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ORIGINAL ARTICLE

Loss of B Cells in Patients with Heterozygous Mutations in IKAROS

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ABSTRACT

BACKGROUND

Common variable immunodeficiency (CVID) is characterized by late-onset hypogammaglobulinemia in the absence of predisposing factors. The genetic cause is unknown in the majority of cases, and less than 10% of patients have a family history of the disease. Most patients have normal numbers of B cells but lack plasma cells.

METHODS

We used whole-exome sequencing and array-based comparative genomic hybridization to evaluate a subset of patients with CVID and low B-cell numbers. Mutant proteins were analyzed for DNA binding with the use of an electrophoretic mobility-shift assay (EMSA) and confocal microscopy. Flow cytometry was used to analyze peripheral-blood lymphocytes and bone marrow aspirates.

RESULTS

Six different heterozygous mutations in *IKZF1*, the gene encoding the transcription factor IKAROS, were identified in 29 persons from six families. In two families, the mutation was a de novo event in the proband. All the mutations, four amino acid substitutions, an intragenic deletion, and a 4.7-Mb multigene deletion involved the DNA-binding domain of IKAROS. The proteins bearing missense mutations failed to bind target DNA sequences on EMSA and confocal microscopy; however, they did not inhibit the binding of wild-type IKAROS. Studies in family members showed progressive loss of B cells and serum immunoglobulins. Bone marrow aspirates in two patients had markedly decreased early B-cell precursors, but plasma cells were present. Acute lymphoblastic leukemia developed in 2 of the 29 patients.

CONCLUSIONS

Heterozygous mutations in the transcription factor IKAROS caused an autosomal dominant form of CVID that is associated with a striking decrease in B-cell numbers. (Funded by the National Institutes of Health and others.)

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The genetic cause of many primary immunodeficiencies remains unknown. Common variable immunodeficiency (CVID) comprises a heterogeneous group of disorders characterized by the late onset of recurrent infections, hypogammaglobulinemia, and poor antibody response to vaccine antigens that cannot be explained by previous exposures, treatment, or infections. Some patients also have autoimmunity, granulomatous disease, or cancer.[1,2]

Genomic approaches, including whole-exome sequencing and high-resolution array-based comparative genomic hybridization (CGH), have accelerated the identification of genetic causes in patients with primary immunodeficiencies, including CVID.[3-5] Mutations in several genes, including *ICOS*, *CD19*, *CD81*, *CD20*, *CD21*, *TWEAK*, *CTLA4*, *LRBA*, *GATA2*, *CXCL12*, *NFKB1*, and *NFKB2*, have been associated with a CVID-like phenotype in a small proportion of patients.[6,7] A number of these new disorders are autosomal dominant with incomplete penetrance. Often, there is marked variation in the clinical severity, even among family members with the same genetic defect.[3-7] We used whole-exome sequencing and high-resolution array-based CGH to evaluate a subset of patients with CVID and low B-cell numbers.

METHODS

STUDY PARTICIPANTS

All study participants or their parents provided written informed consent. The study was approved by the local review boards. The probands in this study, Patients A1, B5, C1, D2, E1, and F2, were first evaluated for immunodeficiency at 3 to 32 years of age, after recurrent or severe bacterial infections that were often due to *Streptococcus pneumoniae*. All six patients had hypogammaglobulinemia and decreased numbers of B cells, findings consistent with the diagnosis of CVID (Table 1, and Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). CVID was subsequently diagnosed in Patients A2, B1, B6, C2, C3, F1, F3, and F4.

After *IKZF1* mutations had been identified in the probands, they were detected in additional affected family members. Patients B1, B2, B3, F5, and F6 had a history of severe bacterial infections. Patients B4, F1, and F6 had recurrent sinusitis, bronchitis, or both. Patient A2 had had two episodes of idiopathic thrombocytopenic purpura. B-cell acute lymphoblastic leukemia (ALL) developed in Patients B7 and F12 at 3 and 5 years of age, respectively. Patient B1 died from pneumonia at 74 years of age, and Patient B7 died from a relapse of B-cell ALL at 5 years of age. No patients showed evidence of increased susceptibility to viral or fungal infections. Most of the patients with hypogammaglobulinemia have done relatively well, despite inadequate treatment with gamma globulin in many of them. Additional clinical data and the methods used for genetic analysis, functional studies, and flow cytometry are described in the Supplementary Appendix.

RESULTS

MUTATION DETECTION

Whole-exome sequencing was performed on DNA samples from Patients A1 and A2 and the unaffected mother of Patient A1; Patients B1 and B6; Patients C1, C2, and C3 and the healthy daughter of Patient C1; and Patients D2 and E1. Mutations in 269 genes known to be associated with immunodeficiency (Table S2 in the Supplementary Appendix), including *BTK* (encoding Bruton's tyrosine kinase), *GATA2*, *CTLA4*, and *TNFRS13B* (encoding TACI [transmembrane activator and calcium-modulator and cyclophilin-ligand interactor]), were not identified in any of these persons. A G→T substitution at c.485 in Family A resulted in a substitution of leucine for arginine at codon 162 (R162L) in *IKZF1*, the gene encoding the transcription factor IKAROS. A G→A substitution at c.485 in Family B resulted in a substitution of glutamine for arginine (R162Q) at the same mutated codon found in Family A. In Family C, an A→G substitution in *IKZF1* at c.500 was found, resulting in a substitution of arginine for histidine at codon 167 (H167R). A G→A substitution at c.551 in Family D resulted in a substitution of glutamine for arginine at codon 184 (R184Q). Sanger sequencing confirmed the mutations in the patients and detected mutations in additional affected family members. The mutations were de novo in Patients A1 and C1; however, Patient D1, the asymptomatic mother of Patient D2, carried the same mutation as her daughter (Fig. 1).

Table 1. Clinical and Laboratory Characteristics of 29 Patients with Heterozygous IKAROS Mutations.*

Patient No.	Clinical Manifestations	Age at Onset	Immunoglobulin			B Cells		T Cells		
			IgG	IgA	IgM	CD19+	CD27+†	CD3+	CD3+CD4+	CD3+CD8+
		yr	*mg/dl*			*% (absolute lymphocyte count/mm³)*				
A1	Infections	9	**<33**	**<7**	**21**	**0.5 (17)**	NA	*95 (3180)*	34 (1128)	*59 (1982)*
A2	ITP Infections	3 6	727‡	**7**	**5**	**0.3 (12)**	NA	*86 (3129)*	*47 (1831)*	*34 (1319)*
B1	Infections	12	**386**	**30**	60	NA	NA	NA	NA	NA
B2	Infections	6	**461**	**36**	**45**	**3.0 (80)**	15 **(12)**	85 *(2350)*	34 (950)	*51 (1410)*
B3	Infections	10	**283**	**15**	71	7.0 (260)	72 (187)	*91 (3330)*	31 (1140)	*61 (2230)*
B4	Infections	43	**433**	**19**	**56**	**2.0 (50)**	45 **(22)**	86 (2030)	23 (540)	*65 (1530)*
B5	Infections	3	1430‡	**21**	**22**	**2.0 (50)**	**3 (1)**	76 (1900)	36 (900)	38 (950)
B6	Infections	1	1260‡	**12**	**21**	**1.0 (20)**	17 **(3)**	79 (1560)	38 (750)	36 (710)
B7	B-cell ALL	3	NA	NA	NA	NA	NA	NA	NA	NA
C1	Infections	30	887‡	73	**11**	**1.0 (26)**	NA	83 (2177)	**31** (806)	*50 (1300)*
C2	Infections	13	1052‡	157	**12**	**0.2 (5)**	NA	83 (2049)	**26** (627)	*50 (1225)*
C3	Infections	4	1030‡	**12**	**<6**	**0.4 (18)**	NA	82 *(3712)*	**26** (1168)	*48 (2147)*
D1	Asymptomatic	—	**637**	**5**	**45**	7.0 (160)	NA	*89* (2022)	**29** (632)	*63 (1397)*
D2	Infections	9	**42**	**<1**	**7**	**0.3 (9)**	NA	*96 (3388)*	40 (1416)	*54 (1888)*
E1	Infections	3	860‡	**<1**	**10**	**0.2 (5)**	NA	*93 (2817)*	**25** (787)	*67 (1966)*
E2	Asymptomatic	—	NA	NA	NA	NA	NA	NA	NA	NA
F1	Infections	57	**113**	**<6**	**14**	**0.8 (11)**	22 **(2)**	66 (866)	**26 (343)**	38 (498)
F2	Infections	29	**<7**	**<4**	**<2**	**1.0 (18)**	NA	*94* (1709)	59 (1068)	35 (640)
F3	Infections	21	1010‡	**<6**	**5**	**0.6 (8)**	24 **(2)**	79 (1020)	40 (522)	38 (486)
F4	Infections	22	1010‡	**<6**	**7**	**0.1 (2)**	58 **(1)**	*95 (2489)*	*31 (787)*	*65 (1686)*
F5	Infections	31	**382**	**29**	**21**	**0.5 (12)**	19 **(2)**	*87* (2078)	35 (822)	*52 (1238)*
F6	Infections	19	**148**	**32**	**39**	**5.5** (138)	**4 (5)**	82 (2115)	48 (1231)	34 (886)
F7	Asymptomatic	—	**528**	**<6**	**18**	**0.5 (9)**	18 **(2)**	84 (1464)	58 (996)	27 (469)
F8	Asymptomatic	—	789	64	46	**2.7 (89)**	NA	86 (2851)	54 (1525)	32 (907)
F9	Asymptomatic	—	844	**28**	**36**	15.0 (354)	**6 (22)**	77 (1940)	34 (866)	40 (955)
F10	Asymptomatic	—	**606**	118	**32**	7.5 (187)	22 (42)	76 (1864)	44 (1068)	31 (758)
F11	Asymptomatic	—	**541**	120	**53**	17.1 (544)	15 (79)	68 (2026)	43 (1286)	22 (656)
F12	B-cell ALL Infections	5 6	**590**	**36**	**13**	13.7 (452)	9 (42)	75 (2292)	34 (1032)	*42 (1269)*
F13	Asymptomatic	—	**629**	**28**	**40**	15.4 (291)	**12 (34)**	78 (1356)	55 (953)	22 (378)

* The most recent, comprehensive, and representative laboratory data are shown for each patient; complete laboratory data are provided in Table S1 in the Supplementary Appendix. Numbers in italics indicate values above the normal range, and numbers in boldface indicate values below the normal range for age-matched controls in the laboratory in which the study was performed: the National Institutes of Health Clinical Center (Families A and C), University Children's Hospital Zurich (Family B), Quest Diagnostics (Family D), Oslo University Hospital and University of Oslo (Family E), and ARUP Laboratories (Family F). ALL denotes acute lymphoblastic leukemia, CVID common variable immunodeficiency, ITP idiopathic thrombocytopenic purpura, and NA not available.

† CD27+ B cells are expressed as the percentage among CD19+ B cells. Values are included only if at least 50 CD19+ B cells were analyzed.

‡ The patient was receiving IgG replacement therapy.

Whole-exome sequencing and customized array-based CGH were performed on a DNA sample from Patient E1. In silico copy-number variation analysis of data from whole-exome sequencing[8] showed an *IKZF1* intragenic heterozygous deletion. Sanger sequencing identified the exact mutation breakpoints (Fig. S1 in the Supplementary Appendix). This 16.8-kb deletion (Chr7 [human assembly GRCh37]:g.50435843_50452713del; NM_006060.5(IKZF1):c.1618388_589+2308del) results in the in-frame deletion of *IKZF1* exons 4 and 5. The same heterozygous deletion was detected in Patient E2 (the son of Patient E1) by means of CGH.

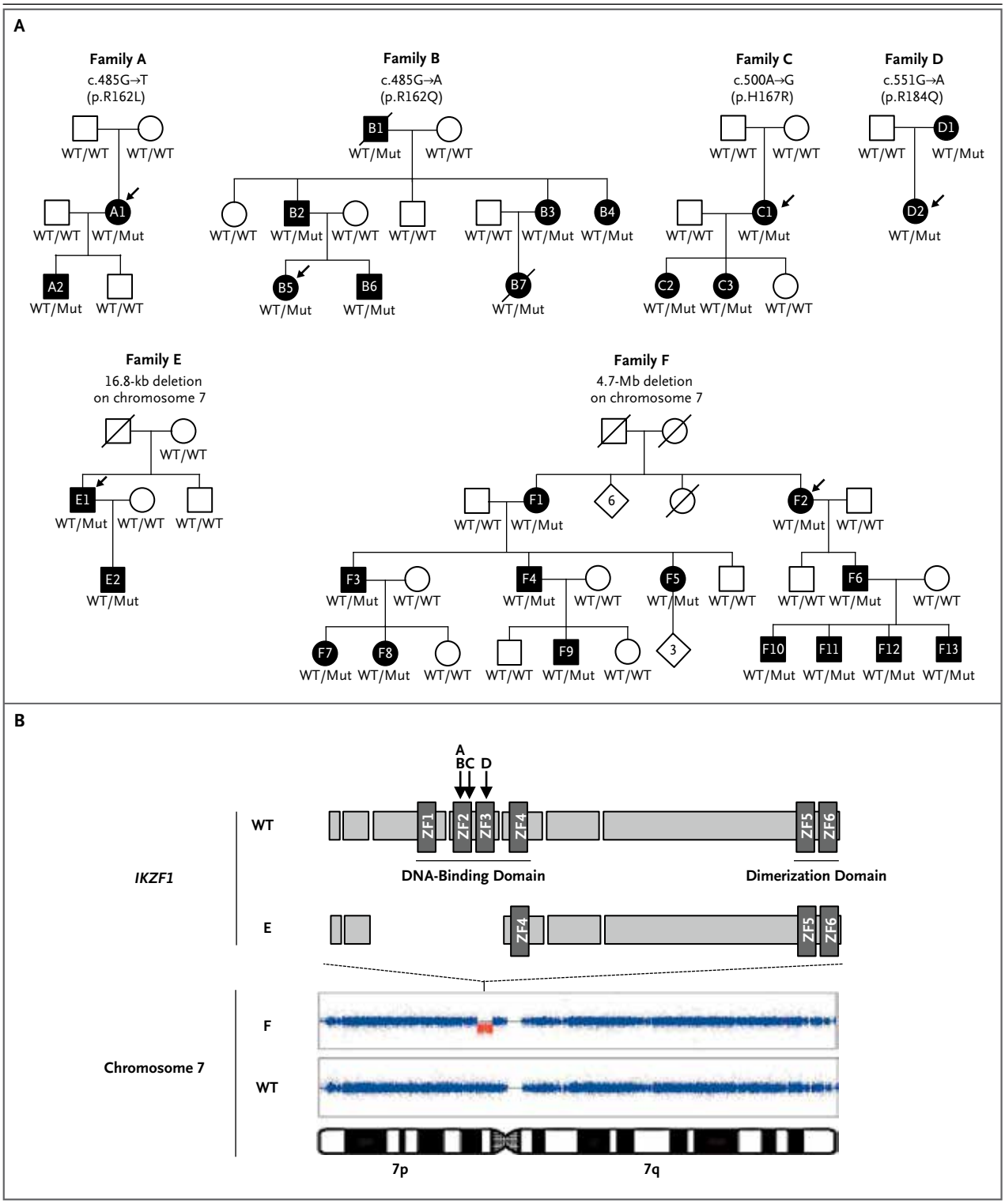
CGH analysis of DNA samples from Patient F1, her husband, and her four children revealed a 4.7-Mb heterozygous deletion on chromosome 7 in Patient F1 and three of her children (Patients F3, F4, and F5). The 7p12.3-p12.1 deletion involves 11 genes *(ABCA13, CDC14C, VWC2, ZPBP, C7orf7, IKZF1, FIGNL1, DDC, GRB10, COBL,* and *POM121L12).* Only *IKZF1* is known to play a role in B-cell development and function. Multiplex ligation-dependent probe amplification revealed the *IKZF1* deletion in nine additional family members (Fig. 1). No germline constitutional deletion of this region was reported in more than 8000 healthy controls.[9] However, patients with complex genetic syndromes associated with total or subtotal chromosome 7p deletions, including one child with Greig cephalopolysyndactyly and ALL, have been described.[10]

IKAROS is a member of a family of hematopoietic zinc-finger transcription factors.[11] It was first identified on the basis of its ability to bind regulatory regions of genes encoding terminal deoxynucleotidyl transferase (TdT) and CD3δ.[12,13] It also binds pericentromeric DNA as part of the NuRD (nucleosome remodeling and histone deacetylase) complex and can both enhance and repress gene transcription.[14-16] The four N-terminal zinc fingers of IKAROS form the DNA-binding domain, and the two C-terminal zinc fingers act as a dimerization domain.[11,17] Multiple splice variants of IKAROS are produced; those that have lost the DNA-binding zinc fingers but have retained the dimerization zinc fingers act as dominant negative regulators of IKAROS family members.[18]

The missense mutations in Families A, B, and C were in zinc finger 2, and the mutation in Family D was in zinc finger 3; these two highly conserved domains (Fig. S1 in the Supplementary Appendix) are essential for DNA binding.[15] The deletion in Patient E1 resulted in the loss of zinc fingers 1, 2, and 3. Previously published structural studies indicate that missense mutations R162L, R162Q, and R184Q occur at DNA contact residues, whereas the H167R mutation involves one of the C2H2 zinc-finger canonical histidines (see Fig. S2 in the Supplementary Appendix for the structure of IKAROS).[15] Furthermore, these four mutations are predicted to be highly damaging to protein function (Fig. S3 in the Supplementary Appendix). These point mutations were not seen in more than 64,000 genomes included in the 1000 Genomes Project and the Exome Aggregation Consortium (ExAC) database. No premature stop codons in *IKZF1* were found in these databases, and the few missense mutations reported were either very rare or predicted to be benign. In addition, *IKZF1* is under strong purifying selection (Fig. S4 in the Supplementary Appendix), meaning that persons carrying dysfunctional variants tend not to survive to reproductive age. Finally, DNA from 132 additional patients with CVID, including 21 patients with low B-cell numbers, was analyzed for mutations in *IKZF1* by means of whole-exome sequencing or Sanger sequencing of exons, but no additional mutations were identified.

FUNCTIONAL ANALYSIS OF *IKZF1* MUTATIONS

Flow cytometry showed that the amount of IKAROS in T cells and B cells from the study participants with amino acid substitutions was equal to that in T cells and B cells from controls, whereas T cells and B cells from the participants with the large deletion had approximately half the normal amount of IKAROS (Fig. S5 in the Supplementary Appendix). To examine the stability of the proteins resulting from the four missense mutations in *IKZF1* (R162L, R162Q, H167R, and R184Q) and the ability of these proteins to dimerize with wild-type IKAROS and enter the nucleus, we transfected HEK293T cells with wild-type and mutant expression vectors. Western blot analysis of extracts from the cytoplasm and nucleus of the transfected cells showed that these mutant proteins were stable,



were dimerized with wild-type IKAROS, and were able to migrate to the nucleus (Fig. S6 in the Supplementary Appendix). The nuclear extracts from transfected cells were used in an electrophoretic mobility-shift assay (EMSA) to evaluate the ability of the mutant proteins to bind an

Figure 1 (facing page). Mutation in IKAROS (*IKZF1*) in Families with Common Variable Immunodeficiency.
Panel A shows the pedigrees of the six families. Circles and squares denote female and male family members, respectively. Black symbols represent family members with a heterozygous mutation in *IKZF1*, and slashes indicate deceased family members. In Family F, the diamond with the number in it indicates the number of family members (male or female) who were not screened. Arrows indicate the probands. The specific complementary DNA (*IKZF1* transcript variant NM_006060) and protein mutation are indicated above each pedigree. Mut denotes mutation, and WT wild type. Panel B, top, shows the exon structure of full-length IKAROS isoform 1 in light-gray boxes (*IKZF1* transcript variant NM_006060), with the zinc fingers (ZFs) indicated in dark-gray boxes. The sites of the mutations in Families A, B, C, and D and the deletion of c.161-8388_589+2308del in *IKZF1* in Family E are shown. The lower part of the panel shows a schematic representation of the 4.7-Mb deletion on the short arm of chromosome 7 (7p12.3-p12.1) in Family F, with the deletion indicated in red.

IKAROS consensus-binding sequence (IK-bs4)[15] and a probe from the pericentromeric region of human chromosome 8 (γSat8).[19] The four proteins resulting from missense mutations failed to bind these probes (Fig. S7A and S7B in the Supplementary Appendix). To determine whether the amino acid substitutions resulted in a dominant negative effect and to mimic the heterozygous state, we performed EMSAs with nuclear extracts from human embryonic kidney 293T (HEK293T) cells transfected with vector expressing 100% wild-type IKAROS or vectors expressing 50% wild-type and 50% mutant IKAROS. DNA binding was reduced by 38 to 74% in the cells transfected with the 50% wild-type and 50% mutant vectors, as compared with the cells transfected with 100% wild-type vector. This finding was consistent with the reduction in the amount of wild-type vector used in transfection (Fig. S7A and S7B).

Confocal microscopy showed that transfected NIH-3T3 cells expressing wild-type IKAROS had the punctate staining pattern that is characteristic of pericentromeric heterochromatin binding and localization (Fig. S7C in the Supplementary Appendix). In contrast, the four proteins resulting from missense mutations had diffuse nuclear staining. When the NIH-3T3 cells were transfected with equal amounts of the vector expressing wild-type IKAROS and the vector expressing mutant IKAROS, the staining pattern was similar to that seen in cells transfected with only the vector expressing wild-type IKAROS (Fig. S7D in the Supplementary Appendix). Similar results were obtained when HEK293T cells were transfected (Fig. S8 in the Supplementary Appendix). The combined results of the EMSAs and confocal microscopy are consistent with the hypothesis that the four proteins resulting from missense mutations do not have a dominant negative effect.

To compare the mutations found in these patients with previously described mutations in *IKZF1*, we performed an EMSA and confocal microscopy with the use of vectors expressing N159A, an experimentally generated in vitro mutation[15]; H191R, an ethylnitrosourea-induced murine mutation[20]; and Y210C, a mutation in zinc finger 4 that was identified in a critically ill premature infant with pancytopenia.[21] The abnormal EMSA results and perinuclear localization for the N159A and H191R mutations were similar to the findings with the R162L, R162Q, H167R, and R184Q mutations. The Y210C mutation showed decreased but not absent DNA binding on an EMSA. Primary T cells from the infant, who was heterozygous for the Y210C allele, were reported to show abnormal subcellular localization of IKAROS; however, we saw normal pericentromeric localization of the protein with the Y210C mutation when expressed alone or in the presence of the wild-type allele in transfected NIH-3T3 cells (Fig. S9 in the Supplementary Appendix).

LABORATORY FINDINGS

There was some variation in laboratory findings among the study participants. However, 26 of the 27 patients for whom pretreatment data were available had a marked decrease in at least two of the three major immunoglobulin isotypes (IgG, IgM, and IgA); 23 had a decrease in all three isotypes, and 6 with panhypogammaglobulinemia had a serum IgG level of less than 150 mg per deciliter before treatment with gamma globulin was started (Table 1). Only 1 patient (at 5 years of age) had normal findings for the three major immunoglobulin isotypes. Assessment of antibodies to vaccine antigens, which was performed in 8 patients, showed an absence of antibodies in 6 patients, progressive loss of antibodies in 1, and normal titers in 1. At their

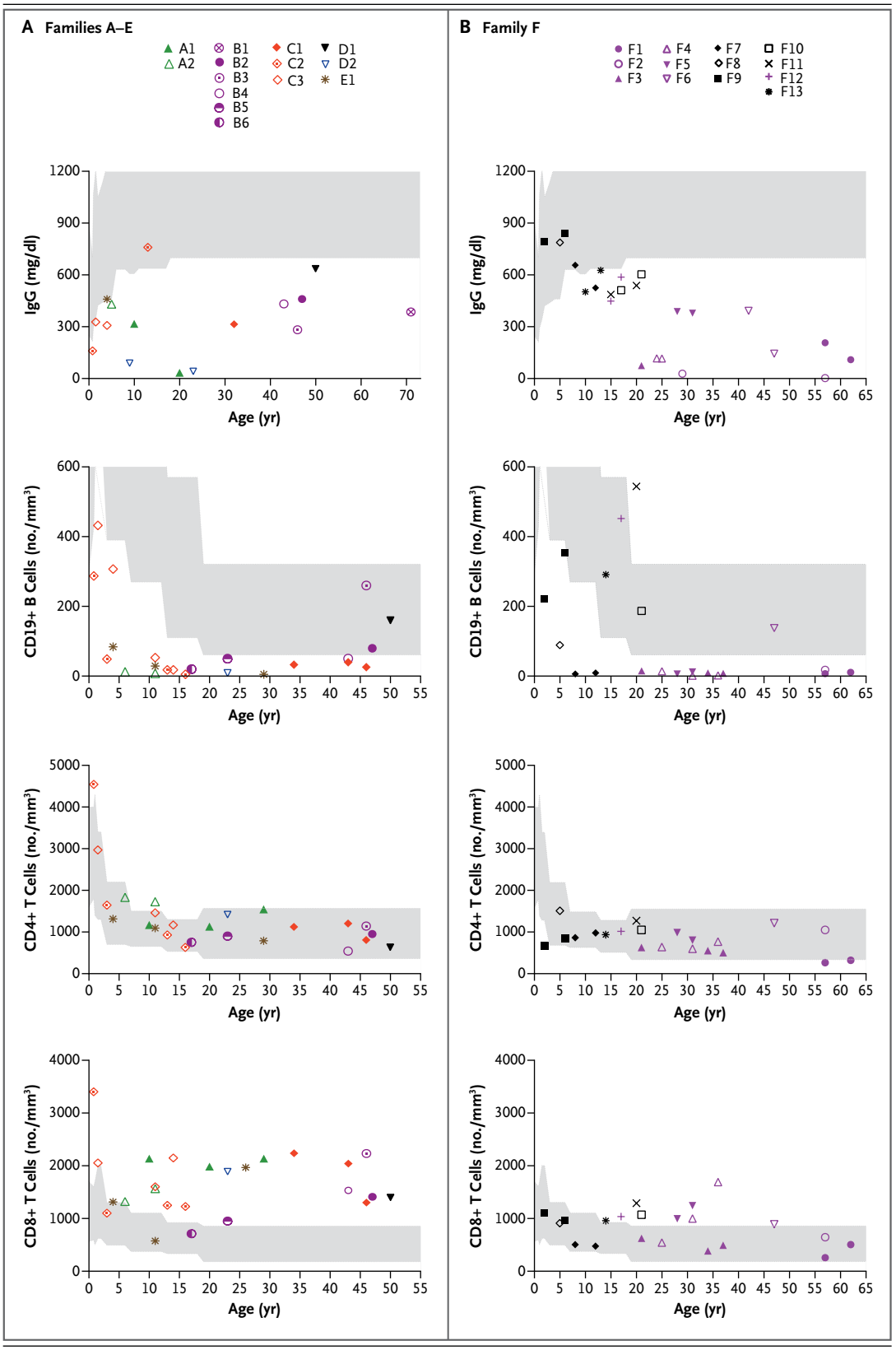


Figure 2 (facing page). Low Serum IgG Levels and Progressive Loss of Peripheral-Blood B-Cell Counts in Patients with *IKZF1* Mutations.

The symbols represent individual family members, with black symbols indicating those who were asymptomatic; gray shading represents mean values ±1 SD in healthy controls. Only patients who were not being treated with gamma globulin are included in the data shown for IgG levels.

most recent evaluation, 14 patients had a CD19+ cell count of less than 1% in peripheral blood; however, CD19+CD27+ memory B cells were readily detectable in the 14 patients in whom they were analyzed. When plotted against age, the serum IgG level showed a progressive decline (Fig. 2). A similar finding was noted when the number of CD19+ B cells was evaluated (Table 1, and Table S1 in the Supplementary Appendix).

T-cell studies showed a consistent increase in CD8+ T cells with reversed CD4:CD8 ratios in 10 of 13 participants with DNA-binding defects in IKAROS (e.g., amino acid substitutions or exon 4 and 5 deletion) and in 3 of 13 patients with complete deletion of *IKZF1* ($P=0.01$ on the basis of a two-tailed Fisher's exact test) (Table 1 and Fig. 2, and Table S1 in the Supplementary Appendix). The CD8+ T cells were predominantly naive cells (CD62L+CD45RA+) and central memory cells (CD62L+CD45RA–) in the 6 patients who were assessed for these markers. Both CD4+ and CD8+ cells appeared to be polyclonal on cytometric analysis of Vβ flow (Fig. S10 in the Supplementary Appendix) and studies of T-cell receptor gamma-chain rearrangement. Proliferation of T-cells and Fas-mediated apoptosis were similar in patients and controls (Fig. S11 in the Supplementary Appendix). Polymerase-chain-reaction assays for cytomegalovirus and Epstein–Barr virus were negative in the 6 patients with reversed CD4:CD8 ratios who were evaluated. The number of natural killer cells ranged from the high end to the low end of the normal range (Table S1 in the Supplementary Appendix).

Bone marrow aspirates were available from two members of Family C (Patient C1, at the age of 45 years, and Patient C2, at the age of 16 years), both of whom had a CD19+ B-cell count of 1% or less in peripheral blood. Normal numbers of CD34+ and CD138+ plasma cells were found in the bone marrow (Fig. 3A), but there was a marked decrease in pro-B cells (defined by coexpression of CD34 and CD19) and earlier precursors (expressing surface CD34 and cytoplasmic TdT in the absence of CD19) (Fig. 3B).[22] Both the number of TdT+ cells and the intensity of TdT expression were decreased. Because TdT is a direct target of *IKZF1*,[12] we compared the number of TdT-mediated additions in immunoglobulin heavy-chain transcripts from complementary DNA obtained from Patients B5 and C1 with the number in four healthy controls and two patients with mutations in *BTK* who had a similar number of peripheral-blood B cells. In the patients and the controls, the number of TdT-mediated additions was within the normal range[23] (Table S3 in the Supplementary Appendix). The small number of CD34–CD19+ cells in bone marrow aspirates from Patients C1 and C2 were equally divided between pre-B cells that were negative for surface immunoglobulin and immature B cells that were positive for surface immunoglobulin (Fig. 3B), indicating that the block in B-cell differentiation was not complete and some precursors were able to differentiate into B cells.

DISCUSSION

CVID is characterized by late-onset hypogammaglobulinemia and a poor antibody response to infectious and vaccine antigens.[1,2] The genetic cause is unknown in the majority of cases, and less than 10% of patients have a family history of the disease. Most patients have normal numbers of B cells but lack plasma cells. This study documents the progressive loss of serum immunoglobulins and B cells in a subset of patients with CVID associated with heterozygous mutations in *IKZF1*. Clinical and laboratory findings varied among these persons, particularly during childhood. However, 13 of the 14 study participants who were older than 25 years of age were clinically symptomatic, and all these adults had laboratory evidence of immunodeficiency. Some of the children and adults had a surprisingly mild clinical course despite low concentrations of serum immunoglobulins, decreased numbers of B cells, and inadequate treatment with gamma globulin. One adult patient (D1) and several chil-

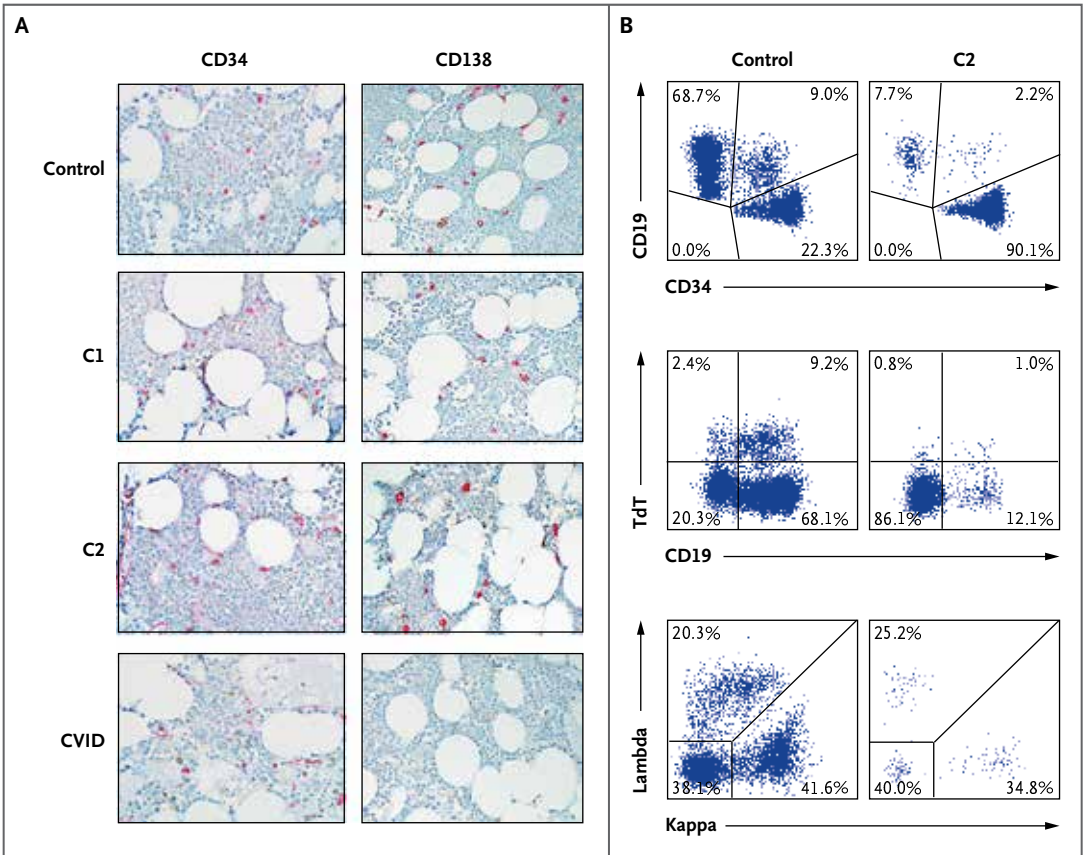


Figure 3. Immunohistochemical Analysis of Bone Marrow–Biopsy Specimens and Flow-Cytometric Analysis of Bone Marrow Aspirates.

Panel A shows immunohistochemical staining for CD34+ cells (hematopoietic stem cells) and CD138+ cells (plasma cells) in bone marrow–biopsy specimens from a healthy control, Patients C1 and C2, and a patient with typical common variable immunodeficiency (CVID). CD34+ and CD138+ cells are present in the control and in Patients C1 and C2 (red staining); CD34+ cells are present but CD138+ cells are absent in the patient with typical CVID. Panel B shows the results of flow-cytometric analysis of bone marrow aspirates from a healthy control and Patient C2. A total of 500,000 events were analyzed for both persons. A gate was used that collected all cells that were positive for CD34, CD19, or both (top and middle graphs) and cells that were negative for CD34 but positive for CD19 (bottom graph). The graph at the top shows a marked decrease in CD34+CD19+ pro-B cells in Patient C2 as compared with the control. The middle graph shows that precursors of pro-B cells that express CD34+ and cTdT+ (cytoplasmic terminal deoxynucleotidyl transferase) but lack CD19 are also decreased. The bottom graph shows that the relative numbers of pre-B cells (CD34–CD19+sIg– [surface immunoglobulin]) to immature B cells (CD34–CD19+sIg+), as documented by the expression of surface kappa or surface lambda, in the patient is similar to that in the control, although the total number of cells in both populations is decreased in the patient.

dren with IKAROS deficiency were asymptomatic, which suggests that penetrance is incomplete (Fig. S12 in the Supplementary Appendix); however, clinical manifestations may appear as late as the sixth decade of life. Nevertheless, several observations indicate that the clinical penetrance of *IKZF1* mutations is very high. The occurrence of de novo mutations in two of the six kindreds and the strong purifying (or negative) selection for *IKZF1* indicate that there is selection against these mutations in evolution. In addition, *IKZF1* ranks second to *RPSA* (and higher than *CTLA4* and *GATA2*) in terms of negative selection among the 15 known autosomal dominant primary immunodeficiencies caused by haploinsufficiency.[24-27]

The mechanism of dominance in Family F is most likely haploinsufficiency on the basis of the complete deletion of one of the *IKZF1* alleles; however, the other genes included in the dele-

tion may contribute to the phenotype. The situation is also complex in Families A, B, C, and D, in which the mutant allele results in a stable protein that is able to dimerize with wild-type IKAROS but is unable to bind target DNA (Fig. S6 and S7, respectively, in the Supplementary Appendix). On the basis of previously reported data,[15] IKAROS protein lacking zinc fingers 1, 2, and 3, as in Family E, behaves similarly to the missense proteins in Families A through D. Because IKAROS functions as a dimer, one might expect these proteins to act in a dominant negative fashion, similar to that recently reported for patients with heterozygous mutations in the DNA-binding domain of E47,[28] another transcription factor required for B-cell development. However, both EMSAs and confocal microscopy showed that the mutant proteins do not inhibit the DNA binding of wild-type IKAROS (Fig. S7). Furthermore, the clinical and laboratory findings in the patients with defects restricted to the DNA-binding domain of IKAROS are similar to those seen in the patients with complete deletion of the gene.

In contrast, the patients with mutations in the DNA-binding domain of IKAROS had a more striking increase in the number of CD8+ cells than did the patients with complete gene deletion. A recent study suggests that murine CD8+ cells that are haploinsufficient for Ikaros produce increased amounts of autocrine interleukin-2 when stimulated.[29] This may explain the increased numbers of CD8+ cells, suggesting a relatively T-cell–specific dominant negative effect in patients with mutations resulting in stable proteins that fail to bind DNA.

Y210C, a different heterozygous de novo mutation in *IKZF1*, was reported in a premature infant with pancytopenia and complete loss of B cells and natural killer cells.[21] The pathophysiological features and severity of the phenotype in this infant cannot be readily explained but may have been due to defects in functions of IKAROS that are unrelated to DNA binding or to additional genetic or nongenetic modifying factors. One of the more striking features of recently identified autosomal dominant genetic defects is the marked heterogeneity in phenotype caused by mutations in the same gene. In some instances, this heterogeneity results from the fact that different mutations in the same gene have very different effects on protein function. In other instances, modifying genetic factors, environmental or infectious exposures, or even the age at which environmental or infectious exposures occur may influence the clinical features.[7]

S. pneumoniae is a common infectious organism in patients with hypogammaglobulinemia, poor antibody function, and low B-cell numbers[30]; it also was common in our patients who had mutations in *IKZF1*. There was no evidence of increased susceptibility to viral or fungal infections in any of these patients. Thus, like other types of CVID, IKAROS deficiency should be viewed as a "predominantly antibody deficiency" on the basis of the International Union of Immunological Societies classification of primary immunodeficiency diseases.[4]

There are several murine models of Ikaros deficiency.[20,31-33] The most severe phenotype is associated with an amino acid substitution (H191R) in zinc finger 3 of the DNA-binding domain.[20] Homozygosity for this mutation is lethal to the embryo because of anemia. Heterozygous mice have normal numbers of peripheral-blood B cells but reduced numbers of B-cell precursors in the bone marrow. Heterozygous *Ikzf1*^{H191R} mice or *Ikzf1*$^{del(ex3\text{-}ex4)}$ mice (mice with a mutation that deletes three of the N-terminal zinc fingers) have a high incidence of T-cell leukemia.[20,34] In contrast, somatic loss-of-function and dominant negative mutations in *IKZF1* are predominantly associated with B-cell leukemias in humans.[35-38] Furthermore, polymorphic variants in *IKZF1* that decrease the number of transcripts constitute a risk factor for both pediatric and adult B-cell ALL.[39] In our study, typical childhood B-cell ALL developed in 2 of the 29 patients with germline heterozygous mutations in *IKZF1*. Thus, the penetrance for acquired hypogammaglobulinemia was higher than that for leukemia. This finding is compatible with data suggesting that mutations in *IKZF1* are not the initiating event in leukemias.[38] Leukemias associated with somatic mutations in *IKZF1* lead to overexpression of hematopoietic stem-cell genes.[37,40] Overexpression of stem-cell genes in patients with germline heterozygous mutations in *IKZF1* may result in stem-cell exhaustion (i.e., excessive proliferation of stem cells, resulting in premature senescence or anergy). This in turn could cause an acceleration of the normal decrease in B-cell production that occurs with age.[41]

We speculate that the relatively mild clinical

phenotype in the patients with heterozygous mutations in *IKZF1* may be due to the production of functional antibodies early in life, with the generation of some CD27+ memory B cells and the persistence of long-lived plasma cells in the bone marrow. The presence of these CD27+ memory B cells and plasma cells, in the context of very low numbers of peripheral-blood B cells, reversed CD4:CD8 ratios, and a family history compatible with autosomal dominant inheritance, distinguishes patients with IKAROS mutations from other patients with CVID.

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APPENDIX

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