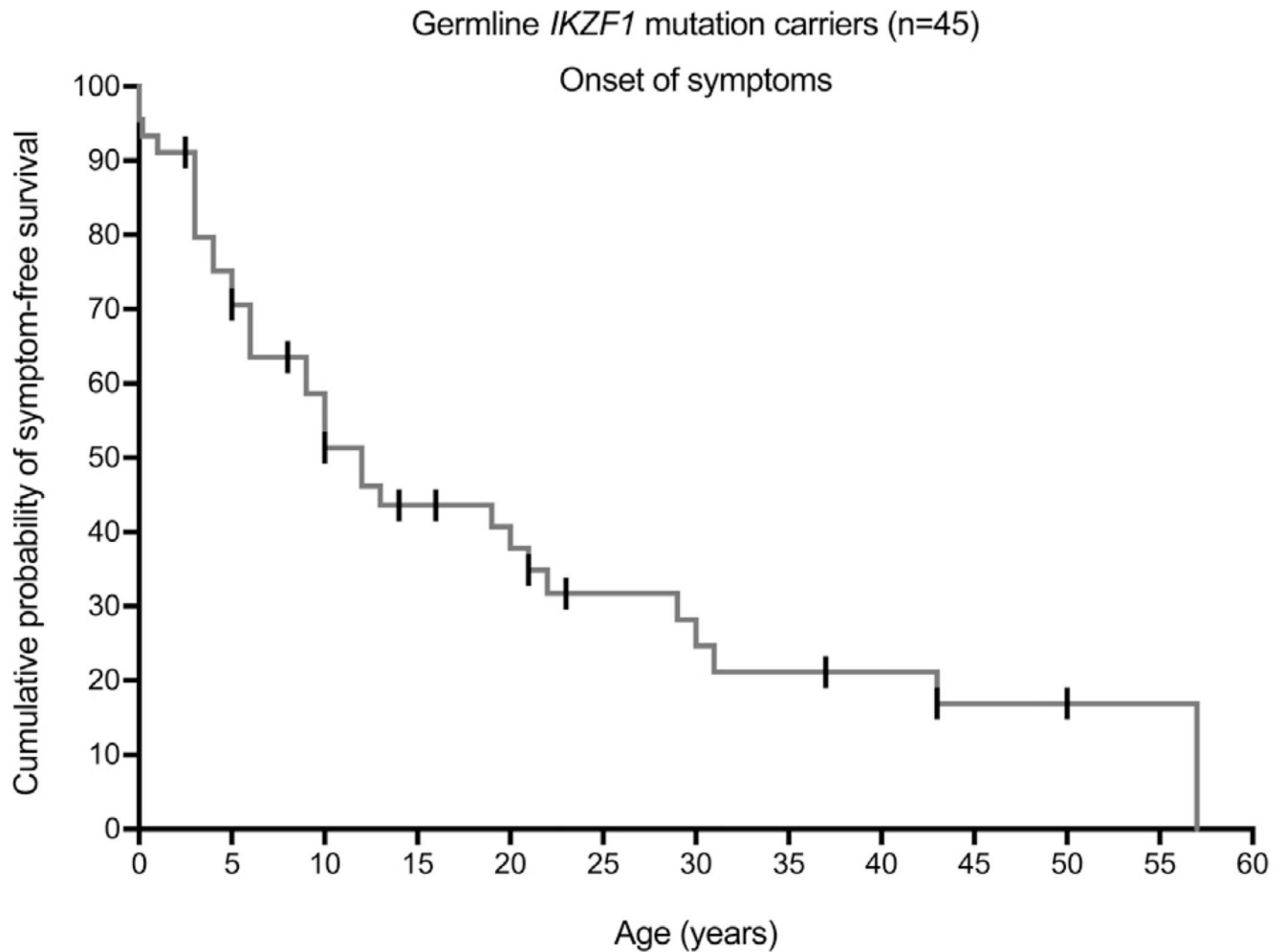


Fig E3.

Flow cytometric analysis of hematopoietic stem and early lineage progenitor cells in the bone marrow. The gating strategy was based on the method of Hoshino et al.^{E10} Percentages are percentages of the parent population, indicated at the top of each plot. *CLP*, Common lymphoid progenitor; *HSC*, hematopoietic stem cell; *MPP*, multipotent progenitor.

**Fig E4.**

Kaplan-Meier curve on symptom-free survival in germline *IKZF1* mutation carriers. The curve was generated based on the presence and age of onset of clinical symptoms in the 42 previously published cases and the 3 reported cases with germline heterozygous *IKZF1* mutations. E4, E10, E11 Manifesting subjects first presented with symptoms up to the age of 57 years. Asymptomatic cases were 50 years or younger at time of publication (ie, censored subjects, as indicated on the curve as *black tick marks*). The Kaplan-Meier symptom-free survival curve estimates that all *IKZF1* mutation carriers will have symptoms by 57 years of age.

Table E1

Routine immunologic laboratory evaluation

	Subject II:3		Subject II:4		Subject I:2	
	Value	Reference range	Value	Reference range	Value	Reference range
	Age: 13 y		Age: 11 y		Age: 42 y	
White blood cells						
Total leukocytes (no./ μ L)	6,820	4,500-12,000	5,050	4,500-12,000	11,320	3,650-9,300
Neutrophils (no./ μ L)	3,580	2,500-8,000	2,240	2,500-8,000	7,850	1,573-6,100
Lymphocytes (no./ μ L)	1,690	1,500-6,500	2,030	1,500-6,500	2,350	1,133-3,105
CD3 ⁺ T cells (no./ μ L)	1,370	800-3,500	1,520	800-3,500	1,930	700-2,100
CD3 ⁺ CD4 ⁺ T _H cells (no./ μ L)	659	400-2,100	771	400-2,100	1,030	300-1,400
CD45RA ⁺ naive CD4 ⁺ T cells (%)	32	33-66 [†]	52	46-77 [†]	24	NA
CD45RO ⁺ memory CD4 ⁺ T cells (%)	60	18-38 [†]	34	13-30 [†]	72	NA
CD3 ⁺ CD8 ⁺ T cytotoxic cells (no./ μ L)	575	200-1,200	508	200-1,200	729	200-1,200
CD45RA ⁺ naive CD8 ⁺ T cells (%)	63	61-91 [†]	80	63-92 [†]	47	NA
CD45RO ⁺ memory CD8 ⁺ T cells (%)	32	4-23 [†]	16	4-21 [†]	53	NA
Ratio CD4/CD8	1.15	0.9-3.4	1.52	0.9-3.4	1.42	1.0-3.6
CD19 ⁺ B cells (no./ μ L)	17	200-600	122	200-600	141	100-500
IgD ⁺ CD27 ⁻ naive B cells (%)	93	51.3-82.5 [‡]	90	51.3-82.5 [‡]	77	48.4-79.7 [‡]
CD24 ⁺⁺ CD38 ⁺⁺ transitional B cells (%)	50	1.4-13.0 [‡]	3	1.4-13.0 [‡]	1	0.9-5.7 [‡]
IgD ⁺ CD27 ⁺ switched memory B cells (%)	1.5	8.7-25.6 [‡]	3	8.7-25.6 [‡]	10	8.3-27.8 [‡]
IgD ⁺ CD27 ⁺ marginal zone B cells (%)	2	4.6-18.2 [‡]	2	4.6-18.2 [‡]	9	7.0-23.8 [‡]
CD21 ^{low} CD38 ^{low} B cells (%)	3	2.7-8.7 [‡]	3	2.7-8.7 [‡]	3	1.6-10.0 [‡]
CD3 ⁺ CD56 ⁺ CD16 ⁺ NK cells (no./ μ L)	304	70-1,200	386	70-1,200	259	90-600
Monocytes (no./ μ L)	730	500-1,000	650	500-1,000	820	247-757
Eosinophils (no./ μ L)	300	100-500	20	100-500	240	28-273
Basophils (no./ μ L)	30	10-100	80	10-100	30	6-50
Immunoglobulins*						
IgG (g/L)	7.0	4.70-11.9	12.7	4.70-11.9	10.7	7.0-16.0
IgG ₂ (g/L)	1.3	0.98-4.8	3.69	0.98-4.8	2.09	1.50-6.40

	Subject II:3		Subject II:4		Subject I:2	
	Value	Reference range	Value	Reference range	Value	Reference range
White blood cells						
IgG ₃ (g/L)	0.668	0.15-1.49	0.309	0.15-1.49	0.617	0.20-1.10
IgM (g/L)	Undetectable	0.27-0.74	0.4	0.27-0.74	0.35	0.40-2.48
IgA (g/L)	Undetectable	0.50-1.66	Undetectable	0.50-1.66	0.98	0.71-3.65
IgE (kU/L)	<4.4	0-90	44.2	0-90	<4.4	0-100
Specific antibody responses*						
<i>Streptococcus pneumoniae</i> polysaccharide IgG (Lab U)	<3	11: immune	3	11: immune	NA	NA
<i>S pneumoniae</i> polysaccharide IgG: specific IgG response to 3 serotypes (8, 9N, 15B)	NA		Insufficient antibody responses	Adequate titer increase for 2 of 3 serotypes [§]	Borderline insufficient antibody responses	Adequate titer increase for 2 of 3 serotypes [§]
Tetanus IgG (IU/mL)	1.0	0.01: immune	0.3	0.01: immune	0.01	0.01: immune
Rubella IgG (IU/mL)	18	>10: immune	68	>10: immune	NA	NA
Measles IgG (mIU/mL)	610	>300: immune	1,000	>300: immune	NA	NA
Mumps IgG (Lab U/mL)	1800	>500: immune	<230	>500: immune	NA	NA
Varicella zoster IgG (mIU/mL)	580	>100: immune	1,900	>100: immune	NA	NA
Lymphocyte proliferation assay						
Response to concanavalin A	Normal	Compared with control	Normal	Compared with control	NA	NA
Response to PHA	Normal	Compared with control	Normal	Compared with control	NA	NA
Response to tetanus toxoid	Moderately reduced	Compared with control	Normal	Compared with control	NA	NA

The most recent, comprehensive, and representative laboratory results are shown for each patient, with patient age at the time of analysis mentioned. Patients II:3 and II:4 were immunized according to the recommended childhood immunization schedule that, among others, included tetanus, measles, mumps, rubella, and 7-valent conjugated pneumococcal vaccines. Subject I:2 received a tetanus booster vaccine within the last 10 years. A polysaccharide (unconjugated) pneumococcal vaccine was given to all 3 subjects at the time of immunologic evaluation; subject I:2 had never received a pneumococcal vaccine before then. Patients II:3 and II:4 were not vaccinated against varicella-zoster virus but had chickenpox in early childhood.

Lab U: Laboratory units; NA, not available.

* Measured before the start of immunoglobulin replacement therapy.

[†]Reference values from Shearer et al.E7

[‡]Reference values from Piatosa et al.E8

[§]In accordance with the recommendations of Orange et al.E9

Acknowledgments

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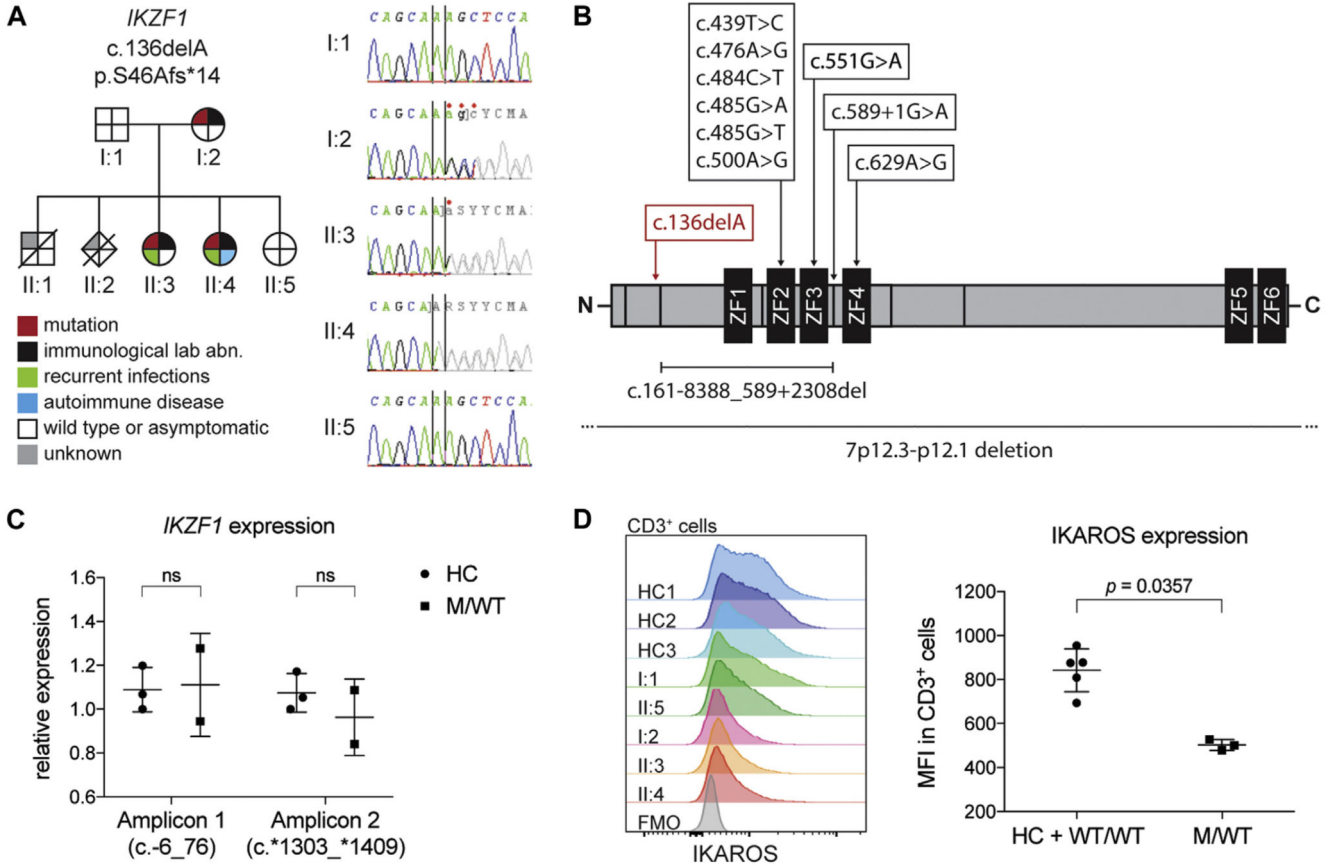


Fig 1. Molecular and functional characterization.

A, Pedigree. Squares, circles, and diamonds indicate male, female, and sex unknown, respectively. Diagonal lines indicate deceased siblings. Corresponding electropherograms are shown on the right. *lab abn.*, Laboratory abnormalities. **B**, Schematic structure of IKAROS isoform 1 (*IKZF1* transcript NM_006060). Gray boxes represent exons. Zinc fingers (ZF) 1 to 4 constitute the DNA binding domain, and ZF5 and ZF6 constitute the protein dimerization domain. Previously published mutations are depicted in black; 1 family had a large chromosomal deletion (7p12.3-p12.1) encompassing the *IKZF1* gene. The reported novel mutation is indicated in red. **C**, *IKZF1* mRNA expression was determined by means of real-time quantitative PCR of 2 amplicons located upstream and downstream of the mutation site, respectively. The cDNA start and end position of each amplicon is shown between brackets (*IKZF1* transcript NM_006060). The 2 studied patients (II:3 and II:4) had similar *IKZF1* mRNA levels compared with healthy control subjects (HC), indicating that the mutant transcripts escape nonsense-mediated mRNA decay. The graph represents the mean \pm SD of 1 experiment. *ns*, Not significant. **D**, IKAROS protein expression in mutant (M/WT) and wild-type (WT/WT) family members and 3 healthy control subjects (HC) analyzed in CD3⁺ T cells by use of flow cytometry. Histograms are depicted on the left. The graph on the right displays mean \pm SDs of the corresponding mean fluorescence intensity (MFI) values. Data shown are representative of 2 replicate experiments. FMO, Fluorescence minus one.

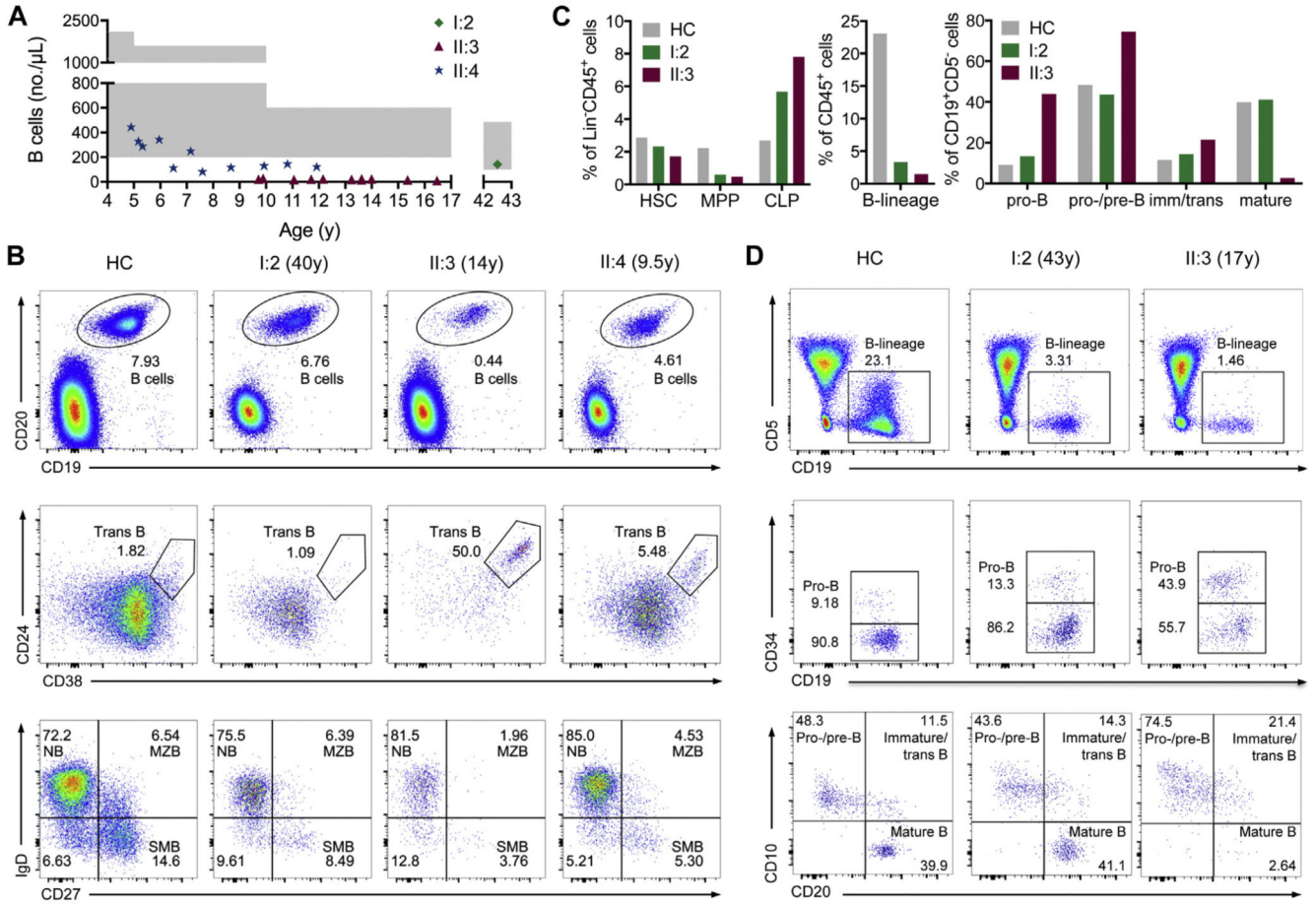


Fig 2. B-cell phenotype.

A, Peripheral blood total B-cell counts. Measurements shown are from the first to the last immunologic laboratory evaluation performed in our hospital. *Gray shading* represents the age-based reference range. **B**, Peripheral blood B-cell subsets. The *top row* depicts total B cells gated as CD19⁺CD20⁺ in alive PBMCs. In the *second and third rows*, B-cell subsets were gated on total CD19⁺CD20⁺ B cells, as indicated. Note that samples shown here were taken at different time points than those in Table E1. **C**, Overview of different precursor stages of B-cell development in bone marrow shown as a proportion of total Lin⁻CD45⁺, CD45⁺, or CD19⁺CD5⁻ cells, as indicated. **D**, Bone marrow B-cell subsets. The *top row* depicts total B-lineage cells gated as CD19⁺CD5⁻ in CD451 cells. In the *second and third rows*, B-lineage subsets were gated within this CD19⁺CD5⁻ population. The *middle graphs* allow discrimination of pro-B cells (CD34⁺CD19⁺) from more mature B-lineage cells (CD34⁻CD19⁺). The *lower graphs* show the development from pro- and pre-B cells (*pro-/pre-B*; CD10⁺CD20⁻) over immature and transitional B cells (*immature/trans B*; CD10⁺CD20⁺) to mature naive B cells (*mature B*; CD10⁻CD20⁺). *CLP*, Common lymphoid progenitors; *HC*, healthy control subject; *HSC*, hematopoietic stem cells; *imm.*, immature B; *MPP*, multipotent progenitors; *MZB*, marginal zone B cells; *NB*, naive B cells; *SMB*, switched memory B cells; *Trans B*, transitional B cells.

	Subject II:3		Subject II:4		Subject I:2	
	Value	Reference range	Value	Reference range	Value	Reference range
White blood cells						
IgG (g/L)	7.0	4.70-11.9	12.7	4.70-11.9	10.7	7.0-16.0
IgG ₂ (g/L)	1.3	0.98-4.8	3.69	0.98-4.8	2.09	1.50-6.40
IgG ₃ (g/L)	0.668	0.15-1.49	0.309	0.15-1.49	0.617	0.20-1.10
IgM (g/L)	Undetectable	0.27-0.74	0.4	0.27-0.74	0.35	0.40-2.48
IgA (g/L)	Undetectable	0.50-1.66	Undetectable	0.50-1.66	0.98	0.71-3.65
IgE (kU/L)	<4.4	0-90	44.2	0-90	<4.4	0-100
Specific antibody responses*						
		Age: 13 y		Age: 11 y		Age: 42 y
<i>Streptococcus pneumoniae</i> polysaccharide IgG (Lab U)	<3	11: immune	3	11: immune	NA	
<i>S pneumoniae</i> polysaccharide IgG: specific IgG response to 3 serotypes (8, 9N, 15B)	NA		Insufficient antibody responses	Adequate titer increase for 2 of 3 serotypes [§]	Borderline insufficient antibody responses	Adequate titer increase for 2 of 3 serotypes [§]
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Varicella zoster IgG (mIU/mL)	580	>100: immune	1,900	>100: immune	NA	
Lymphocyte proliferation assay		Age: 9 y		Age: 5 y		
Response to concanavalin A	Normal	Compared with control	Normal	Compared with control	NA	
Response to PHA	Normal	Compared with control	Normal	Compared with control	NA	
Response to tetanus toxoid	Moderately reduced	Compared with control	Normal	Compared with control	NA	

The most recent, comprehensive, and representative laboratory results are shown for each patient, with patient age at the time of analysis mentioned. Patients II:3 and II:4 were immunized according to the recommended childhood immunization schedule that, among others, included tetanus, measles, mumps, rubella, and 7-valent conjugated pneumococcal vaccines. Subject I:2 received a tetanus booster vaccine within the last 10 years. A polysaccharide (unconjugated) pneumococcal vaccine was given to all 3 subjects at the time of immunologic evaluation; subject I:2 had never received a pneumococcal vaccine before then. Patients II:3 and II:4 were not vaccinated against varicella-zoster virus but had chickenpox in early childhood.

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In accordance with the recommendations of Orange et al.E9

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