

Abnormal hematopoiesis and autoimmunity in human subjects with germline *IKZF1* mutations

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Background: Ikaros, which is encoded by *IKZF1*, is a transcriptional factor that play a critical role in hematopoiesis. Somatic *IKZF1* alterations are known to be involved in the pathogenesis of leukemia in human subjects. Recently, immunodeficiency caused by germline *IKZF1* mutation has been described.

Objective: We sought to describe the clinical and immunologic phenotypes of Japanese patients with heterozygous *IKZF1* mutations.

Methods: We performed whole-exome sequencing in patients from a dysgammaglobulinemia or autoimmune disease cohort and used a candidate gene approach in 4 patients. Functional and laboratory studies, including detailed lymphopoiesis/hematopoiesis analysis in the bone marrow, were performed.

Results: Nine patients from 6 unrelated families were identified to have heterozygous germline mutations in *IKZF1*. Age of onset was 0 to 20 years (mean, 7.4 years). Eight of 9 patients presented with dysgammaglobulinemia accompanied by B-cell deficiency. Four of 9 patients had autoimmune disease, including immune thrombocytopenic purpura, IgA vasculitis, and systemic lupus erythematosus. Nonautoimmune pancytopenia was observed in 1 patient. All of the mutant Ikaros protein demonstrated impaired DNA binding to the target sequence and abnormal diffuse nuclear localization. Flow cytometric analysis of bone marrow revealed reduced levels of common lymphoid progenitors and normal development of pro-B to pre-B cells. **Conclusions:** Germline heterozygous *IKZF1* mutations cause dysgammaglobulinemia; hematologic abnormalities, including B-cell defect; and autoimmune diseases. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Acute lymphoblastic leukemia, autoimmune disease, B-cell deficiency, common lymphoid progenitor, DNA binding, dysgammaglobulinemia, germline mutation, Ikaros, *IKZF1*, nuclear localization

Lymphopoiesis and hematopoiesis are controlled by numerous transcription and signaling factors, which act at distinct stages. Loss of activity of these factors results in developmental arrest and leukemic transformation. Ikaros, which is encoded by *IKZF1*, is an essential transcription factor broadly expressed in hematopoietic cells.^{1,2}

In human subjects a critical role for Ikaros in B-cell development is well recognized. Somatic alterations in *IKZF1* have been frequently observed in patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL), especially in patients with Philadelphia-positive (Ph⁺) BCP-ALL, which represents more than 80% of cases.³ Ikaros-null (*Ikzf1*^{null/null}) mice are characterized by a lack of hematopoietic stem cells (HSCs), an absence of B cells and their progenitors, and development of T-cell leukemia/lymphoma with high penetrance.^{1,4,5} Several murine models have been reported with various degrees of abnormal lymphopoiesis/hematopoiesis. Differential gene regulation by individual zinc finger (ZF)-containing proteins contributes to phenotypic heterogeneity.⁶ Although functional analysis of mutant Ikaros was not comprehensively performed, an infant who died of congenital pancytopenia was reported to carry a constitutive heterozygous *IKZF1* mutation.⁷ As for the immune system, genome-wide association studies revealed a significant association between *IKZF1*

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Abbreviations used

BCP-ALL:	B-cell precursor acute lymphoblastic leukemia
CLP:	Common lymphoid progenitor
CMP:	Common myeloid progenitor
EMSA:	Electrophoresis mobility shift assay
GMP:	Granulomonocyte progenitor
HSCT:	Hematopoietic stem cell transplantation
HSC:	Hematopoietic stem cell
ITP:	Immune thrombocytopenic purpura
LMPP:	Lymphoid-primed multipotent progenitor
Ph ⁺ :	Philadelphia positive
SLE:	Systemic lupus erythematosus
WES:	Whole-exome sequencing
WT:	Wild-type
ZF:	Zinc finger

variants and systemic lupus erythematosus (SLE).⁸ These findings suggest the possibility of an association between various phenotypes in human subjects and a germline *IKZF1* mutation. Very recently, a series of 6 families with germline mutations in *IKZF1* associated with common variable immunodeficiency reported low B-cell numbers.⁹ The report described a progressive loss of B cells and serum immunoglobulins and a predisposition to BCP-ALL. The identified *IKZF1* mutations revealed a loss-of-function mutation in *IKZF1*, leading to impairment of its target sequence. This report clearly showed that an autosomal dominant heterozygous germline mutations in *IKZF1* could be responsible for a novel immunodeficiency predominantly characterized by an antibody deficiency. However, an in-depth description of clinical manifestations, together with the effect of *IKZF1* mutations on B-cell development and autoimmunity, remains to be elucidated.

Here we describe 9 patients from 6 families with heterozygous *IKZF1* mutations. The patients are characterized by a defect in B cells, which is caused by aberrant development of common lymphoid precursors (CLPs). Notably, some patients have pancytopenia or autoimmune diseases, including immune thrombocytopenic purpura (ITP) and SLE.

METHODS**Study approval**

Informed consent was obtained from patients or their parents. The study was conducted in accordance with the Helsinki Declaration and approved by the ethics boards of the University of Toyama and Tokyo Medical and Dental University.

Genetic analysis

For whole-exome sequencing (WES), exome capture was carried out with a SureSelect Human All Exon V5 Kit (Agilent Technologies, Santa Clara, Calif), and massively parallel sequencing was performed with a HiSeq 2000 platform (Illumina, San Diego, Calif) with 100-bp paired-end reads. Data were processed with an in-house-constructed analysis pipeline (Genomon-exome: <http://genomon.hgc.jp/exome/en/>), which aligned the reads by using the Burrows-Wheeler aligner 0.5.8, counted variant allele numbers by using Samtools, and annotated genes by using ANNOVAR. Variant filtering steps are described in Table E1 in this article's Online Repository at www.jacionline.org. Sanger sequencing was performed to validate the results. The crystal structure of a C2H2 ZF protein (Protein Data Bank 1G2D) was produced by using Chimera.^{10,11}

Flow cytometry

PBMCs were stained with fluorochrome-conjugated antibodies. Stained cells were analyzed with the BD LSRFortessa (BD Biosciences, San Jose, Calif), and data were processed with FlowJo software (Tree Star, Ashland, Ore). For lymphocyte phenotyping, mAbs were used to stain the cell surface and are listed in Table E2 in this article's Online Repository at www.jacionline.org. For intracellular forkhead box P3 staining, PBMCs were labeled with anti-CD4 (Miltenyi Biotec, Bergisch Gladbach, Germany) and CD25 (Dako, Tokyo, Japan). Then cells were fixed and permeabilized with a Fixation/Permeabilization Kit (eBioscience, San Diego, Calif), washed in permeabilization buffer (eBioscience), and stained with anti-forkhead box P3 (clone 259D/C7; BD PharMingen, San Diego, Calif). For T-cell receptor repertoire analysis, an IOTest Beta Mark TCR-Vβ Repertoire Kit (Beckman Coulter, Brea, Calif) was used. Bone marrow-derived mononuclear cells were used for evaluation of hematopoiesis in bone marrow. Cells were stained with the mAbs listed in Table E2 and divided as follows: HSCs (CD34⁺CD38[−]Lin[−]CD90⁺CD45RA[−]), multipotent progenitors (CD34⁺CD38[−]Lin[−]CD90[−]CD45RA[−]), lymphoid-primed multipotent progenitors (LMPPs; CD34⁺CD38⁺Lin[−]CD45RA⁺CD10[−]CD62^{hi}), CLPs (CD34⁺CD38⁺Lin[−]CD45RA⁺CD10⁺), common myeloid progenitors (CMPs; CD34⁺CD38⁺Lin[−]CD45RA[−]CD135⁺CD10[−]CD62L^{−or+}), granulomonocyte progenitors (GMPs; CD34⁺CD38⁺Lin[−]CD45RA⁺CD135⁺CD10[−]CD62L^{−or+}), and megakaryocyte-erythroid progenitors (CD34⁺CD38⁺Lin[−]CD45RA[−]CD135[−]CD10[−]CD62L^{−or+}). For B-cell lineage analysis, cells were stained with anti-CD19 (Miltenyi Biotec), IgM (BD PharMingen) and VpreB (clone HSL96; BioLegend, San Diego, Calif) after fixation and permeabilization, as previously described.¹²

Immunoblot and electrophoresis mobility shift assay

The entire coding region of *IKZF1* was amplified from wild-type (WT) cDNA by using primers containing *EcoRI* and *XhoI* restriction sites. PCR products and pcDNA3 vector (Invitrogen, Waltham, Mass) were digested with *EcoRI* and *XhoI*, and each fragment was ligated with the Quick Ligation Kit (New England Biolabs, Ipswich, Mass). The mutant Ikaros expression vectors were generated by means of site-directed mutagenesis. The plasmids containing WT or mutant *IKZF1* genes were transfected into HEK293T cells by using Lipofectamine LTX Reagent (Thermo Fisher Scientific, Waltham, Mass), according to the manufacturer's protocol. At 24 hours after transfection, the cells were subjected to immunoblot analysis and nuclear extraction for the electrophoresis mobility shift assay (EMSA). We used NE-PER Nuclear and Cytoplasmic Extraction Regents (Thermo Fisher Scientific) for nuclear extraction. We incubated nuclear extract with ³²P-labeled (α-dATP) IK-bs4 probe (forward: 5'-gtc aTG ACA GGG AAT ACA CAT TCC CAA AAG C-3' and reverse: 5'-gtc aGC TTT TGG GAA TGT GTA TCC CCT GTC A-3'), which contains a high-affinity Ikaros binding site.¹³ We used anti-Ikaros antibody (ab26083; Abcam, Cambridge, United Kingdom) and anti-V5 antibody (Sigma-Aldrich, St Louis, Mo) for immunoblotting and the EMSA supershift assay. Primer sequences and PCR conditions are available on request.

Immunofluorescence staining

NIH3T3 cells were plated at a density of 5 × 10⁴ cells/mL in 6-well culture dishes with a cover glass. Twenty-four hours later, pcDNA3 vector (1.0 μg per plate for single-plasmid transfection or 1.5 μg per plate and 0.75 μg for each plasmid for cotransfection experiments) containing HA-tagged WT, C147R, N159S, R162Q, or Y210C alleles of *IKZF1* and/or V5-tagged WT alleles of *IKZF1* were introduced by means of lipofection with Lipofectamine 3000 Reagent, according to the manufacturer's guidelines (Invitrogen). Immunostaining was performed, as previously described.¹⁴ Briefly, 24 hours after transfection, the cells were fixed in the presence of 4% paraformaldehyde (pH 7.4) for 15 minutes and permeabilized with PBS containing 0.5% NP-40 for 10 minutes. They were then blocked by means of incubation overnight with 10% goat serum in PBS in the presence of an anti-V5 mouse monoclonal antibody (1:200 dilution; Medical & Biological Laboratories, Nagoya, Japan)

TABLE I. Clinical and immunologic features of the symptomatic patients

	A.1	B.1	C.I.1	C.II.1	C.II.2	D.I.1	D.III.1	E.II.1	F.2
Age, sex	7 y, M	16 y, F	37 y, M	12 y, F	10 y, M	68 y, F	11 y, M	8 y, M	5 mo, M
Age of onset	5 y	2 mo	10 y	12 y	10 y	20 y	6 y	3 y	0 mo
Sinopulmonary bacterial infections	—	+	+	—	—	—	+	—	—
Other infections	—	PCP	—	—	—	—	—	—	—
Autoimmune disease	ITP	—	—	—	—	ITP	IgA vasculitis	SLE, Evans syndrome	—
Nonautoimmune cytopenia	—	—	—	—	—	—	—	—	+
Other clinical findings	—	—	—	—	—	—	Premature birth, intracranial hemorrhage, epilepsy	—	Premature birth, intracranial hemorrhage
T cells	CD4/CD8 ↓ CD4 ⁺ naive ↓ Treg ↓ Skewed TCR Vβ repertoire	CD4/CD8 ↓ CD4 ⁺ memory ↓ CD8 ⁺ memory ↓ DNT ↑, NKT ↓, Treg ↓ Proliferation ↓	CD4/CD8 ↓ CD4 ⁺ naive ↓	CD4 ⁺ naive ↓	CD4 ⁺ naive ↓	CD4/CD8 ↓ T _{FH} ↑	CD8 ⁺ naive ↓ NKT ↓	CD4/CD8 ↓ CD4 ⁺ naive ↓ CD8 ⁺ naive ↓	γδ T cell ↓ Proliferation ↑
B cells	↓	↓	↓	↓	↓	NL	↓	↓	↓
NK cells	CD16 ⁺ CD56 [−] ↑ NK cell activity ↓	↓	CD16 ⁺ CD56 [−] ↑	NL	NL	NL	NL	↓	NL
Other abnormal values	Platelet aggregation activity ↓	No	No	No	No	No	No	No	No

DNT, Double-negative T cells; F, female; M, male; ND, not determined; NK, natural killer; NL, normal; PCP, *Pneumocystis jirovecii* pneumonia; TCR, T-cell receptor; T_{FH}, T follicular helper cells; Treg, regulatory T cells.

and/or an anti-HA rabbit polyclonal antibody (1:200 dilution; Sigma-Aldrich). Alexa Flour 594–conjugated goat anti-mouse IgG antibody (Invitrogen, Eugene, Ore) and/or Alexa Flour 488–conjugated goat anti-rabbit IgG antibody (Invitrogen) were used as secondary antibodies. Cell nuclei were stained with Hoechst 33342 (Invitrogen). Images were acquired with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan), with identical exposure times to optimize the comparisons.

PBMCs, which were not stimulated or stimulated with anti-CD3/CD28 activating Dynabeads (Life Technologies, Oslo, Norway) for 24 hours, were used for immunofluorescence staining by using standard methods.¹⁵ Briefly, cytospin preparations were fixed with 4% paraformaldehyde, permeabilized with acetone, washed, and incubated with anti-Ikaros antibody (Abcam). 4'-6-Diamidino-2-phenylindole (Vector Laboratories, Burlingame, Calif) was used for counterstaining. Slides were analyzed with a fluorescence microscope (FV10i; Olympus, Tokyo, Japan) equipped with Fluoview viewer and review station (Olympus).

Proliferation assays

PBMCs were labeled with carboxyfluorescein succinimidyl ester (3 μmol/L; eBioscience) for 5 minutes at room temperature and stimulated for 3 days with anti-CD3 and anti-CD28 antibodies (1, 0.3, or 0.1 μg/mL; BD PharMingen). Then cells were stained for CD4 and CD8 and analyzed by means of flow cytometry.

Splicing product analysis

PCR was performed with cDNA from PBMCs prepared by using SuperScript VILO (Invitrogen). PCR products, which included the entire

coding region on *IKZF1*, were cloned with the TOPO TA cloning kit (Life Technologies), and independent clones were sequenced. Primers are listed in Table E3 in this article's Online Repository at www.jacionline.org.

RESULTS

Identification of heterozygous mutations in *IKZF1*

Initially, we identified heterozygous *IKZF1* mutations in 3 families from a dysgammaglobulinemia cohort (Table I, Fig 1, and see the Supplemental Note in this article's Online Repository at www.jacionline.org). A 7-year-old boy (A.1) had ITP and dysgammaglobulinemia with low B-cell counts at 5 years of age. Patient B.1, a 16-year-old girl, had recurrent sinopulmonary bacterial infections since 2 months of age and was treated with intravenous immunoglobulin. She also had a history of *Pneumocystis jirovecii* pneumonia. Patient D.III.1 was an 11-year-old boy who had bacterial pneumonia with agammaglobulinemia at 6 years of age and IgA vasculitis at 3 years of age. After excluding the diagnosis of dysgammaglobulinemia with known causes, we performed WES using DNA samples from patients and unaffected family members and identified heterozygous *IKZF1* mutations (p.C147R, p.N159S, and p.R162Q; see Table E1). These mutations were confirmed by means of Sanger sequencing (see Fig E1 in this article's Online Repository at www.jacionline.org). p.C147R and p.N159S were *de novo* mutations. Interestingly, the unaffected mother (D.II.1) of patient D.III.1 had the same mutation at 43 years of age. His

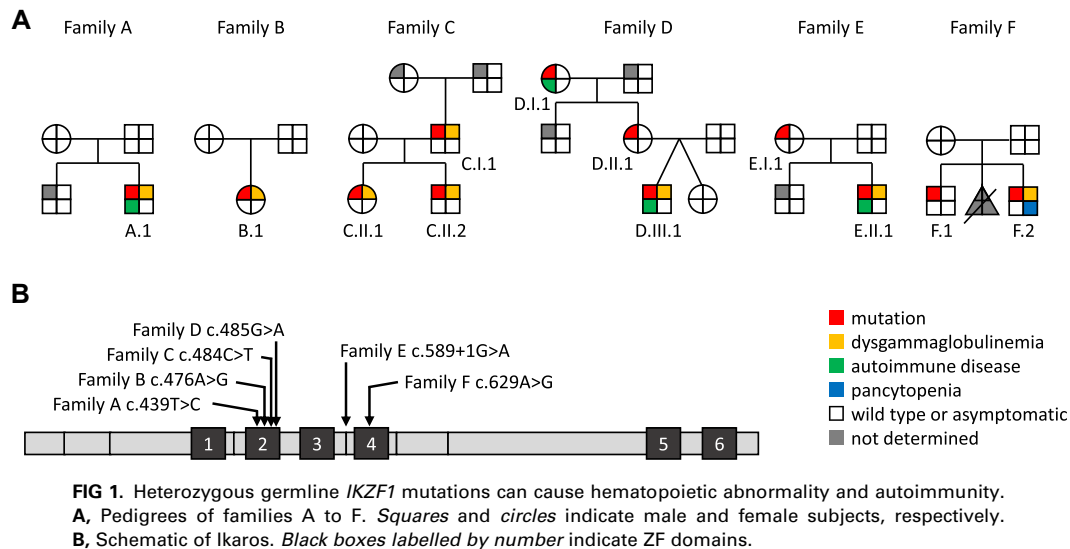


FIG 1. Heterozygous germline *IKZF1* mutations can cause hematopoietic abnormality and autoimmunity.

A, Pedigrees of families A to F. Squares and circles indicate male and female subjects, respectively.

B, Schematic of Ikaro. Black boxes labelled by number indicate ZF domains.

maternal grandmother (D.I.1) with a history of ITP without dysgammaglobulinemia also had the same mutation at 68 years of age. These findings prompted us to examine the possibility that heterozygous *IKZF1* mutations caused immunodeficiency with or without autoimmune diseases. We sequenced the *IKZF1* coding exons in a family (C.I.1, C.II.1, and C.II.2) with hypogammaglobulinemia of autosomal dominant inheritance, and we identified a heterozygous p.R162W mutation in *IKZF1*. All 4 missense mutations described above were located within the ZF2-coding sequence. One missense mutation (p.C147R) was found and affected cysteine, which coordinates zinc conferred rigidity to the finger. Three missense mutations (p.N159S, p.R162W, and p.R162Q) were also identified to affect amino acids that interact with DNA,¹⁶ suggesting a deleterious effect for maintaining the finger structure and DNA-binding activity (Fig 2).

At the same time, we performed WES in a patient (E.II.1) from an autoimmune disease cohort and identified a heterozygous *IKZF1* mutation in the exon 5 donor splice site (c.589+1G>A). The patient, an 8-year-old boy, was given a diagnosis of SLE at 3 years of age. Although his serum immunoglobulin levels were high at diagnosis, he has since developed hypogammaglobulinemia. His asymptomatic mother (E.I.1) carried the same mutation at 37 years of age. This mutation caused numerous abnormal splicing events, including insertions, deletions, or both in various locations, which are predicted to severely impair the function of ZF2, ZF3, or ZF4 (Fig 3).

Patient F.2, a 5-month-old boy, showed pancytopenia with extremely low B-cell counts just after birth. Sanger sequencing with DNA samples from whole blood and nails uncovered the reported mutation (p.Y210C).⁶ His unaffected brother (F.1) had the same mutation at 2 years and 6 months of age, but DNA from whole blood of his parents revealed no mutation, suggesting germinal mosaicism. This missense mutation affected one of the essential residues for structural integrity in ZF4 (Fig 2, A).¹⁶

Impaired DNA binding activity and diffuse nuclear localizations of Ikaro

We characterized the functional defects in mutant Ikaro by means of immunoblotting and EMSA with HEK293T cells

transiently expressing WT or mutant Ikaro. As shown in Fig 4, A, the mutant Ikaro protein was expressed at the expected molecular weight of 63 kDa. We then analyzed the intrinsic DNA-binding ability of the Ikaro proteins using EMSA with the IK-bs4 probe that contained a high-affinity Ikaro binding site (Fig 4, B). We observed DNA binding to the IK-bs4 sequence in WT Ikaro-expressing cells. The specificity of binding was confirmed by using a supershift assay (see Fig E2 in this article's Online Repository at www.jacionline.org). In contrast, all Ikaro mutants, except for the Y210C mutant, were unable to bind DNA. Intriguingly, the Y210C Ikaro mutant showed weak residual binding to the target sequence, which is consistent with previous reports.^{7,9} Taken together, all of the *IKZF1* mutations identified in the current study were loss-of-function mutations because of their inability to bind to DNA.

Localization of Ikaro to pericentromeric heterochromatin is important for its normal function in chromatin remodeling and regulation of gene expression. To investigate nuclear localization of WT or mutant Ikaro, we performed immunostaining with NIH3T3 cells transiently expressing WT and/or mutant *IKZF1*. Both HA-tagged and V5-tagged WT Ikaro localized the nucleus and showed the punctate staining pattern (Fig 4, C and D). On the other hand, HA-tagged mutant Ikaro located the nucleus but showed abnormal diffuse nuclear localization (Fig 4, C). We then investigated NIH3T3 cells coexpressing HA-tagged WT or mutant Ikaro and V5-tagged WT Ikaro (Fig 4, E). All of the HA-tagged mutant Ikaro, as well as HA-tagged WT Ikaro, displayed the punctate staining pattern when they were coexpressed with V5-tagged WT Ikaro. Therefore, similar to the previous study,⁹ abnormal diffuse nuclear localization in mutant Ikaro-expressing cells was corrected by coexpression with WT Ikaro. Interestingly, our study showed abnormal pericentromeric localization of Y210C Ikaro, whereas the previous study demonstrated a normal localization pattern of Y210C protein when expressed alone.⁹ This difference might be influenced by the experimental procedure. Immunofluorescence microscopy with anti-Ikaro antibody was performed in cells from patients (see Fig E3 in this article's Online Repository at www.jacionline.org). Lymphocytes from patients exhibited a punctate staining pattern of Ikaro in response to CD3/CD28 stimulation, as did those from healthy control subjects, which

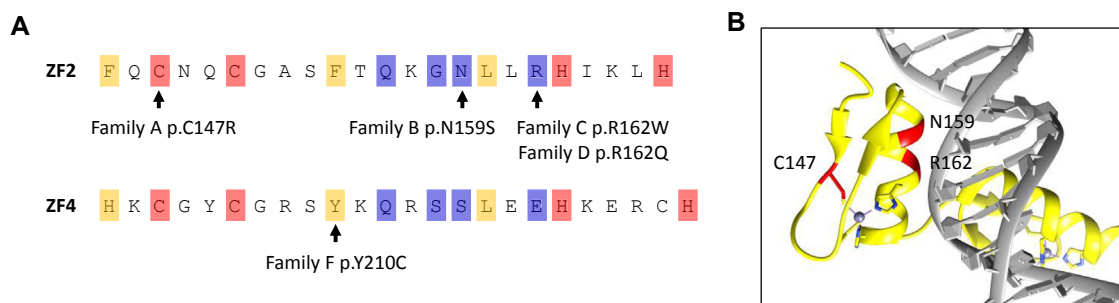


FIG 2. Genetic analysis of heterozygous mutation in *IKZF1*. **A**, ZF2 and ZF4 sequences. Cysteine (C) and histidine (H) residues, which coordinate the zinc atom, are highlighted in red. The -1, 2, 3, and 6 positions, which are the DNA-interacting residues, are highlighted in blue. The -12, -3, and 4 positions, which are essential residues for structural integrity, are highlighted in yellow. **B**, Crystal structures of *IKZF1* (yellow). C147, N159, and R162 are shown in red.

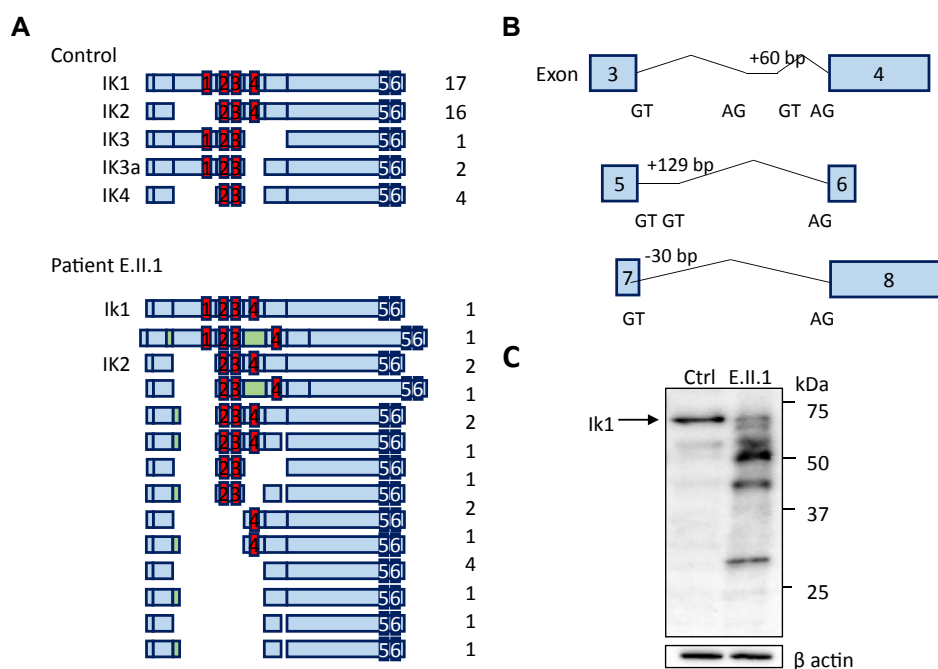


FIG 3. Splicing variants in patient E.II.1. **A**, Schematic representation of the alleles obtained by direct cloning and their numbers. **B**, Insertions, deletions, or both. **C**, Ikaros protein expression in nonstimulated PBMCs.

indicates localization to pericentromeric heterochromatin. These findings were consistent with the results of coexpression of WT and mutant Ikaros with NIH3T3 cells.

Impaired B-cell development

The recurrent observation of aberrant *IKZF1* in patients with BCP-ALL suggested its critical role in B-cell development in human subjects.⁵ Indeed, all symptomatic patients but patient D.I.1 had extremely low B-cell counts (Fig 5, A, and see Table E4 in this article's Online Repository at www.jacionline.org). The increased frequency of transitional B cells in patients C.I.1, D.III.1, E.II.1, and F.2, and the abnormal B-cell subset (CD10⁺CD24⁺CD38⁻IgD⁺IgM⁻ B cells) in patient B.1 also suggest abnormal B-cell development (Fig 5, C, and see Fig E4, A, in this article's Online Repository at www.jacionline.org). Notably,

asymptomatic subjects had normal B-cell counts, suggesting an incomplete penetrance similar to that seen in patients with other dominantly inherited immunodeficiency diseases.^{17,18} The patients, except B.1 and F.2, had infantile-onset progressive hypogammaglobulinemia, and interestingly, normal B-cell counts were confirmed in early infancy (Fig 5, B, and see Fig E4, B and C). Autoantibodies were detected at the onset of autoimmune disease. Patient F.2, with affected ZF4 function, had a lack of B cells only just after birth (Fig 6, A). His B cells spontaneously recovered, although he remained to have hypogammaglobulinemia. No somatic reversion in *IKZF1* was identified in the purified B cells (data not shown). In mouse models B-cell counts were reduced to a various degree, or B-cell recovery was also described, suggesting a mechanism of compensation.^{6,19}

To investigate hematopoiesis, including B-cell development, we evaluated bone marrow cells from patients A.1 and D.III.1

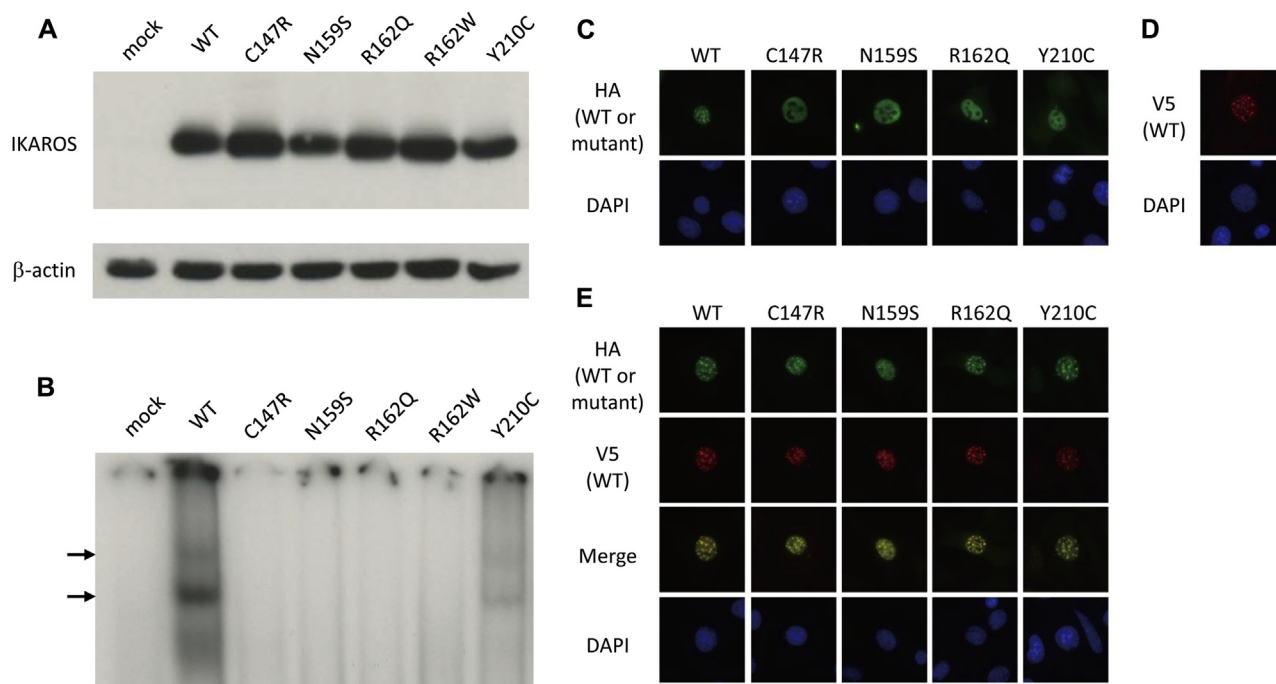


FIG 4. Functional analysis of Ikaros mutants. **A**, Immunoblot. *Upper and lower panels* indicate anti-Ikaros and anti- β -actin, respectively. Ikaros mutants are shown to be normal at 63 kDa. **B**, EMSA. Ikaros mutants (C147R, N159S, R162Q, and R162W) completely abolish DNA binding to the IK-bs4 sequence, whereas the Y210C mutant shows weak residual binding. *Arrows* indicate Ikaros-containing complexes. **C**, Subcellular localization of Ikaros protein in NIH3T3 cells expressing HA-tagged WT or mutant Ikaros. **D**, Subcellular localization of Ikaros protein in NIH3T3 cells expressing V5-tagged WT Ikaros. **E**, Subcellular localization of Ikaros protein in NIH3T3 cells coexpressing HA-tagged WT or mutant Ikaros and V5-tagged WT Ikaros.

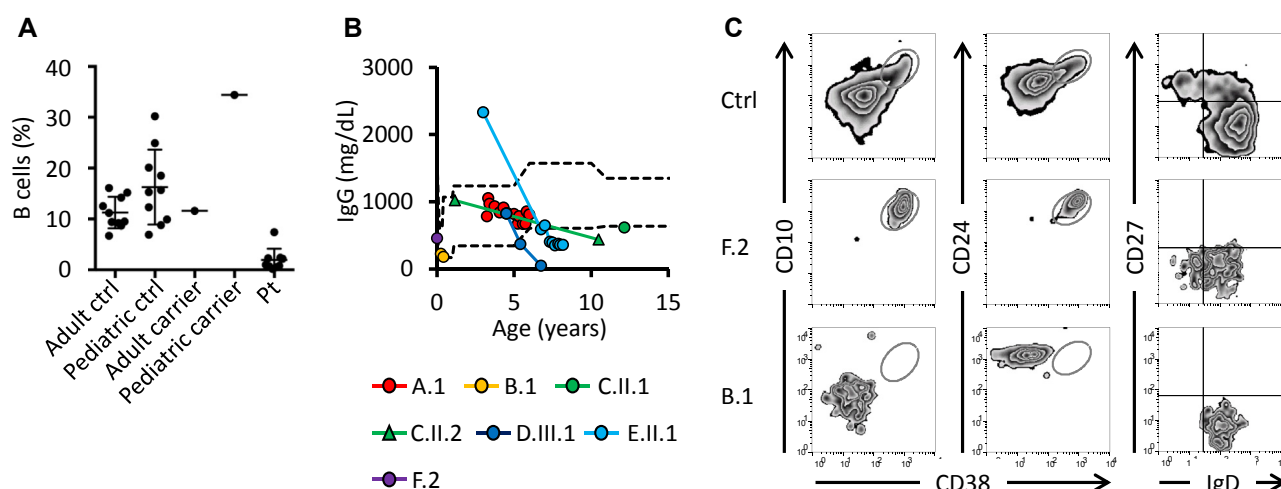


FIG 5. Immunologic studies of patients with *IKZF1* mutations. **A**, B-cell counts in lymphocytes. *Ctrl*, Control subjects; *Pt*, patients. **B**, Sequential changes of serum IgG levels before starting immunoglobulin replacement therapy. *Dotted lines* indicate upper and lower healthy control values. **C**, B-cell subsets. *Circles* indicate $CD10^+CD38^+$ or $CD24^+CD38^+$ transitional B cells.

(Fig 7). These patients showed reduced HSC and CLP counts and increased LMPP counts. Relatively normal numbers of GMPs suggested increased generation from LMPPs. The B-cell lineage cell number was 10-fold lower in the patients than in healthy control subjects, and it most likely resulted from reduced CLP counts. The relative frequencies of pro-B cells, pre-B cells, and B cells in

B-lineage cells were shown to be normal. These findings indicate that lack of peripheral B cells in patients might be caused by aberrant CLP development and not by developmental arrest of pre-B cells. Consistent with those data, previous studies in mice demonstrated that *Ikzf1* promoted HSC self-renewal, CLP development from LMPPs, and GMP development from CMPs (see Fig E5 in

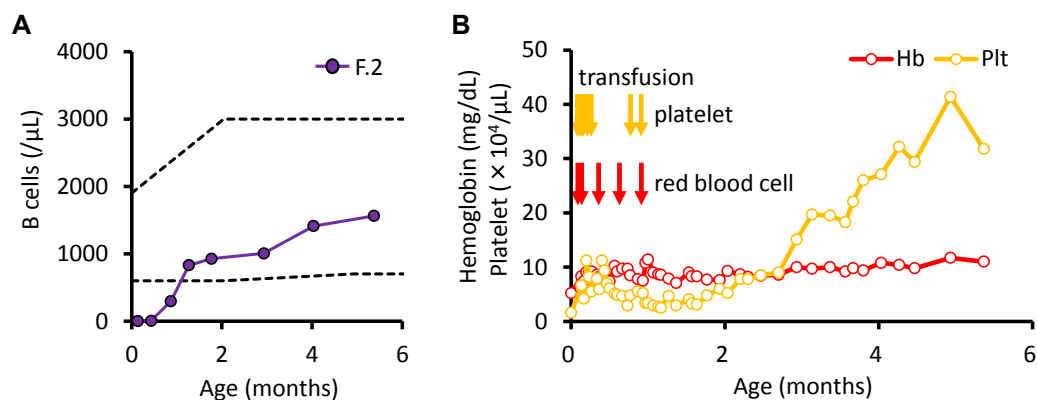


FIG 6. Sequential change of laboratory data in patient F.2. **A**, Course of B-cell counts. **B**, Sequential changes of red blood cell and platelet counts.

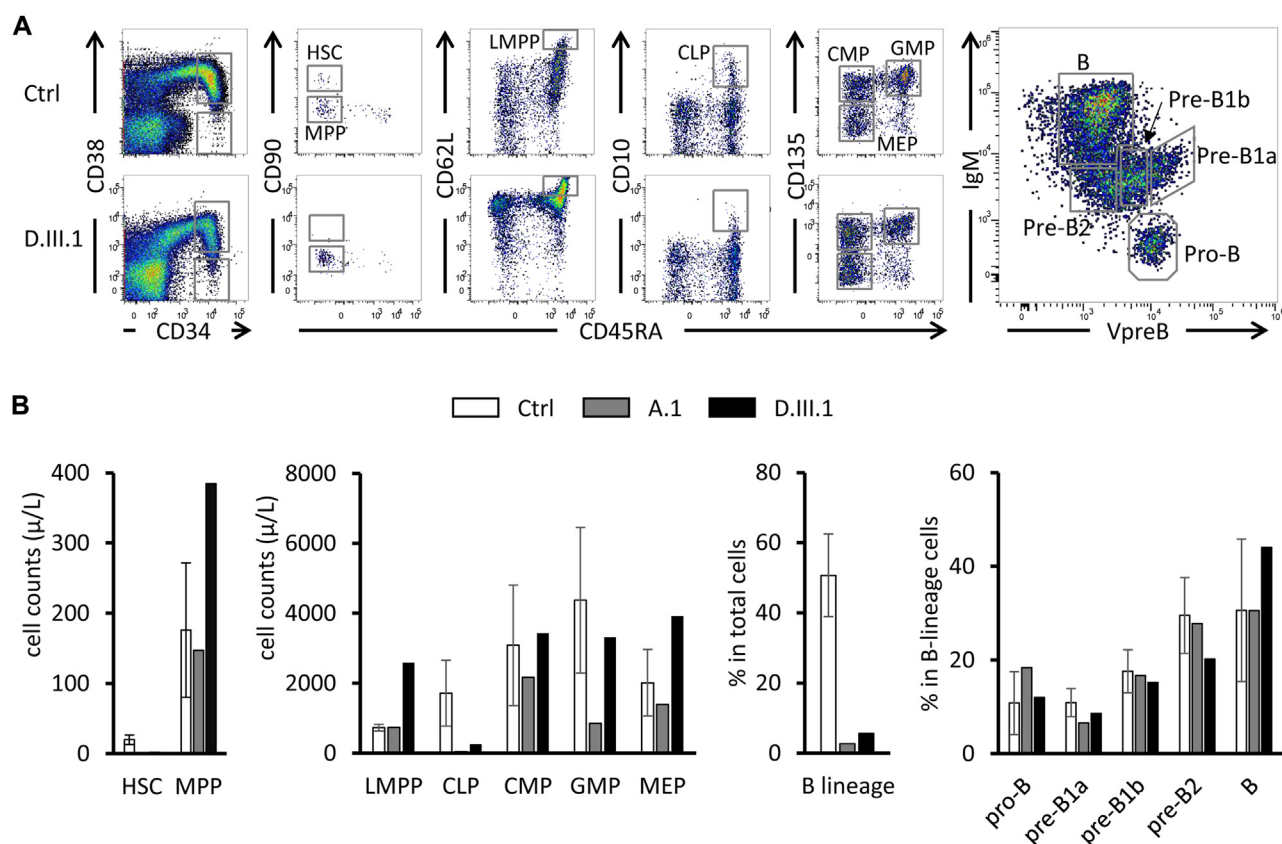


FIG 7. Hematopoietic cell and B-cell development in bone marrow. **A**, Evaluation of hematopoiesis and B-cell development in bone marrow by using multicolor flow cytometry. **B**, Comparison of each component in hematopoiesis and B-cell development in bone marrow. White bars indicate means $\pm$ SDs of the control subjects (HSCs and progenitors, $n = 4$; B lineage, $n = 10$).

this article's Online Repository at www.jacionline.org).^{4,19} Although Ikaros is essential for development from pro-B to pre-B cells in mice,²⁰ the relative frequencies of pre-B cells were normal in human subjects. In a previous study patients with *IKZF1* mutation had normal numbers of CD138⁺ plasma cells in bone marrow, which were lacking in a patient with common variable immunodeficiency.⁹ However, we could not evaluate CD138 expression in the bone marrow samples.

Other hematologic abnormalities

Immunophenotyping revealed that all patients except patient D.I.1 had increased T-cell counts and that very patient except patient F.2 had increased CD8⁺ T-cell counts (see Table E4), results that are consistent with a previous study.⁹ T-cell maturation and function were heterogeneous, including reduced regulatory T-cell counts, skewed T-cell receptor repertoire, and increased T-cell receptor $\alpha\beta^+\text{CD}4^-\text{CD}8^-$ double-negative T-cell counts,

which were found in some patients with autoimmune diseases or primary immunodeficiencies mostly associated with autoimmunity (see Table E4 and Fig E6, A, in this article's Online Repository at www.jacionline.org).^{21,22} Lack of $\gamma\delta$ T cells and a lower threshold of T-cell proliferation were observed, which were also described in mice (see Fig E6, B).^{1,6,23} T cells from patient B.1, who had a history of *Pneumocystis jirovecii* pneumonia, exhibited a predominance of naive phenotype and a poor response to anti-CD3/CD28 antibody, which were different features from those of the other patients (see Table E4 and Fig E6, B). Other hematologic abnormalities, including low natural killer cell activity, anemia, and thrombocytopenia, were observed in a small fraction of the patients. Patient F.2, with affected ZF4 function, had nonautoimmune pancytopenia, which required frequent red blood cell and platelet transfusion just after birth. The infant reported the same phenotype and underwent allogeneic hematopoietic stem cell transplantation (HSCT) early in life but died of multiorgan failure at 40 days after HSCT.⁷ Notably, patient F.2, who planned to undergo HSCT, recovered from pancytopenia spontaneously in 1 month and has survived without HSCT (Fig 6, B).

DISCUSSION

Here we described 9 patients from 6 families with heterozygous *IKZF1* mutations. We identified 5 missense mutations and 1 splice site mutation in the *IKZF1*. Two missense mutations (p.R162Q and p.Y210C) were previously reported,^{7,9} but 4 mutations (p.C147R, p.N159S, p.R162W, and c.589+1G>A) were novel. Interestingly, the p.R162L mutation was described by Kuehn et al,⁹ and p.R162 might be recurrently mutated in *IKZF1*. All but 1 patient presented with dysgammaglobulinemia accompanied by a decreased number of peripheral B cells. Despite this finding, 3 asymptomatic carriers were found to have a normal number of peripheral B cells. Intriguingly, 4 patients had autoimmune diseases, including ITP, IgA vasculitis, and SLE. In addition, nonautoimmune pancytopenia was observed in 1 patient. A germline mutation in *IKZF1* was originally identified in an infant with pancytopenia and absence of B cells.⁷ In addition, 29 patients from 6 unrelated families have been reported thus far.⁹ However, the clinical manifestation of autoimmunity, which was only observed in 1 patient with ITP, was not a focus in the previous study.⁹ The current study also demonstrated that the patients showed aberrant CLP development, probably causing a lack of peripheral B cells. Therefore our study expands our understanding of the features of autosomal dominant Ikaros deficiency. In this cohort 3 of 12 patients were asymptomatic, and the penetrance of this disease was 75% (9/12), which was similar to the value of 72% (21/29) in a previous study.⁹ The rate might be unaffected by ethnic background.

Although *Ikzf1*^{null/null} mice exhibit various abnormal hematopoiesis, including a lack of B-lineage cells, *Ikzf1*^{null/wt} mice have normal lymphocyte development.^{6,20} In contrast, the identification of mutations in *IKZF1* in B cell-deficient patients in this study, together with a previous report, provides genetic evidence for a heterozygous *IKZF1* mutation causing dysgammaglobulinemia with or without autoimmune disease in human subjects. This observation is supported by heterozygous *IKZF1* alterations in patients with BCP-ALL and, notably, the heterozygous p.N159S and p.R162W mutations that were previously described in Ph⁺ BCP-ALL cells.²⁴ These clinical and

experimental observations suggest that Ikaros might play a more important role in B-cell development in human subjects than in mice.

A detailed evaluation of bone marrow from the patients revealed that aberrant CLP development can cause a lack of peripheral B cells. Consistent with our data, Kuehn et al⁹ described reduced pro-B cells and early precursors (CD19⁺CD34⁺ and CD19⁺TdT⁺ cells, respectively) in patients with germline *IKZF1* mutations. Although the patients had reduced CLP counts, they had normal peripheral T-cell counts, which is consistent with the idea that T-cell lineage cells can directly develop from LMPPs beyond CLPs (see Fig E5).²⁵ However, mouse studies have revealed that Ikaros influenced not only CLP development but also pre-B-cell development. The cells from patients with Ph⁺ BCP-ALL with somatic *IKZF1* alterations evade normal pre-B-cell differentiation.²⁶ The arresting of B cells in different stages of development might be due to differences in species or age.²⁰ We also speculate that *IKZF1* mutations can affect pre-B-cell receptor signaling and the cell cycle in certain leukemic cells with pre-existing genetic changes. This is supported by the fact that *IKZF1* alterations are found as secondary genetic changes in Ph⁺ BCP-ALL cells.²⁷ The current and previous studies showed an increase in CD8⁺ T cells and other T-cell dysfunctions in most of the patients.⁹ These data implied that Ikaros might play some role in T-cell development, as well as B-cell development. Further investigation for T cells would be required.

Patient F.2 with the Y210C mutation had pancytopenia, resulting in hemorrhage just after birth. This is a similar phenotype reported in a patient with the Y210C mutation who underwent HSCT before hematologic recovery.⁷ The Y210C mutation or possibly mutations affecting ZF4 might contribute to pancytopenia before and just after birth. Mouse models have shown phenotypic heterogeneity caused by individual mutations, especially those affecting individual ZFs,⁶ and the differential requirements of Ikaros during fetal and adult hematopoiesis.¹ The residual DNA-binding activity was observed in Y210C Ikaros. Although normal pericentromeric localization of Y210C protein was found by using an artificially transfected cell line in the previous report,⁹ our *in vitro* experiment demonstrated abnormal subcellular localization of Ikaros, as well as the case report of the Y210C mutation.⁷ The difference among the reports might depend on use of a dominant normal allele or experimental procedure. In any case the Y210C mutation was considered to be a hypomorphic mutation, and it might be associated with a less severe clinical phenotype with the hematologic recovery, including B-cell counts.

In a previous study 2 of the 29 patients had BCP-ALL at 3 and 5 years of age.⁹ However, there were not any patients who had neoplastic disorders, including BCP-ALL, in our study. The patients, especially those with p.N159S and p.R162W mutations, might have increased risk of BCP-ALL because the same somatic mutations were found in patients with Ph⁺ BCP-ALL.²⁴

Four of 9 patients had autoimmune diseases, although only 1 presented with ITP in a previous study.⁹ Heterozygous germline mutations in *IKZF1* might be associated not only with B-cell deficiencies but also with autoimmune diseases. The clinical interest is that autoimmune disease can be presented even if dysgammaglobulinemia does not develop. Although genome-wide association studies are providing support for the relationship

between Ikaros and SLE,⁸ its pathogenesis remains unclear. Further investigation is required.

In conclusion, we identified germline heterozygous *IKZF1* mutations that caused dysgammaglobulinemia; hematologic abnormalities, including B-cell defects; and autoimmune diseases. This study provides new evidence for the involvement of *IKZF1* in human hematopoiesis and immunology.

We thank the patients and their families for their participation in this study.

Clinical implications: Heterozygous germline mutations in *IKZF1* are associated with a novel human immunodeficiency characterized by B-cell deficiency. Peripheral B-cell deficiency can be caused by aberrant CLP development.

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Supplementary materials for "**Abnormal hematopoiesis and autoimmunity in humans with germline *IKZF1* mutations**" in the Journal of Allergy and Clinical Immunology

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Supplemental Note

Clinical presentation of the patients

Family A

A.1 is a seven-year-old boy with a history of immune thrombocytopenic purpura (ITP). He developed thrombocytopenia (platelet count 11,000/ μ L) at the age of five years. Bone marrow aspiration revealed normocellular marrow with increased megakaryocytes, and increased levels of platelet-associated IgG (75 ng/ 10^7 cells, reference value 9.0-25.0 ng/ 10^7 cells) were found. He received intravenous immunoglobulin (IVIG, total dose 2 g/kg) infusion. Although platelet counts remained between 50,000 and 100,000/ μ L for three years, his platelet counts spontaneously increased to 300,000/ μ L at age 7. He had a bleeding tendency due to reduced platelet aggregation activity. Dysgammaglobulinemia (IgG 784 mg/dL, IgA 2 mg/dL and IgM 9 mg/dL) was found at diagnosis of ITP. Although measles and rubella-specific antibodies were undetectable after vaccinations, he had no recurrent or severe infection without IVIG.

Family B

B.1 is currently a 16-year-old female, who presented with lung abscess at the age of two months. She had normal T-cell counts and neutrophil function. Because agammaglobulinemia was found, IVIG was initiated. At ages two and three, she developed *pneumocystis jirovecii*

pneumonia. Although IVIG switched to subcutaneous immunoglobulin and trough IgG levels were maintained at greater than 1,400 mg/dL, she had recurrent sinopulmonary bacterial infections and developed bronchiectasis. She suffered from chickenpox without severe clinical findings at age 10.

Family C

C.I.1 is a 37-year-old male, who was diagnosed with common variable immunodeficiency at the age of 10 years. He had reduced B-cell counts and received IVIG. Serum immunoglobulin levels were sometimes examined in his daughter (C.II.1) and son (C.II.2) because of their family history. Hypogammaglobulinemia was recently revealed at the ages of 12 and 10 years old in the daughter and son, respectively.

Family D

D.III.1 is currently an 11-year-old boy with a history of premature birth at gestational age of 33 weeks and subependymal hemorrhage. He was associated with IgA vasculitis with normal clinical course at three years of age. Normal B-cell counts and serum immunoglobulin levels were confirmed at this time. At four years of age, he was diagnosed with epilepsy. He developed mumps meningitis with increased levels of mumps IgM. When he was six years old, he developed bacterial pneumonia accompanied with agammaglobulinemia. After IVIG was started, he had no

recurrent infections. His mother (D.II.1) had no clinical findings. His grandmother (D.I.1), who is currently 68 years old, developed ITP at the age of 20. She received prednisolone for 2 years with a resolving thrombocytopenia. She had no recurrent or severe infection with normal levels of serum immunoglobulins (IgG 1,136 mg/dL, IgA 204 mg/dL and IgM 79 mg/dL).

Family E

E.II.1 is an eight-year-old boy, who was diagnosed with systemic lupus erythematosus (SLE) at the age of three years. He presented with fever, skin rash, arthritis, systemic lymphadenopathy and renal disease. He had increased levels of anti-nuclear antibodies ($\times 1,280$, reference value <40), anti-double-stranded DNA antibodies ($> 8,000$ IU/mL, reference value < 12 IU/mL), anti-cardiolipin antibodies (22 U/mL, reference value < 10 U/mL), anti-ribonucleoprotein antibodies (12.2 U/mL, reference value < 10 U/mL), and decreased complement levels (CH50 <12.0 CH50/mL, reference value 25-48 CH50/mL). He also had Evans syndrome. Although his disease activity was severe (SLE disease activity index scores, 29), it had improved after immunosuppressive therapies including methylprednisolone pulse, immunoadsorption, prednisolone, and mycophenolate mofetil. Although high levels of serum immunoglobulins (IgG 2329 mg/dL, IgA 154 mg/dL and IgM 97 mg/dL) were found at diagnosis of SLE, they gradually decreased. Although he currently had hypogammaglobulinemia (IgG 361 mg/dL, IgA < 3 mg/dL, IgM 5 mg/dL), he had no recurrent or

severe infection without IVIG. His mother (E.I.1) had no clinical findings.

Family F

F.2 is currently a five-month-old boy with a history of premature birth at gestational age of 35 weeks. He was admitted to neonatal intensive care unit, and peripheral blood examination revealed pancytopenia with hemoglobin of 5.2 g/dL, 1,500/ μ L white blood cell with 1,200/ μ L neutrophils and 150/ μ L lymphocytes, and 16,000/ μ L platelets. Recurrent red blood cell and platelet transfusion was required, and he developed subcutaneous and intracranial hemorrhage. Although hematopoietic stem cell transplantation was recommended as inherited bone marrow failure, pancytopenia spontaneously recovered in one month. The current laboratory data revealed hemoglobin of 12.5 g/dL, 9,720/ μ L white blood cell with 2,770/ μ L neutrophils and 6,030/ μ L lymphocytes, and 315,000/ μ L platelets at six months of age. His brother (F.1) had no clinical findings.

Table E1. Filtering and numbers of called variants

Patients	A.1	B.1	D.II.1	E.II.1
Total variants in the patient	24,900	25,589	25,250	36,109
Nonsynonymous variants	12,529	13,056	12,799	21,058
dbSNP131-based filtering	2,661	3427	2,984	10,712
In-house SNP database-based filtering	2,103	2,872	2,457	10,033
Ranking by allele frequency*	365	358	309	503
Inheritance model filtering	79	2	–†	–†
Common variant	1	1	1	1

SNP, single nucleotide polymorphism.

*Exclude variants having allele frequencies of less than 0.25 (SNVs) or 0.10 (indels).

†Whole exome sequencing was not performed using DNA samples from the families.

Table E2. Monoclonal antibodies information

Specificity	Conjugation	Clone	Manufacture
CD3	VioGreen	BW264/56	Miltenyi Biotec
CD4	VioBlue	M-T466	Miltenyi Biotec
CD8	PE-Vio770	BW135/80	Miltenyi Biotec
CD10	APC-Vio770	97C5	Miltenyi Biotec
CD11c	PC5	BU15	Beckman Coulter
CD16	FITC	3G8	Beckman Coulter
CD19	VioBlue	LT19	Miltenyi Biotec
CD20	VioGreen	LT20	Miltenyi Biotec
CD24	PE	32D12	Miltenyi Biotec
CD25	FITC	ACT-1	Dako
CD27	PC7	1A4CD27	Beckman Coulter
CD31	APC	AC128	Miltenyi Biotec
CD34	PE	BirmaK3	Dako
CD38	APC	IB6	Miltenyi Biotec
CD45RA	ECD	2H4LDH11LDB9	Beckman Coulter
CD45RO	APC-Vio770	UCHL1	Miltenyi Biotec
CD56	PC5	N901 (NKH-1)	Beckman Coulter
CD62L	PC5	DREG56	Beckman Coulter
CD90	VioBlue	DG3	Miltenyi Biotec
CD123	PE	AC145	Miltenyi Biotec
CD135	PE-Vio770	BV10A4H2	Miltenyi Biotec
CD185	PerCP/Cy5.5	J252D4	BioLegend
HLA-DR	ECD	Immu-357	Beckman Coulter
IgD	FITC	IA6-2	BD Pharmingen
IgM	PE-Cy5	G20-127	BD Pharmingen
Lin	FITC		BD Pharmingen
TCR $\alpha\beta$	PE	BW242/412	Miltenyi Biotec
TCR $\gamma\delta$	FITC	11F2	Miltenyi Biotec
TCR V α 24	FITC	C15	Beckman Coulter
TCR V β 11	PE	C21	Beckman Coulter

Table E3. Primers information

	Sequence
Forward primer (5' to 3')	GCTGATGAGGGTCAAGACAT
Reverse primers (5' to 3')	TCGTTCTCCTTCTCGTAGCT

	A.I.†	B.I‡	C.I.1 ‡	C.II.1 †	C.II.2 †	D.I.1 ‡	D.II.1 ‡	D.III. 1†	E.II.1 †	F.1†	F.2*	normal values*	normal values†	normal values‡
T cells, % CD3 ⁺ /Lym	85.0	87.1	83.8	80.8	80.1	80.4	77.1	88.2	93.5	47.5	89.8	53.6-86.0	58.6-77.2	64.9-80.6
Helper T cells, % CD4 ⁺ /Lym	25.9	39.5	19.9	42.2	39.7	30.4	32.1	44.1	26.9	26.6	76.1	36.3-64.3	31.7-54.7	34.5-47.9
Naïve CD4 ⁺ T cells, % CD45RA ⁺ /CD3 ⁺ CD4 ⁺	51.2	81.4	24.1	52.7	57.6	ND	ND	72.3	41.1	95.6	91.1	84.6-98.5	62.1-82.5	53.1-70.5
Memory CD4 ⁺ T cells, % CD45RO ⁺ /CD3 ⁺ CD4 ⁺	46.7	17.9	71.2	36.8	37.1	86.4	ND	18.7	59.1	4.1	6.9	1.5-15.4	17.5-37.9	29.5-46.9
Recent thymic emigrants, % CD31 ⁺ /CD3 ⁺ CD4 ⁺ CD45RA ⁺	97.8	97.9	98.9	95.9	95.1	ND	ND	99.7	90.7	92.7	97.1	86.7-96.3	65.9-95.6	58.8-81.3
T follicular helper cells, % CD45RO ⁺ CXCR5 ⁺ /CD3 ⁺ CD4 ⁺	2.1	1.5	1.4	6.4	7.85	21.1	ND	1.2	1.5	2.0	0.3	0.2-1.0	1.6-5.1	1.4-6.7
Regulatory T cells, % FOXP3 ⁺ CD25 ⁺ /CD3 ⁺ CD4 ⁺	1.6	1.5	ND	ND	ND	ND	ND	6.5	3.1	ND	ND	1.0-3.6	3.1-7.7	4.9-9.2
Cytotoxic T cells, % CD8 ⁺ /Lym	48.3	41.1	53.1	30.9	35.7	41.2	40.6	34.7	59.1	12.3	12.3	10.3-26.3	15.7-28.9	17.6-26.4
Naïve CD8 ⁺ T cells, % CD45RA ⁺ /CD3 ⁺ CD8 ⁺	69.7	92.3	74.7	82.1	82.8	ND	ND	64.0	44.7	97.7	97.7	81.2-99.4	71.0-91.0	68.0-83.8
Memory CD8 ⁺ T cells, % CD45RO ⁺ /CD3 ⁺ CD8 ⁺	28.2	7.8	25.4	16.3	17.6	ND	ND	37.7	54.9	3.5	3.5	0.6-18.8	9.0-29.0	16.2-32.0

TCRγδ T cells, % TCRαβ ⁺ TCRγδ ⁺ /CD3 ⁺	16.1	2.4	20.8	7.7	5.2	8.8	3.3	11.7	7.6	18.4	0.6	3.0-8.5	3.6-13.2	2.0-13.5
Double negative T cells, % CD4 ⁺ CD8 ⁻ /CD3 ⁺ TCRαβ ⁺	0.7	6.5	0.7	0.6	0.9	0.9	1.3	0.7	0.8	0.5	0.1	0.1-0.5	0.3-1.8	0.3-1.3
invariant NKT cells, % TCR Vα24 ⁺ TCR Vβ11 ⁺ /CD3 ⁺	0.004	0.000	0.004	0.016	0.014	0.007	0.007	0.000	0.006	0.045	0.017	0.008-0.058	0.010-0.049	0.009-0.040
B cells, % CD19 ⁺ CD20 ⁺ /Lym	2.3	0.5	0.7	2.4	2.2	7.4	11.6	1.0	0.2	34.4	0.8	11.3-30.2	7.7-19.2	2.9-12.3
Transitional B cells, % CD24 ⁺ CD38 ⁺ /CD19 ⁺	20.3	0.2	88.7	47.3	34.3	3.6	ND	87.2	78.9	9.4	85.2	8.8-46.7	5.6-22.1	6.7-12.4
Memory B cells, % CD27 ⁺ /CD19 ⁺	7.2	0.7	2.6	9.5	7.0	16.4	38.0	3.5	13.1	11.0	2.6	1.2-3.3	11.4-22.1	18.2-42.0
Class-switched memory B cells, % IgD ⁻ CD27 ⁺ /CD19 ⁺	4.1	0.7	0.8	3.4	2.8	52.6	60.0	0.3	9.8	5.5	0.7	0.5-3.8	5.3-15.5	8.4-23
NK cells, % CD16 ⁺ CD56 ⁺ /Lym	7.0	4.8	9.1	11.4	12.4	9.8	10.1	9.1	3.8	10.2	1.9	2.7-10.9	2.4-12.4	7.8-14.4
myeloid dendritic cell, % Lin ⁻ HLA-DR ⁺ CD11c ⁺ CD123 ⁻ /CD45 ⁺	0.18	0.09	0.18	0.32	0.56	0.05	0.36	0.13	0.19	ND	0.33	0.19-0.55	0.13-0.81	0.14-0.77
plasmacytoid dendritic cell, % Lin ⁻ HLA-DR ⁺ CD11c ⁻ CD123 ⁺ /CD45 ⁺	0.06	0.02	0.01	0.03	0.04	0.29	0.08	0.05	0.02	ND	0.36	0.04-0.70	0.02-0.64	0.02-0.44

* 0-1 years.

† 2-15 years.

‡ 16- years.

Values outside the normal range are shown in bold. Values more than the normal range are shown in red, and values less than the normal range are shown in blue.

ND, not determined; TCR, T-cell receptor; NKT, natural killer T.

FIG E1. Genetic analysis of heterozygous mutation in *IKZF1*

Sanger sequencing of the families.

FIG E2. Supershift assay

Supershift assay was performed using HEK293T cells transiently expressing the untagged WT IKAROS. The bands are supershifted by the addition of anti-IKZF1 antibody but not by anti-V5 antibody.

FIG E3. Subcellular localization of IKAROS protein in lymphocytes

CD3/CD28-stimulated lymphocytes are shown in upper panel. Unstimulated lymphocytes are shown in lower panel. IKAROS and the overlays with 4,6-diamidino-2-phenylindole are shown. Ctrl means control.

FIG E4. The characterization of B cells, red blood cells and platelets

(A) Abnormal B-cell subset, CD10⁺CD24⁺CD38⁻IgD⁺IgM⁻ B cells in patient B.1. (B) Sequential changes of serum IgA and IgM levels. (C) The course of B-cell counts in patient A.1.

FIG E5. Hematopoiesis and the involvement of Ikaros

Previous studies in mice demonstrated that *Ikzf1* is required for hematopoietic stem cell self-renewal, common lymphoid progenitors development, granulo-monocyte progenitors development from common myeloid progenitors and erythrocyte development.

B, B cell; CLP common lymphoid progenitor; CMP, common myeloid progenitor; Ery, erythrocyte; Gra, granulocyte; GMP, granulo-monocyte progenitors; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multi-potent progenitors; Mono, monocyte; MEP, megakaryocyte-erythroid progenitor cell; MKa, megakaryocyte; MPP multipotent progenitor; T, T cell.

FIG E6. T cell maturation and function

(A) T-cell receptor repertoire analysis. Patient A.1 shows skewed Vb11 expression in CD8⁺ T cells. White bars indicate mean $\pm$ SD of the controls, which were provided by the manufacture. (B) Proliferation assays of T cells. Threshold of proliferation is decreased in patient F.2. T cells respond poorly to anti-CD3/CD28 antibodies in patient B.1.

Fig. E1

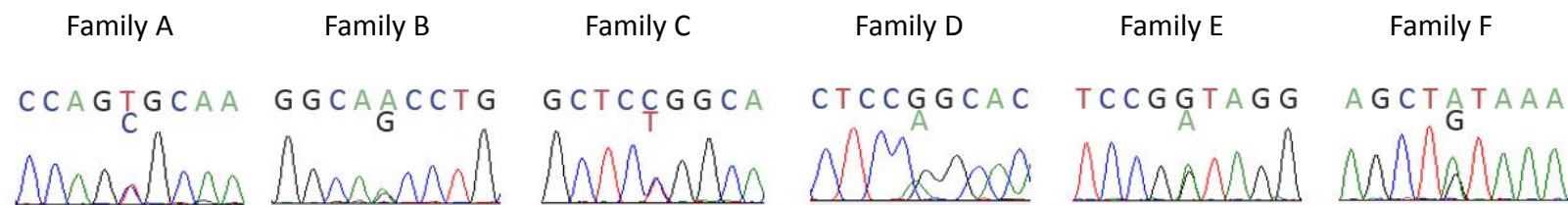


Fig. E2

	no antibody	anti- IKZF1	anti- V5
antibodies			
plasmids	WT	WT	WT



Fig. E3

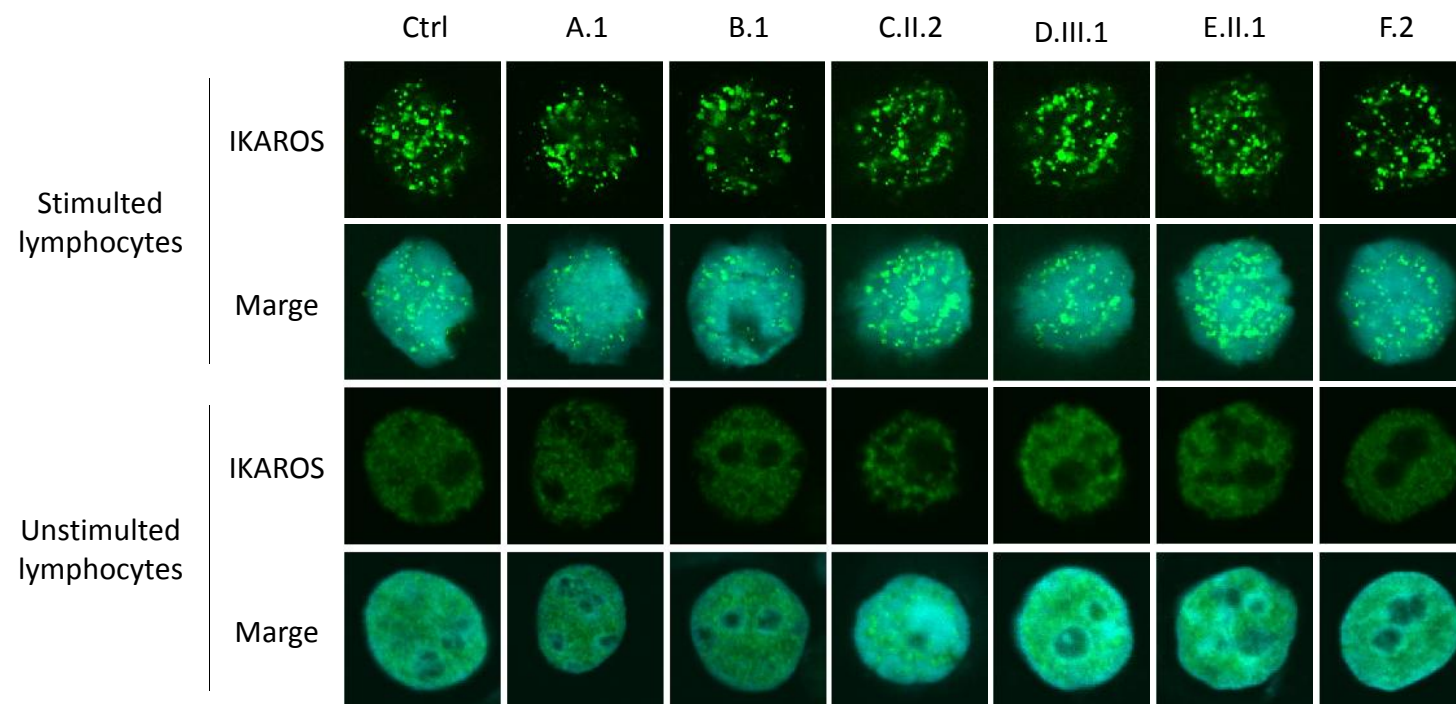


Fig. E4

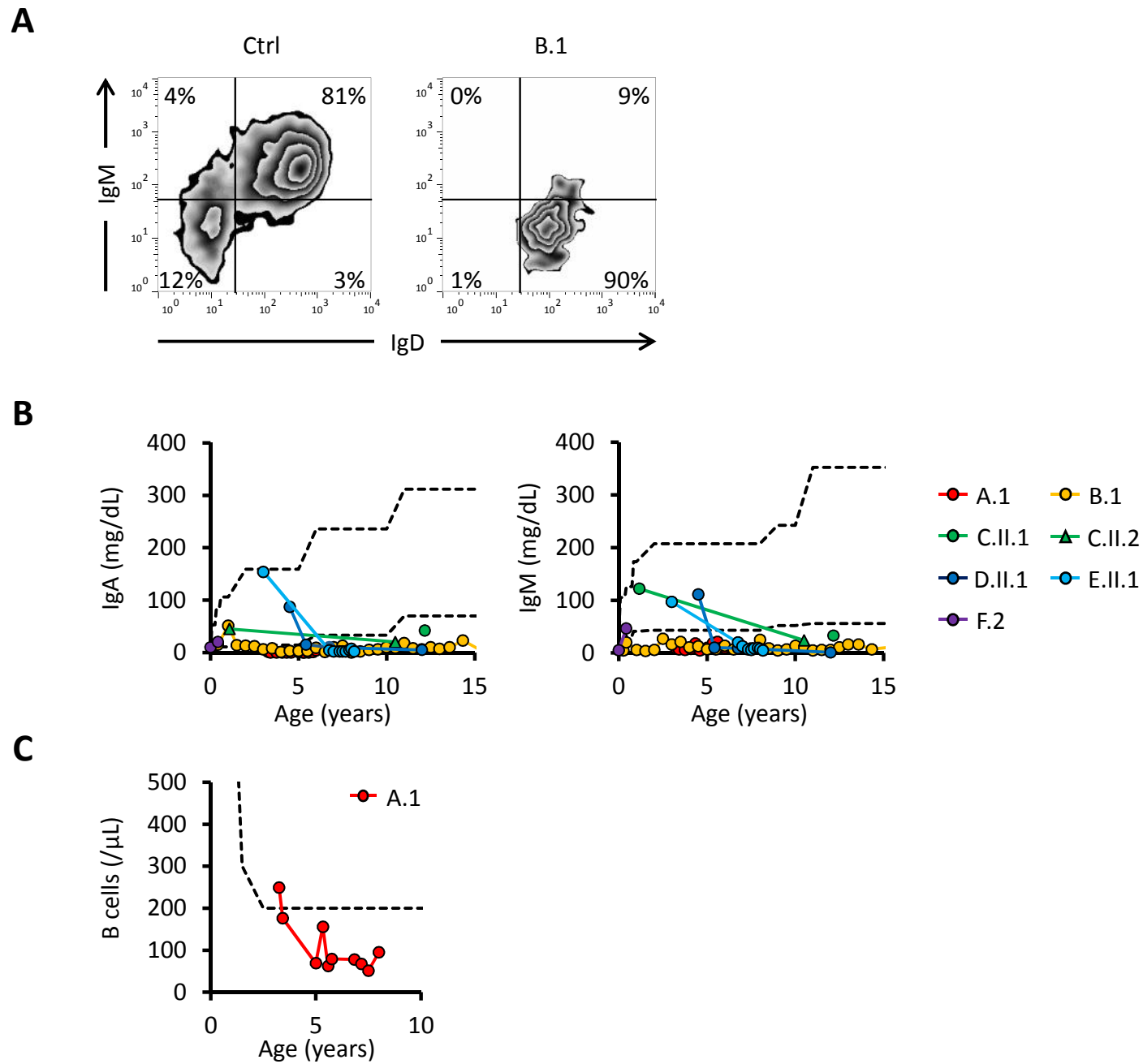


Fig. E5

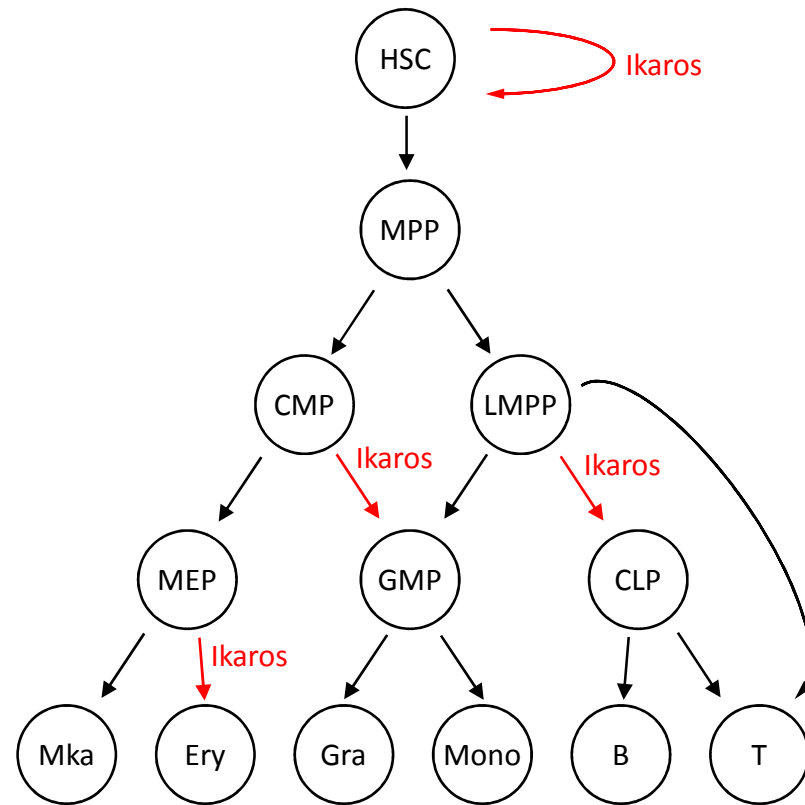
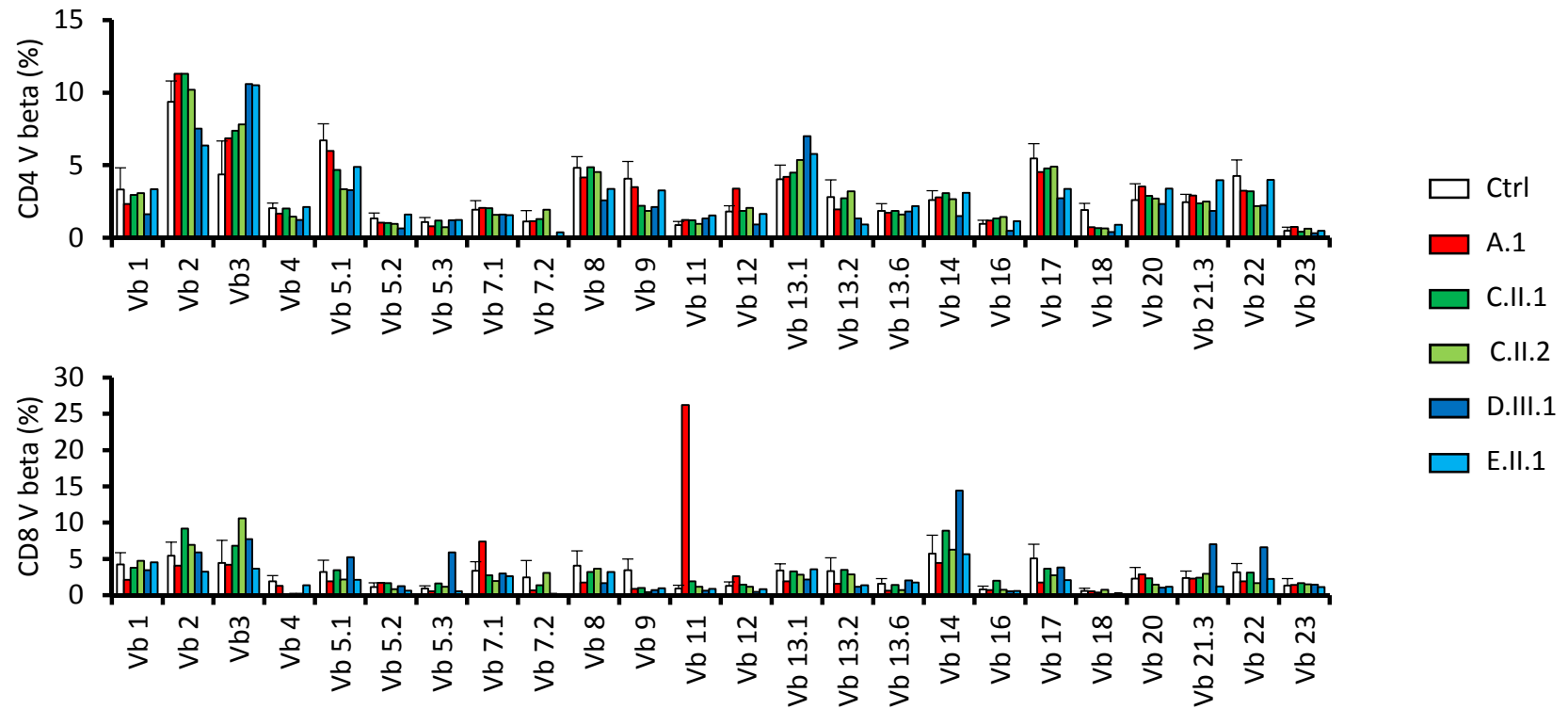


Fig. E6

A



B

