

## Letter to the Editor

**A novel mutation in the *POLE2* gene causing combined immunodeficiency**

To the Editor:

Early lymphocyte development requires the orchestrated interplay of pathways to maintain genomic integrity and accurate DNA repair during the proliferative bursts associated with antigen receptor rearrangement.<sup>1</sup> Inborn errors in replication control or DNA repair can lead to primary immunodeficiency.<sup>2</sup> We report the case of a patient with combined immunodeficiency, facial dysmorphisms, and autoimmunity with a novel mutation in the DNA polymerase epsilon subunit 2 (*POLE2*) gene. A, 5-year-old boy born to related parents of Saudi origin was referred to our center with a history of omphalitis and erythroderma in the neonatal period, systemic *Bacillus Calmette-Guerin* infection after immunization, and multiple respiratory infections subsequently. Diabetes mellitus was diagnosed at age 5 months. At 8 months, he developed severe dyspnea with hypoxia, hepatomegaly, and hypothyroidism. Laboratory investigation disclosed agammaglobulinemia, absence of circulating B cells, T-cell lymphopenia, and neutropenia. Replacement therapy with intravenous immunoglobulins was initiated. At 3 years, he developed generalized lymphadenopathy and a

lymph node biopsy showed effaced architecture with lack of follicles, an increased number of CD163<sup>+</sup>-activated macrophages, and activated (CD45RO<sup>+</sup>) T lymphocytes. He continued to have recurrent respiratory infections with pulmonary atelectasis. When evaluated at our center, his height and weight were at the third percentile and head circumference was  $48 \pm 2.4$  cm. Dysmorphic features (low anterior hairline, flat supraorbital ridges, downturned corners of the mouth, and a short philtrum) were noticed. Laboratory evaluation (Table I) confirmed lymphopenia with absence of B lymphocytes, markedly increased proportion of effector memory T cells, undetectable T-cell receptor excision circles (TRECs), and a reduced number of natural killer cells, 30% of which were CD56<sup>bright</sup> (our internal laboratory normal reference range,  $8.4\% \pm 3.8\%$ ). *In vitro* proliferative response to mitogens was normal, but response to antigens was abrogated (Table I). Upon stimulation of PBMCs with anti-CD3  $\pm$  anti-CD28 mAbs, an increased proportion of cells were found to be positive for Annexin V staining by fluorescence-activated cell sorting, consistent with increased cell death. Flow-cytometric analysis of expression of 24 T-cell receptor (TCR) V $\beta$  families revealed normal representation in CD4<sup>+</sup> cells, whereas some skewing was observed among CD8<sup>+</sup> cells, with 4 families being underrepresented and 2 overrepresented. Bone marrow examination disclosed a low proportion of

**TABLE I.** Hematologic and immunologic features

| Laboratory parameter                                                                                 | Patient's age |          |          |           | Normal controls |
|------------------------------------------------------------------------------------------------------|---------------|----------|----------|-----------|-----------------|
|                                                                                                      | 10 mo         | 4 y 6 mo | 5 y 8 mo | 6 y 10 mo |                 |
| ANC ( $\times 10^9/L$ )                                                                              | 0.15          | 3.27     | 2.28     | 3.88      | 2.49-5.96       |
| ALC ( $\times 10^9/L$ )                                                                              | 1.90          | 2.47     | 0.70     | 0.76      | 1.33-3.47       |
| CD3 <sup>+</sup> (cells/ $\mu L$ )                                                                   | 2,030         | 2,341    | 763      | 809       | 1,400-6,200     |
| CD3 <sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> (%)                                                  |               | 97.3     |          |           | 88-99.4         |
| CD3 <sup>+</sup> TCR $\gamma\delta$ <sup>+</sup> (%)                                                 |               | 2.7      |          |           | 0.6-12.0        |
| CD4 <sup>+</sup> (cells/ $\mu L$ )                                                                   | 1,370         | 1,114    | 403      | 290       | 700-2,200       |
| Naive CD4 (CD45RA <sup>+</sup> CCR7 <sup>+</sup> ) (% of CD4 <sup>+</sup> )                          | 21.9*         | 25.7     |          | 21.6      | 57.1-84.9       |
| Central memory CD4 (CD45RA <sup>-</sup> CCR7 <sup>+</sup> ) (% of CD4 <sup>+</sup> )                 |               | 0.9      |          | 9.9       | 11.3-26.7       |
| Effector memory CD4 (CD45RA <sup>-</sup> CCR7 <sup>-</sup> ) (% of CD4 <sup>+</sup> )                |               | 72.1     |          | 67.7      | 3.3-15.2        |
| CD4 <sup>+</sup> CD45RA <sup>+</sup> CCR7 <sup>-</sup> (% of CD4 <sup>+</sup> )                      |               | 1.3      |          | 0.8       | 0.4-2.6         |
| Recent thymic emigrants (CD45RA <sup>+</sup> CD31 <sup>+</sup> ) (% of CD4 <sup>+</sup> )            |               | 16.2     |          |           | 19.4-60.9       |
| CD8 <sup>+</sup> (cells/ $\mu L$ )                                                                   | 543           | 1,262    | 328      | 505       | 490-1,300       |
| Naive CD8 (CD45RA <sup>+</sup> CCR7 <sup>+</sup> ) (% of CD8 <sup>+</sup> )                          | 2.8*          | 6.6      |          | 2.1       | 28.4-80.6       |
| Central memory CD8 (CD45RA <sup>-</sup> CCR7 <sup>+</sup> ) (% of CD8 <sup>+</sup> )                 |               | 0.7      |          | 4.1       | 1.0-4.5         |
| Effector memory CD8 (CD45RA <sup>-</sup> CCR7 <sup>-</sup> ) (% of CD8 <sup>+</sup> )                |               | 86.0     |          | 85.1      | 6.2-29.3        |
| T <sub>EMRA</sub> (CD8 <sup>+</sup> CD45RA <sup>+</sup> CCR7 <sup>-</sup> ) (% of CD8 <sup>+</sup> ) |               | 6.8      |          | 8.6       | 9.1-49.1        |
| CD3 <sup>+</sup> CD16/56 <sup>+</sup> (cells/ $\mu L$ )                                              | 60            | 1        |          | 30        | 130-720         |
| CD19 <sup>+</sup> (cells/ $\mu L$ )                                                                  | 17            | 0        |          | 3         | 390-1,400       |
| IgG (mg/dL)                                                                                          | <150          | 516†     | 556†     | 740†      | 639-1,344       |
| IgA (mg/dL)                                                                                          | <25           | <7       | <7       | <7        | 50-150          |
| IgM (mg/dL)                                                                                          | <18           | <5       | <5       | <5        | 22-100          |
| IgE (IU/mL)                                                                                          |               | <1       | <1       | <1        | 0-200           |
| <i>In vitro</i> proliferation to mitogens (cpm)                                                      |               |          |          |           |                 |
| Medium                                                                                               |               | 938      | 779      | 461       | 321-2,510       |
| PHA                                                                                                  |               | 179,226  | 74,035   | 96,355    | 104,415-319,780 |
| <i>In vitro</i> proliferation to antigens (cpm)                                                      |               |          |          |           |                 |
| Medium                                                                                               |               | 1,721    | 2,605    | 2,398     |                 |
| Tetanus toxoid                                                                                       |               | 1,157    | 7,166    | 3,422     |                 |
| Diphtheria toxoid                                                                                    |               | 1,071    | 1,908    |           |                 |
| <i>Candida albicans</i>                                                                              |               |          | 1,993    | 2,735     |                 |

ALC, Absolute lymphocyte count; ANC, absolute neutrophil count.

\*At this time point, naive CD4 and CD8 lymphocytes were defined as CD45RA<sup>+</sup> CD62L<sup>+</sup>.

†On intravenous immunoglobulin replacement therapy.

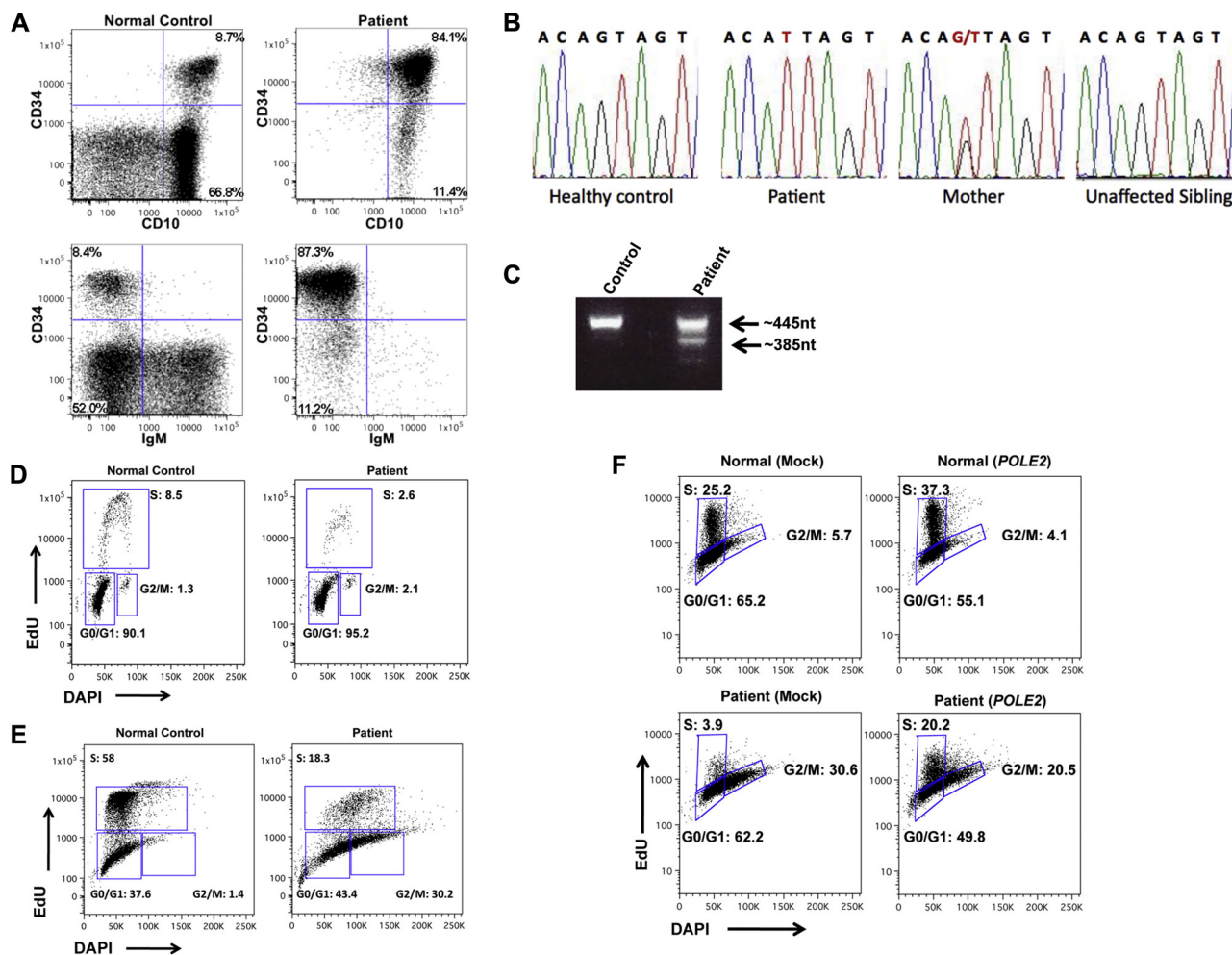


**FIG 1.** A, FACS analysis of bone marrow from a healthy control and the patient. Upon gating on CD19<sup>+</sup>/CD45<sup>+</sup> positive cells, an increased proportion of B-cell precursors (CD10<sup>+</sup>/CD34<sup>+</sup>) and a decreased proportion of mature IgM<sup>+</sup> B cells were demonstrated in the patient. B, Sequence chromatogram depicting sequence variance at position -1 of intron 13 in a healthy control, the patient, his mother, and an unaffected sibling. C, RT-PCR spanning from exon 12 through exon 16 of *POLE2*. A single band of 445 nucleotides (nt) is detected in the normal control, whereas 2 bands are observed in the patient: a 442nt product (resulting from the use of a cryptic splice site at position +3 of exon 14) and a 385nt product with skipping of exon 14. D, Peripheral blood lymphocytes from a healthy control (left) and the patient (right) stimulated for 96 hours with PHA and then stained with EdU and DAPI. A decreased proportion of cells in the S phase are observed in the patient. E, Cell cycle analysis of fibroblasts grown asynchronously 24 hours after pulsing with EdU and DAPI. Patient's fibroblasts show accumulation of cells in G2/M and decreased proportion of cells in the S phase. F, Cell cycle analysis of fibroblasts transfected with either empty- or wild-type *POLE2*-containing vector 6 hours after release from growth synchronization. Partial rescue of cell cycle progression is observed in patient's *POLE2*-transduced fibroblasts. These data are representative of 2 experiments. DAPI, 4',6-Diamidino-2-phenylindole; EdU, 5-Ethynyl-2'-deoxyuridine; FACS, fluorescence-activated cell sorting.

CD19<sup>+</sup> cells (8.2% of the cells in the lymphocyte gate), most of which (89%) were CD34<sup>+</sup>. By staining for CD19, CD34, CD10, and specific IgM, we observed a severe block at pre-BII stage and virtual absence of mature CD19<sup>+</sup> IgM<sup>+</sup> cells (Fig 1, A). The clinical course of the patient was complicated by a severe episode of hemophagocytic lymphohistiocytosis that failed to respond to etoposide, dexamethasone, alemtuzumab, and anakinra, and led to death at the age of 7 years and 11 months.

In an attempt to identify the underlying cause of the disease, we performed whole exome sequencing (see this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). A homozygous splice-site mutation (g>t) at position -1 of intron 13 of the *POLE2* gene was identified, within a region of chromosome 14, where single nucleotide

polymorphism (SNP) analysis demonstrated homozygosity. This splice-site mutation was confirmed by Sanger sequencing (Fig 1, B), and has not been previously reported in dbSNP or 1000Genome. Both parents were heterozygous for the mutation, and none of the 4 healthy siblings was homozygous for the mutation. RT-PCR analysis yielded 2 transcripts in the patient: a normal-sized transcript with a deletion of 3 nucleotides resulting from the usage of a cryptic acceptor splice site at position +3 in exon 14 and a shorter transcript due to skipping of exon 14 (Fig 1, C). The product with the 3-nt deletion is predicted to result in loss of a single highly conserved amino acid residue, Ser 359 (NP\_001184260.1). Immunoblot analysis for *POLE2* protein revealed similar expression in patient and control samples (data not shown).

DNA polymerases help achieve replication of DNA with remarkable accuracy through conserved pathways that repair DNA damage and correct nucleotide misincorporation during DNA replication.<sup>3</sup> The DNA polymerase  $\epsilon$  (POLE) is a large, multisubunit polymerase that is conserved throughout evolution in eukaryotes. Of the 4 genes that encode for the POLE complex, *POLE1* encodes for a 261-kDa protein that contains the catalytic activity and is complexed with the POLE2 (59kDa) subunit. POLE3 (17kDa) and POLE4 (12kDa) represent 2 additional subunits in the complex.<sup>4,5</sup> The functional role of the POLE holoenzyme includes leading strand synthesis required for DNA replication and proofreading activity required for the repair of DNA damage and for maintenance of sequence (epigenetic) inheritance.<sup>4,5</sup> The exact function of the POLE2 subunit is not known, but is thought to involve protein-protein interactions, including dimerization with POLE1 and influencing the C-terminal part of the catalytic subunit. Given the role of POLE in DNA replication, we investigated cell cycle progression after stimulation of the patient's PBMCs with PHA, and cells with failed DNA replication in the S phase were demonstrated (Fig 1, D). Similarly, asynchronous culture of skin-derived primary fibroblasts from the patient showed a reduced number of cells in the S phase and an increased proportion of cells in G2/M (Fig 1, E) as compared with control fibroblasts. Furthermore, patient's fibroblasts had stunted growth, and karyotype analysis demonstrated that 66% of the cells in culture were tetraploid. Upon transduction of patient and control fibroblasts with a lentiviral vector expressing wild-type POLE2, followed by growth synchronization, there was a partial rescue of cell cycle progression in wild-type POLE2-transduced patient fibroblasts, with a significant increase in the number of cells in the S phase (Fig 1, F).

Somatic mutations in human POLE have been demonstrated in colon cancer, and are thought to contribute to genomic instability.<sup>6,7</sup> Recently, a mutation in *POLE1*, a member of the DNA polymerase pathway, has been found in a large consanguineous family, leading to a syndrome with facial dysmorphism, immunodeficiency with hypogammaglobulinemia, livedo reticularis, and short stature.<sup>8</sup> A variable decrease in the absolute number of B cells, and to a lesser degree naive T cells, was detected among the affected individuals, with impaired G1- to S-phase progression in primary T cells and in lymphoblastoid cell lines generated from B cells. In contrast, our patient had a more severe block in B-cell development than did patients with *POLE1* mutation, a difference that may reflect the distinct nature of mutations or functions of the POLE subunits. Furthermore, clinical evolution of the patient described here was also more severe than for patients with *POLE1* mutations. To our knowledge, this is the first case in which germline mutations in a replicative DNA polymerase have been associated with an early block in lymphocyte development in humans.<sup>1,9</sup> Altogether, identification of *POLE1* and *POLE2* mutations in patients with immunodeficiency and other extraimmune manifestations indicates an important role for the POLE holoenzyme in cell proliferation, and in the lymphoid lineage in particular. The relative contribution of impaired lymphocyte proliferation due to defective DNA replication, altered cellular survival, and/or defective repair of lymphoid-specific DNA alterations in patients with POLE defects remains to be addressed.<sup>6</sup> Finally, longitudinal follow-up studies are needed to uncover the potential role of germline *POLE1* and *POLE2* mutations in cancer susceptibility.

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## METHODS

### Patient

Blood samples from the patient and his parents were obtained either at Boston Children's Hospital or were shipped from collaborators, with informed consent according to our institutional review board.

Genomic DNA was extracted from whole blood by using the Gentra Puregene Blood Kit (Qiagen, Valencia, Calif).

### Fluorescence-activated cell sorting analysis

Flow cytometry was used to measure the percentages of lymphocyte populations in whole blood using the mAb conjugates (6 Color TBNK Reagent, CD3-peridinin-chlorophyll protein [PerCP]-Cy5.5, CD4-allophycocyanin [APC], CD8-V450, CCR7-fluorescein isothiocyanate [FITC], CD45RA-PE, CD19-PerCP-Cy5.5, CD27-phycoerythrin [PE], IgD-FITC, CD24-APC, and CD38V450, all from BD Biosciences, Franklin Lakes NJ). The percentage of each patient's lymphocyte subset was compared with normal control ranges for age that have been established independently in the Boston Children's flow cytometry laboratory. Lymphocyte proliferation assays were performed in 3 independent experiments.

### Sequencing

Genomic DNA extracted from the patient was subjected to whole exome sequencing. DNA was sheared with a Covaris S2 Ultrasonicator (Covaris, Waburn, Mass), and an adapter-ligated library was prepared with the Paired-End Sample Prep Kit V1 (Illumina, San Diego, Calif). Subsequently, exome capture was performed with the SureSelect Human All Exon Kit (Agilent Technologies, Santa Clara, Calif) and single-end sequencing was performed with an Illumina Genome Analyzer IIx (Illumina), generating 72-base reads. The sequences were aligned with the human genome reference sequence (hg18 build), with BWA aligner. Three open-source packages were used for

downstream processing and variant calling: Genome analysis toolkit (GATK), SAMtools, and Picard Tools (<http://picard.sourceforge.net/>). Substitution calls were made with GATK UnifiedGenotyper, whereas indel calls were made with GATK IndelGenotyperV2. All calls with a read coverage of 4× or less and a phred-scaled SNP quality of 30 or less were filtered out. Together with whole genome SNP analysis, we identified a stretch of homozygosity in chromosome 14.

### Cell cycle analysis

Cell cycle progression in fibroblasts from patients and controls was analyzed upon pulsing with 10 mM Edu and analyzed using the Click-iT Edu Alexa Fluor 488 Imaging Kit (Invitrogen). For cell synchronization experiments, fibroblasts were arrested with double thymidine block and nocodazole release, as described in Bermudez et al<sup>E1</sup> and then analyzed for cell cycle progression. Three independent experiments were performed. Annexin V staining for lymphocytes was performed twice using the Apoptosis Detection Kit 1 (BD Biosciences, San Jose, Calif) as per the manufacturer's protocol.

**Generation of lentiviral vectors.** The *POLE2* cDNA was cloned into a lentiviral vector backbone containing the SFFV U3 promoter and an iresEGFP cassette (kind gift of Dr Axel Schambach, Hannover, Germany). Lentiviral vector production was performed in 293T for packaging. Two independent experiments were performed.

**Karyotyping.** Karyotyping (G-banding) was performed by Cell Line Genetics Inc (Madison, Wis).

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